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Review Article

Lipids and NMR: More Than Mere Acquaintances

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Abstract. Recognising the paramount importance of lipids in cell physiology and function, there is an analytical need to measure the composition of lipids within the cell and how different lipid species interact. In this review, we will explore the role NMR spectroscopy can have in this. We will show how the technique can be used to measure lipid concentrations, but we will also provide evidences of its importance to characterise lipid interactions with other molecules, such as proteins, and to measure lipoproteins, the transporters of triglycerides and cholesterol, discussing advantages and limitations. Furthermore, we will highlight its potential for quality control analysis, particularly in food science and industry, if further development of benchtop instruments continues. Complementary to liquid chromatography mass spectrometry, which is able to measure numerous lipids in a complex mixture, NMR is an invaluable tool for fulfilling this need of better characterising lipids.

Keywords: NMR spectroscopy, lipids, lipidomics, ligand binding site

Lipids have a rich and diverse set of roles in the cell including the regulation of certain nuclear hormone receptors, maintenance of electrochemical gradients across lipid bilayers, subcellular partitioning, first- and second-messenger cell signalling at cell surface receptors, energy storage, and protein trafficking and membrane anchoring [1]. The molecules covering all these functions are diverse and include fatty acids, triglycerides, phospholipids, ceramides, and sterols, to name but a few. This diverse and large number of different types of molecules underpins the difficulty in defining what a lipid is. To be able to orderly classify them, lipids have recently been defined as "hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acids, polyketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.)" [2].

Given the complexity of their definition, it is perhaps not surprising that it can be challenging to measure lipids. These molecules span a range of properties in chemical space as well as concentration, and therefore it has proved a technical challenge to develop a comprehensive method covering all of the classes. In recent years, lipidomics has arisen with the ambitious aim to provide "the comprehensive understanding of the influence of all lipids on a biological system with respect to cell signalling, membrane architecture, transcriptional and translational modulation, cell-cell and cell-protein interactions, and response to environmental changes over time." [1].

The leading technique used in lipidomics is mass spectrometry which is most often hyphenated with liquid chromatography (LC-MS) [3, 4]. This is due to mass spectrometry's high sensitivity, ease with being coupled

to chromatography and relatively good reproducibility. In addition to LC-MS, mass spectrometry has been coupled with ion mobility, to further improve separation between the different lipid species after LC [5], or even used on its own in terms of direct infusion shotgun lipidomics [6] and mass spectrometry imaging [7].

However, mass spectrometry is not the only technique that has been used to measure lipids [8, 9]. Raman and infrared spectroscopy have been applied, as well as Nuclear Magnetic Resonance (NMR) spectroscopy.

NMR spectroscopy is a powerful technique for measuring small molecules. Many reviews have been previously written about its use [10-12] in this context. The main advantages of NMR are being reproducible, non-destructive, non-invasive, cost effective on a per sample basis and the fact that it's relatively easy to identify unknown compounds. Its main drawback, however, is its lack of sensitivity- being limited to detecting species down to a limit of detection between 100-1 μM, depending on the magnetic field used, the type of probe, the acquisition time and the molecule detected [13]. NMR spectroscopy was one of the first techniques to be used in metabolomics/metabonomics to profile metabolites in a tissue extract or biofluid despite its relatively high limit of detection [14, 15]. However, its use has been more limited in terms of looking at lipid metabolism, in part because of the difficulty of discriminating between different lipids using this approach. Nonetheless, the technique does provide some unique insights, in particular concerning lipoprotein measurements in blood plasma and serum. In this review, we will give an overview of the different contributions NMR spectroscopy can provide not only in measuring lipids, but also in defining their physiological role.

1. NMR Spectroscopy As an Analytical Tool

NMR is a spectroscopic technique which relies on the nuclear property of spin [16]. When certain isotopes are placed in a magnet field their nuclei either align or oppose the magnetic field. A radiofrequency wave can cause a transition between these states, and in the NMR experiment we observe the relaxation of this transition. As the exact frequency is dependent on the chemical environment, as well as the isotope under observation, this makes for an incredibly powerful tool for structure elucidation.

