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Title: Ins and outs of interpreting lipidomic results

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ABSTRACT

Membrane lipids are essential for life; however, research on how cells regulate cell lipid composition has been falling behind for quite some time. One reason was the difficulty in establishing analytical methods able to cope with the cell lipid repertoire. Development of a diversity of mass spectrometry-based (MS) technologies, including imaging MS, has helped to demonstrate beyond doubt that the cell lipidome is not only greatly cell-type dependent but also highly sensitive to any pathophysiological alteration such as differentiation or tumorigenesis. Interestingly, the current popularization of metabolomic studies among numerous disciplines has led many researchers to rediscover lipids. Hence, it is important to underscore the peculiarities of these metabolites and their metabolism, which are both radically different from protein and nucleic acid metabolism. Once differences in lipid composition have been established, researchers face a rather complex scenario, to investigate the signaling pathways and molecular mechanisms accounting for their results. Thus, a detail often overlooked, but of crucial relevance, is the complex networks of enzymes involved in controlling the level of each one of the lipid species present in the cell. In most cases, these enzymes are redundant and promiscuous, complicating any study on lipid metabolism, since the modification of one particular lipid enzyme impacts simultaneously on many species. Altogether, this review aims to describe the difficulties in delving into the regulatory mechanisms tailoring the lipidome at the activity, genetic, and epigenetic level, while conveying the numerous, stimulating, and sometimes unexpected research opportunities afforded by this type of studies.

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1. The stunning lipid multidiversity of cell membranes

There is no doubt that the formation of a lipid bilayer in an aqueous medium was one of the most crucial events in life evolution. The possibility of sustaining two different chemical environments enabled the appearance of the first protocells, which went on to evolve into current live forms. Although it would be hard to imagine how cells could be viable in the absence of lipids, it is surprising how both membrane lipid composition and its metabolism have often been simplified and overlooked. Fortunately, this scenario is changing and there is a growing understanding among researchers that membranes are not just barriers or static scaffolds, but that their composition and dynamics actually play a crucial role in cell physiology. In fact, according to the Lipid Maps Structure Database®, the membrane lipid repertoire consists of more than 40,000 unique lipid structures (Fig. 1), to which eukaryotic cells commit approximately 5% of their genes [1]. Bearing this in mind, the obvious question would be: if an isolating barrier can be generated using few, or even a single, phospholipid species, why do cells generate such a complex lipid repertoire?

For a long time, one of the factors accounting for the lack of advancements in lipid biology was the delay in the development of analytical techniques powerful enough to cope with lipid complexity. That is, in addition to being quantitatively numerous, many membrane lipid species are very similar to each other at the level of chemical structure, which increases the analytical challenge. At first glance, cholesterol and phosphatidylcholine (two common membrane lipid components) are clearly very different, both structurally and metabolically, and as such are easy to classify. However, on many occasions, differences between lipid components are much subtler. As shown below, any phospholipid or sphingolipid encompasses dozens of different molecules depending on the fatty acid(s) present in each specific lipid species. In some cases, differences might be due solely to the position of a simple double bond, such as between oleic acid (18:1n-9) and vaccenic acid (18:1n-7). Importantly, this *simple* distinction is frequently associated with very different biosynthetic pathways, and can also determine differences in tissue or cell distribution. All of this makes any lipid extract a complex mixture somewhat difficult to analyze, as many of the components are closely related at the chemical level. This complexity is one of the reasons, if not the main one, why lipid research has fallen behind compared to protein and gene research [2]. However, the systematic application of mass spectrometry (MS) methods to lipid analysis has changed this scenario, leading lipid research to the “omics” age, thus providing the ability to accurately investigate changes at the molecular level. This is of great importance, since no other analytical method (thin layer chromatography, gas chromatography, ELISA, nuclear magnetic resonance…) enables the simultaneous detection of thousands of lipids while acquiring detailed chemical structure (nature of acyl chains, position of double bonds, precise stereoisomery…). Furthermore, this rapid evolution in the field coincides with a sudden rise in the number of publications on lipids, which has increased 3-fold over the last two decades (from 20,000 in 1980 to 60,000 in 2018, according to PubMed).