What nuclei can be detected by NMR spectroscopy? The possibility of detecting a nucleus by NMR spectroscopy depends on the number of protons and neutrons in the nucleus. Nuclei where both the number of protons and neutrons are even have no spin, and cannot be detected by NMR. Examples of nuclei belonging to this group are ^{12}C, ^{16}O, ^{32}S. Nuclei where either the number of protons or neutrons is odd have half-integer spin and can be detected by NMR. Examples include ^{1}H, ^{13}C, ^{31}P. Nuclei where both the number of protons and the number of neutrons are odd have integer spin and can be detected by NMR, although the spectra they produce are both more complicated and highly dependent on the environment they are found in. Examples include ^{2}D, ^{10}B, ^{14}N. This means that every element will have at least one isotope that can be detected by NMR spectroscopy, but how abundant that isotope is can be highly variable. From the point of view of measuring lipids, the relevant elements for detecting are H, C, O, N and P, with ^{1}H, ^{13}C and ^{31}P being the most accessible isotopes, as they are naturally the most abundant ones and spin 1/2.

How is the signal originated? A spin-half nucleus has an interaction with a magnetic field which gives rise to two energy levels [16]. The difference in population between the two states is proportional to the difference in energy. At room temperature that difference is low, explaining in part why NMR spectroscopy is a relatively insensitive technique.

A radio frequency can excite nuclei from the lower to the higher energy state, making their populations the same; removing the frequency will result in the system coming back to the original difference in population, as the extra nuclei promoted go back to the lower energy level emitting a signal in the radio frequency range. The signal is registered in the time domain, and then transformed into the frequency domain through a Fourier transform.

How can the pattern of the resonances be characterised? The pattern of lines that make up the NMR signal is the result of many factors, which give important information about the nuclei originating them. Here we describe them, exemplifying them with the specific case of the fatty acids depicted in Figure 1.

- The **position** of the signal depends and gives us information about the electronic cloud around the nucleus (i.e. the type of bond the nucleus is involved in). The more the electronic cloud is close to the nucleus, the more it is "shielded" and it is close to the zero on the chemical shift scale used by NMR, examples of this type of nuclei are protons attached to aliphatic carbons. On the other hand, if the electron cloud is attracted away from the nucleus, it is "unshielded" and it is farther away on the opposite extreme of the scale, an example here is the proton found adjacent to an aldehyde group. In the case of fatty acids, saturated carbons have electronic clouds closer to them than unsaturated ones, therefore they are more shielded and closer to zero than unsaturated ones. Furthermore, saturated carbons far away from heteroatoms, such as terminal $–CH_3$ are more shielded than saturated carbons close to heteroatoms, such as the $–CH_2$- directly bonded to the carboxylic group, therefore they are closer to zero.
- The **multiplicity** depends on the adjacent atoms and gives us information about the neighbouring groups. The presence of protons that have a distance of three bonds gives rise to a splitting of the signal, which depends on the number of adjacent protons. A singlet

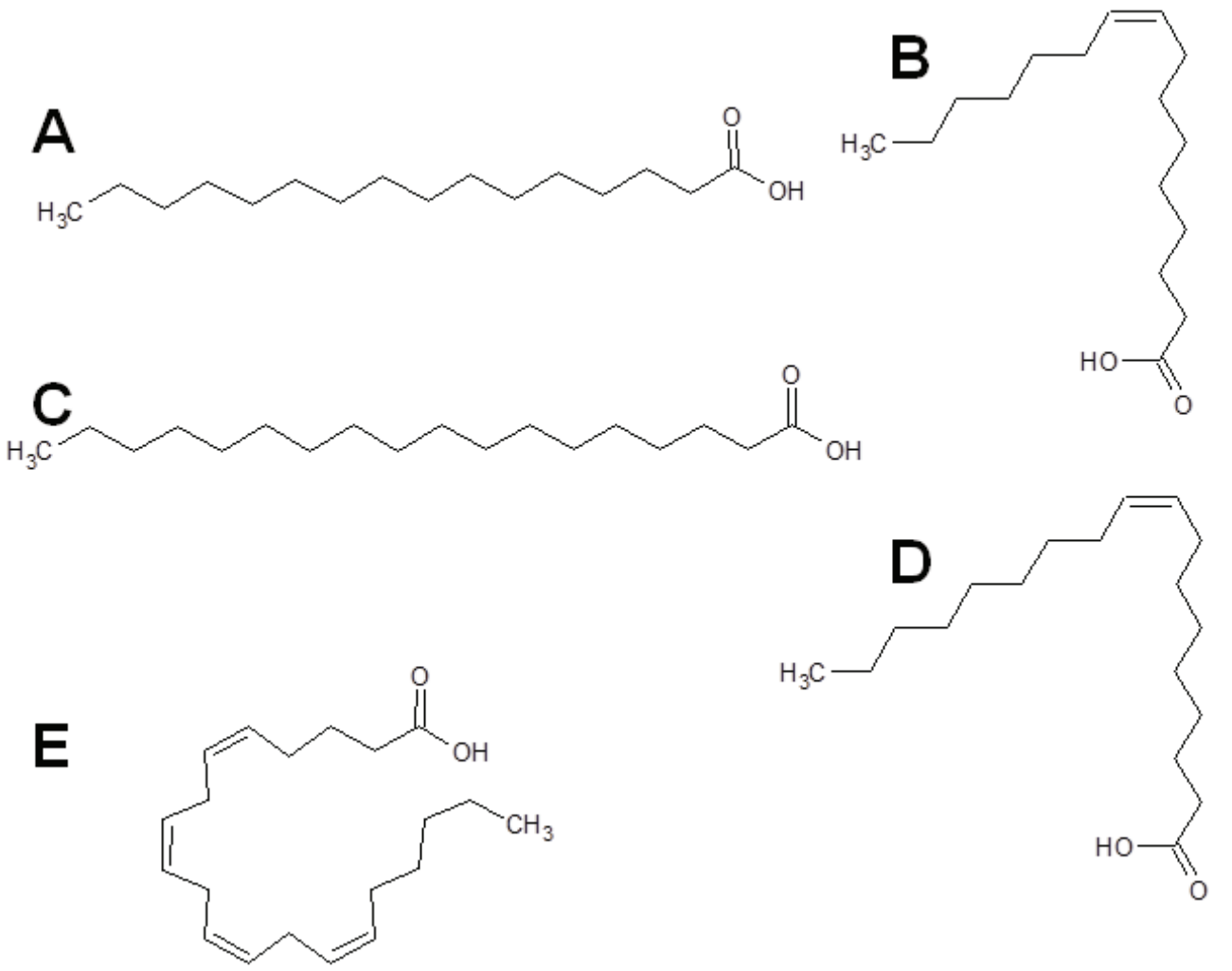

Figure 1: Chemical structures of five common fatty acids: **A.** palmitic acid (16 Cs, no double bonds); **B.** palmitoleic acid (16 Cs, 1 double bond); **C.** stearic acid (18 Cs, no double bonds); **D.** oleic acid (18 Cs, 1 double bond); **E.** arachidonic acid (20 Cs, 4 double bonds).

means that there is no other group containing proton directly linked to its C; a doublet means that there is a group containing one proton directly linked to its C, and so on. In the case of fatty acids, for example, terminal $-CH_3$s usually have two protons on the group directly bonded, therefore they appear as triplets.

- The **intensity** depends on and gives us information about two characteristics, the number of equivalent proton and the concentration of the molecule. The more concentrated the molecule is, the higher is the signal. The classical example to explain this is lactate (shown in Figure 2), originating two signals, one for the CH_3 group and the other for the CH. Under fully relaxed conditions, the signal of the CH_3 group will have an intensity three times higher than the CH, because they belong to the same molecule, but there are three protons in the first case and only one in the second.

Having surveyed the NMR basis, we are now in a position to see how this can be put to use to understand lipid biology.