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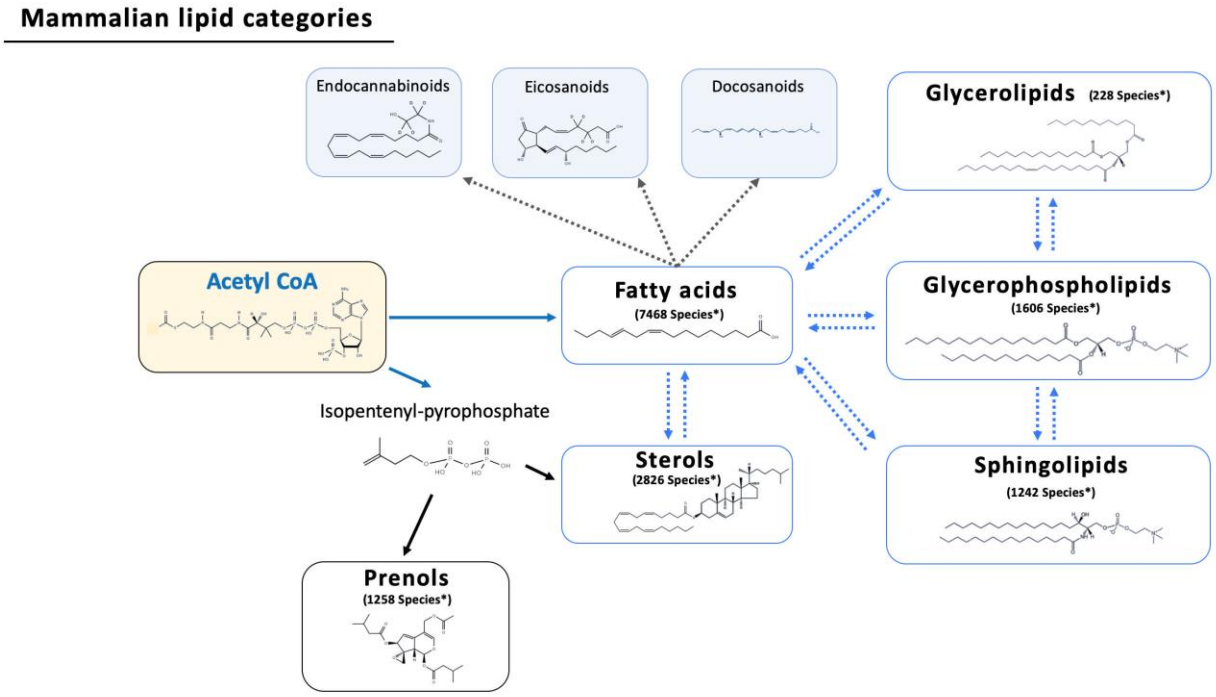

Figure 1. Mammalian lipid categories. Metabolic interactions between the relevant lipid categories in mammals (in white), including the number of lipid species defined so far in each class. The information on lipid species was obtained from the Lipid Maps Structure Database® (last accessed June 10th 2019) [3]. The rise of lipidomics has made it possible to establish a comprehensive classification including the following eight categories: fatty acids, glycerophospholipids (or phospholipids), sterols, sphingolipids, glycerolipids, prenol lipids, saccharolipids, and polyketides [4]. All these categories derive from a common precursor, acetyl-CoA (orange). In blue, three examples are given of fatty acid-derived molecules widely studied because of their role in cell physiology [5,6]. Herein, we focus only on the major lipid components present in mammalian membranes: phospholipids and sphingolipids. Readers may find a summary of the key features of these categories in the Supplemental Information. Those interested in the other categories are referred to the following reviews for more detailed information: sphingolipids [7], glycerolipids [8], sterols [9,10], prenol lipids [11], saccharolipids [4], and polyketides [12].

As if this were not complex enough, the issue has turned out to be even more complicated after the development of Imaging Mass Spectrometry (IMS) techniques. Thus, using state-of-the-art MS-based molecular imaging approaches, it has been possible to demonstrate how hundreds of different lipid species are precisely distributed within tissues according not only to the type of cell but also to its pathophysiological state (see Section 2, Fig. 2). These results corroborate what lipid analysts had previously stated after analyzing many cell type extracts: the cell lipidome profile would be equivalent to its ID, unique and entirely cell type-dependent. Together with the continuous improvement in MS techniques, there is no doubt that IMS images are helping to convince, day by day, those that still remain skeptical regarding the essential role that lipid diversity plays in cell sustainability. Unfortunately, our knowledge concerning how this specificity is achieved is rather scant and once lipidomic data are generated, researchers face something similar to a “death valley” when it comes to investigating the underlying molecular mechanisms and signaling pathways acting at the activity, genetic, and epigenetic level and accounting for the observed differences [13]. Hence, despite the fact that at least one gene has been attributed to almost all the steps in lipid synthetic pathways (except for

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plasmalogen and LBPA synthesis) [14], there is a shortage of information as to how any of these enzymes are regulated at both the activity and transcriptional level.