2. Lipid-protein Binding

One key aspect in studying of nuclear hormone receptors is to understand their regulators and how such regulation occurs. To fully explain these mechanisms, one needs first to identify the ligand binding site and then to determine the conformation the receptor assumes to interact with such ligand. NMR has been extensively used to study protein structure as it allows recording of small and large-scale protein dynamics. Therefore, information about hydrogen bonds, allosteric processes and protein-ligand interactions can be captured. Here, instead of briefly listing a series

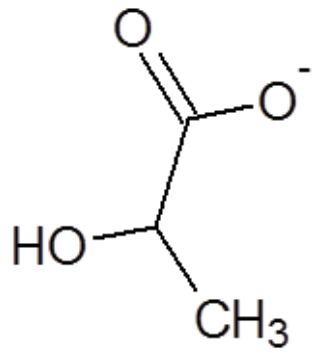

Figure 2: Chemical structures of lactate.

of application, we present in more details one specific case, the nuclear receptor-related 1 protein (Nurr1/NR4A2), recently published [17], as a concrete demonstration of the contribution the technique can give to this field.

For long time, Nurr1 was classified as a ligand-independent transcription factor, following the determination by crystal structure of the absence of a ligand-binding pocket, and also of the presence of a bulky hydrophobic residues region where its putative canonical pocket was expected [18, 19]. However, NMR spectroscopy has since shown that the putative Nurr1 ligand binding domain is dynamic, accessible from the solvent and in exchange between two or more conformations. The peaks of both the amide and the side-chain methyl groups of the amino acids in the putative ligand binding region were compared with the peaks belonging to amino acids away from that region, revealing features indicative of dynamics in the μs-ms timescale, not captured by the crystal structure. Such dynamics are most likely to be an exchange between two or more conformation of the protein in solution.

This was not the sole contribution that NMR gave to a better understanding of how Nurr1 functions. *In vitro* metabolomics studies had previously shown that unsaturated fatty acids bind to the ligand binding domain of Nurr1 and of another orphan nuclear receptor related to it [20]. Exploring the behaviour of Nurr1, first in the presence of docosahexaenoic acid [21], then in the presence of arachidonic acid, linoleic acid and oleic acid [17], it was possible to show that, in every case, all the more affected NMR peaks map to the putative ligand binding pocket. These results strongly suggest that the interaction between the fatty acid and the receptor occurs in that area. Overall, this body of work shows how the putative ligand binding pocket can expand from the crystallographic structure and highlights how powerful NMR is in capturing the exchange between two or more conformations on the μs-ms timescale. NMR spectroscopy well complements crystallography and electron microscopy, the other two leading techniques in the field. In fact, many cases exist of proteins that can be analysed only by one method, for example giving good NMR data, but no crystallization, or vice-versa; furthermore, adding the dynamic information obtained by NMR spectroscopy to the crystal structure enriches the understanding of the behaviour of a protein. In conclusion, when taken together, these techniques allow an optimal description of the characteristics of a protein and pipelines have been developed and tested for their combined use in the case of small proteins [22, 23].

Other notable examples of the use of NMR spectroscopy to clarify the interaction between lipids and proteins include:

- examining the interaction between palmitic acid and bovine β-lactoglobuline in the pH range between 8.4 and 2.1. The results show that, at neutral pH, the methyl end of the fatty acid is bound deep within the central cavity of the protein. Furthermore, the ligand binding is completely reversible and change according to pH, with the release of the ligand starting at pH 6 and being completed at acidic pH [24];
- studying the mechanism through which the fatty acid-binding protein 1 (FABP1) mediates peroxisome proliferator-activated receptor α (PPARα) activation. Substituting key residues adjacent to the ligand-binding portal region of FABP1 showed that the binding of some ligands, such as oleic acid or PPARα activator GW7647, both helps the transport of poorly water-soluble compounds through the cell cytoplasm and stabilizes a conformation of FABP1, able to increase its nuclear localization and the activation of PPARα [25];
- understanding the structure of the complex between arachidonic acid and the odour binding protein 22 of *Aedes Aegypti*. Odour binding proteins are key in regulating the feeding behaviour of female mosquitoes, an important step in the transmission of certain viruses. NMR spectroscopy detected a complex between arachidonic acid and an odour binding protein, and also the region where the binding occurs. This region undergoes a significant conformational change in the presence of the fatty acids [26].

3. NMR-based Lipidomics

In addition to give us information about where and how a lipid interacts with another molecule NMR can help us also determining changes in classes and concentrations of lipids as the characteristics making it a good technique for the measure of aqueous metabolites remain true also for lipids.

3.1. ^{1}*H-NMR use in lipidomics.* Recent examples of the use of ^{1}H-NMR for lipidomics are varied and include:

- the dose-dependent increase in docosahexaenoic acid and the change in the n-6/n-3 ratio in lipid extracts from adipose tissue, following an increased concentration of polyunsaturated n-3 fatty acids in the diet. With this study, the authors aimed to prove that it is possible to evaluate dietary interventions from biopsies and therefore, have a suitable tool to tease out in the future the nutritional part of the risk associated to breast cancer and the evolution of such a contribution [27];
- the decrease of hepatic triglycerides in db/db mice and its correlation with the attenuated development of fatty liver, following a dietary intervention with leucine [28];
- the deregulation of membrane constituent-lipids, such as glycerophospholipids, cholesterol and sphingolipids, and the accumulation of energy storage lipids, such as triglycerides, in fish from wetland contaminated from metals and metalloids [29];
- the measurement of lipid classes in extracts from rodent furs to monitor sebaceous gland atrophy following the administration of a stearoyl-CoA desaturase 1 inhibitor [30].

Beyond the sensitivity issue already outlined as a drawback, the other characteristic that makes ^{1}H-NMR sub-optimal for lipidomics when compared with mass spectrometry is the high overlapping of the signals. There are two main contributing reasons in the particular case of the lipids, one intrinsic to the technique, the other more relevant for these molecules. Proton signals span a small range of chemical shift (10 ppm) and are highly complex in shape, and thus, many resonances overlap making it difficult to determine what species are being observed. Furthermore, lipids have a high degree of similarity in their structures, therefore making the signals highly similar to each other. In Figure 1, we have depicted five common fatty acids with different chain length (16, 18 and 20 C atoms) and levels of unsaturation (0, 1 or 4 double bonds) as examples, to show this similarity in practice. It is useful here to remember again that NMR detects all non-equivalent protons as separated signals, therefore creating complex and similar spectra even for these common cases. The consequence is that it is really challenging (if not utterly impossible) to identify univocally and quantify a single lipid even in relatively simple mixtures.

3.2. ^{31}*P-NMR use in lipidomics.* ^{31}P is a very interesting isotope from lipidomics point of view as it has 100% abundance, a high gyromagnetic ratio (that make it sensitive even at low field) and a wide chemical shift dispersion (that minimize the signal overlapping). Therefore, it is immediately apparent how the detection of this nucleus can be helpful for molecules containing it.

In the case of lipidomics, it is particularly relevant for the identification of the different classes of phospholipids [31]. For example, ^{31}P spectra of lipid extracts from chocolate were shown to provide information on the type and amount of emulsifier used [32]. One particular attraction of this application is that the analysis can potentially be implemented on a benchtop NMR, making the approach very cost effective and easy to use. Benchtop NMR instruments have a low field (usually 1 Tesla or 2 Tesla) [33] and do not require cryogen fluids, and therefore are transportable, cheaper and easier to access and maintain compared to traditional NMR. All these characteristics make these instruments ideal for process controls in industry. In another recent paper [34], the potential of using low-field ^{31}P for lipidomics applications was explored, with an evaluation of the analytical performances and the proposal of two methods to measure absolute concentrations of phospholipids. The authors concluded that it was possible to detect phospholipids in 2 h with a limit of detection of 0.5 mM at 1 Tesla and 0.2 mM at 2 Tesla, unambiguously assign the headgroups of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol, and obtain absolute quantifications for them. In Figure 3, the head groups for these families of lipids are represented, to help understanding the capabilities of the techniques. These new applications demonstrate how the technique is still useful and can be particularly appropriate for quality control, especially in food science and industry.