The main aim of this review is to explain the complexity and challenges of investigating the causes of the altered lipidome at the molecular level. As is discussed below (see Fig. 7), even if only two lipid species are affected (which is rarely the case), the number of enzymes involved in regulating their levels increases rapidly, hence the difficulty of these studies. Although herein we focus our discussion on membrane lipids, conceptually all the points raised apply – to a greater or lesser extent – to any lipid category or life form (bacteria, yeasts...). Before turning to the substance of the review, we first devote the next section to IMS techniques, a relatively new method which has turned out to be particularly suitable for lipid analysis [13].

2. Multidiverse yet strikingly specific: characteristics of the lipidome clearly *visualized* by IMS

Mass spectrometry is an analytical technique that enables the identification of chemical species according to their mass-to-charge ratio (m/z). Briefly, the sample is ionized with the conversion of the molecules into gaseous ions, with or without fragmentation, and characterized by their m/z. Initially (60's-70's), only a few lipids were amenable for direct MS analysis as they did not ionize in those conditions. The introduction of fast atom bombardment ionization changed this scenario [15] and non-volatile lipids could be directly analyzed [16]. Yet, it was the development of electrospray ionization (ESI) [17] and MALDI [18] which made the definitive contribution to being able to analyze a large number of biological lipids by MS. Currently, there are two major "-omic" strategies to characterize the lipid composition of any fluid sample (including tissue homogenates): shotgun lipidomics, and chromatography based lipidomics (LC-MS and tandem LC-MS/MS). Targeted chromatography could be somehow considered as a third strategy, although it is in fact a variation of the above, typically carried out in the chromatography based-mode [19]. It is beyond the scope of this review to deal in depth with the numerous MS techniques developed so far, and for this reason readers are referred to numerous reviews covering this topic [20–24]. In any event, each of the strategies has its own advantages and disadvantages, and the type of strategy to follow depends very much on the aim of the study. Nevertheless, it is most important to bear in mind the limitations of each methodology, as there is no single method able to detect, measure, and provide full chemical structure information of all lipid species at once. That said, it would be rather safe to say that chromatography based-MS (or MS/MS) techniques are the best in terms of high sensitivity and specificity, high throughput, and high accuracy; furthermore, regarding lipid analysis, they are the most precise in the identification of lipid species [25]. The success of these methodologies has resulted in a surge in the number of lipid species identified and characterized, leading consequently to an increase in research lines in which lipids are the aim of study.

However, once lipids are extracted, the possibility of establishing a relationship between tissue morphology and composition vanishes, leading to the loss of very valuable information. It is precisely this aspect that IMS techniques overcome, by enabling researchers to describe the two- and even three-dimensional distribution of hundreds of analytes [13]. Importantly, in contrast to other imaging techniques, such as immunohistochemistry, IMS is a label-free and non-invasive approach. Hence, IMS allows the

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multiplexed detection and localization of biological and synthetic molecules (proteins, peptides, lipids, metabolites, drugs, etc.) by acquiring position-correlated spectra along a tissue section [26]. Once spectra are acquired, specialized software transforms the variable "peak intensity" into a pixel, which is colored according to a scale, one per coordinate. Finally, once all pixels are generated and placed at their coordinates, a final image of the compound distribution within a tissue section is generated (Fig. 2C).