The limitations of ^{31}P NMR are similar to the ones highlighted above for ^{1}H NMR spectroscopy. It is still challenging (if not almost impossible) to distinguish among the many lipid species: ^{31}P NMR permits to obtain information about the different classes of lipids, but not about the individual species in each class. Furthermore, obviously the lack of sensitivity remains a relevant issue as it can limits the applications it can be implemented for, especially when low fields are considered.

4. Measuring Lipoproteins By NMR Spectroscopy

As cholesterol and triglycerides are insoluble in water, these lipids are transported in the organism in complexes with

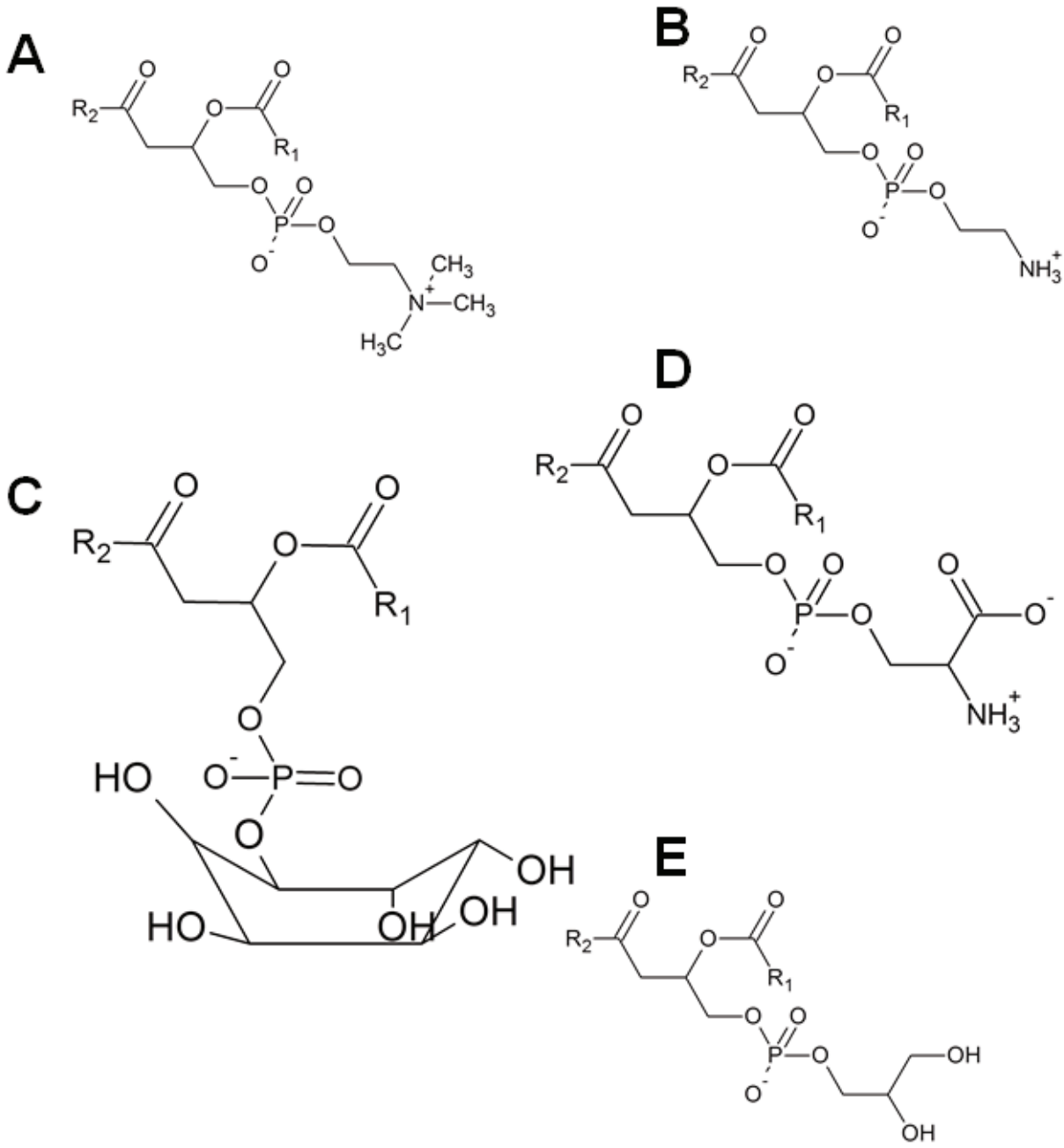

Figure 3: Chemical structures of phospholipids: **A.** phosphatidylcholine, **B.** phosphatidylethanolamine, **C.** phosphatidylinositol, **D.** phosphatidylserine and **E.** phosphatidylglycerol. $-R_1$ and $-R_2$ indicate the two fatty acid moieties.

proteins and other phospholipids. These resulting particles are called lipoproteins and are divided in different classes according to their size and composition [35]. Extensive studies highlighted the crucial role of lipoproteins in the developing of cardiovascular diseases, including type 2 diabetes [36], coronary heart disease [37] and atherosclerosis [38, 39]. Moreover, a role for specific lipoproteins, in particular apolipoproteins, in neurological diseases such as Alzheimer [40] and Parkinson [41] has long been recognised. Therefore, it is really important both for clinical practice and research purposes, to measure these particles confidently. NMR has become a widespread tool in epidemiological studies for measuring lipoproteins in plasma [42]. A pioneer in the use of the technique in this context is Ala-Korpela [43], who developed a method combining ^{1}H NMR spectroscopy and a line-fitting analysis, on the

basis of measurements for each of the ultracentrifuged lipoprotein fractions obtained from human plasma. The results, validated by comparison with the values from traditional biochemical methods, showed that it was possible to obtain "absolute concentrations of phospholipids, total cholesterol, free cholesterol, esterified cholesterol, total proteins, and total masses were estimated for very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) fractions" [43]; moreover VLDL and LDL triglycerides were also quantified. The method has been further extended to include low-molecular-weight metabolites together with lipoproteins, allowing the measure of 225 molecules at the same time in each sample [44].

More recently, a second way to measure lipoproteins from NMR, based on the spectral deconvolution of the plasma methyl lipid resonances originally introduced by Otvos [45], has been automated and standardised [46], showing "a high level of reproducibility and accuracy across the individual platforms".

The number of studies where this technique was used demonstrates its importance [42]. Recent applications include:

- showing how reduced plasma levels of small HDL particles were associated with poor outcomes in patients with idiopathic and heritable pulmonary arterial hypertension [47];
- examining the association of lipoproteins and other blood metabolites with risk of incident myocardial infarction, ischemic stroke and intracerebral haemorrhage. The results demonstrate that myocardial infarction and ischemic stroke have broadly similar strengths of association with concentrations of lipoprotein and lipid constituents (with very low-, intermediate-, and low-density lipoproteins positively associated with the diseases and high-density lipoproteins inversely associated with them), but for intracerebral haemorrhage the associations were substantially weaker. In contrast, certain non–lipid-related metabolites, such as glycoprotein acetyls and glucose, showed similar strengths of association for all 3 subtypes of diseases [48].
- comparing statin treatment with the genetic inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9), as a naturally occurring trial of PCSK9 inhibitors: both lower blood low-density lipoprotein cholesterol levels and therefore reduce risk of cardiovascular events. The results show similar metabolic and lipid changes, with noteworthy discrepancies, however, observed for very low-density lipoproteins, suggesting that PCSK9 inhibitors could have a smaller effect in reducing cardiovascular events compared to statins [49];
- comparing diets with different fatty acids composition showed that both monounsaturated fatty acids and the Mediterranean diet decreased exactly the same fractions of LDL, including particle number, lipid, phospholipid and free cholesterol fraction; however the Mediterranean diet also decreased the larger subclasses of VLDL, several related VLDL fractions, VLDL-triglycerides, and serum-triglycerides [50];
- comparing diets rich in saturated fatty acids and n-6 polyunsaturated fatty acids, in people supplemented with eicosapentaenoic and docosahexaenoic acid, and revealing that both diets reduced the concentration of total very-low-density lipoprotein (VLDL) particles, and their subclasses and increased VLDL and LDL particle size [51];
- studying psoriasis patients and showing increased lipoprotein(a), oxidized lipoprotein(a), and oxidized HDL compared to controls; furthermore a significant association of oxidized LDL and oxidized HDL with noncalcified burden was found [52];
- comparing the composition of the HDL fractions in patients with low, normal or high HDL-cholesterol. It is, in fact, not only the quantity of the different lipoproteins in the blood that can increase the risk of specific illnesses, but also their composition. Analysing the NMR spectra of the extract obtained from the HDL fractions only, it was possible to highlight that cholesterol was not the only lipid that was altered. In patients with low cholesterol-HDL, it was found that sphingomyelins and phosphatidylcholines are also lower than for other patients, while triglycerides are higher. Furthermore, a lower degree of unsaturation in the fatty acids esterifying all the different classes of lipids characterise these patients [53];
- a simultaneous characterization of the metabolic profiles in the maternal, as well as cord blood samples, to increase the knowledge about foetal growth restriction. This condition, affecting up to 10% of pregnancies, can have consequences after the birth, in addition to causing severe problems to the foetus. Significantly lower plasma concentrations of cholesterol-intermediate density lipoprotein (IDL), triglycerides-IDL and HDL were found in mothers of growth-restricted foetuses compared to controls; on the other hand, growth-restricted foetuses had significantly higher plasma concentrations of cholesterol and triglycerides transporting lipoproteins, as well as increased VLDL particle types [54].

In Table 1, we have summarised all the applications presented throughout this review.

Table 1: Summary of the studies, where NMR spectroscopy has been applied to characterise lipids, presented in this review.

Authors	Application	Reference
Ligand-Protein Interaction		
de Vera et al.	Determination of Nurr1 protein conformations and interaction with binding fatty acids	17,21
Ragona et al.	Interaction between β-lactoglobuline and palmitic acid in a range of pHs	24
Patil et al.	Mechanism through which FABP1 mediates PPARα activation	25
Jones et al.	Interaction between arachidonic acid and odour binding protein 22 in *Aedes Aegypti*	26
^{1}H-NMR based lipidomics		
Ouldamer et al.	Dose-dependent changes in polyunsaturated fatty acids in lipid extracts from adipose tissue	27
Chen et al.	Decrease of hepatic triglycerides, following a dietary intervention with leucine	28
Melvin et al.	Deregulation of membrane constituent-lipids and the accumulation of energy storage lipids in fish contaminated from metals and metalloids	29
Khandelwal et al.	Lipid classes in extracts from rodent furs following the administration of a stearoyl-CoA desaturase 1 inhibitor	30
^{31}P-NMR based lipidomics		
Malmos et al.	Quantification of the different types of emulsifiers used for chocolate	32
Gouilleux et al.	Proposal and evaluation of two methods to measure absolute concentrations of phospholipids	34
Lipoprotein measures		
Harbaum et al.	Lipoproteins association with outcomes in patients with idiopathic and heritable pulmonary arterial hypertension	47
Holmes et al.	Association of lipoproteins and other blood metabolites with risk of incident myocardial infarction, ischemic stroke and intracerebral haemorrhage	48
Sliz et al.	Comparison of lipoprotein profiles in cases of statin treatment and genetic inhibition of PCSK9	49
Michielsen et al.	Comparison of lipoprotein profiles for diets with different fatty acids composition	50
Dias et al.	Comparison of lipoprotein profiles for diets rich in SFA and n-6PUFA, in people supplemented with eicosapentaenoic and docosahexaenoic acid	51
Sorokin et al.	Lipoprotein profiles in patients with psoriasis	52
Kostara et al.	Comparison of the composition of the HDL fractions in patients with low, normal or high HDL-cholesterol	53
Miranda et al.	Simultaneous characterization of the metabolic profiles in maternal and cord blood samples, to increase the knowledge about foetal growth restriction	54

5. Conclusions

Notwithstanding some limitations largely associated with sensitivity, NMR spectroscopy remains a powerful technique both to measure lipids and to characterise their physiological role in the cell, with many recent examples demonstrating the versatility of the technique. Furthermore, the continuous development of cryogen-free magnets is expanding the range of its industrial applications for quality control and including the measurement of lipids.

Competing Interests

The authors declare no competing interests.

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