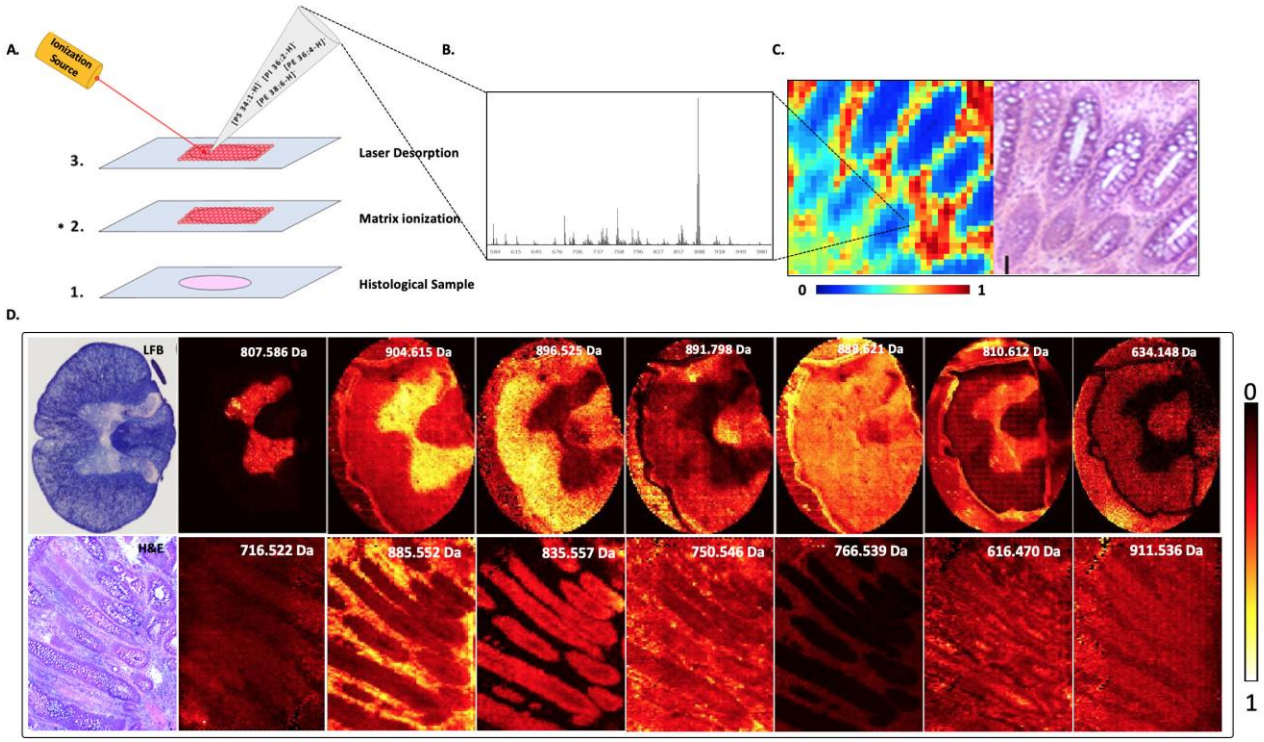

Figure. 2. Simplified workflow for fresh frozen tissue sample IMS analysis. A) Sample pretreatment steps include cutting (1) and mounting the tissue section on a slide (3). The ionization source can be an ion beam (in SIMS), a laser (in MALDI), or a solvent of charged droplets (in DESI) [19,20]; B) Mass spectra are generated at each x,y coordinate. C) The intensity of each m/z peak is integrated, and the value is represented according to a color scale vs. the coordinates where the spectra were recorded, allowing the visualization of the distribution of a single molecule within the tissue (image). Hence, each image is generated by the addition of thousands of recorded spectra (one per pixel). In turn, each pixel is associated to a complete chromatogram containing 200-300 peaks of different intensities; D) IMS analysis of a rat spinal cord section and a human colonoscopic biopsy. First column: hematoxylin and eosin staining of a consecutive section included for comparison. The remaining columns show MALDI-MS images of selected lipid species obtained after analysis of a rat spinal cord section in positive-ion mode and at 25 µm of spatial resolution, and of a human colonoscopic section in negative-ion mode at 10 µm of spatial resolution. *Only in the case of MALDI (Matrix Assisted Laser Desorption/Ionization) techniques is a matrix (chemical compound) applied to the tissue section (2). Adapted from [27]

Unexpectedly, the combination of soft-ionization and MS methods turned out to be particularly suitable for lipid analysis. Currently, it is possible to obtain images of hundreds of lipids species at once with a spatial resolution that may range from micrometers to nanometers depending on the specific technique (Fig. 2D) [13]. This has resulted in numerous studies identifying specific lipid-fingerprints for a wide range of tissues, including brain [28–34], spinal cord [35], lung [36–39], kidney [40,41], colon [42–45], prostate [46], breast [47–49], thyroid [50], ovary [51], placenta [52], arteries [53], skin [54], pancreas [55], bone [56], and retina [57] in both healthy and pathological conditions, such as cancer and neurodegenerative diseases. The findings of all these studies, mostly focused on identifying differences at the composition level, confirm that the high

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specificity of the lipidome may certainly be used to classify a cell according to its origin and its pathophysiological state, reinforcing membrane lipids as excellent biomarkers [58]. However, a thorough and comprehensive analysis of IMS generated data can also be used to directly study complex processes such as differentiation, tumorigenesis, and chronic inflammation. The unexpected results obtained while studying human colon lipidome help to illustrate this concept well.

Colon epithelium consists of a single monolayer of colonocytes that invaginates into the stroma, generating the functional units called crypts (Fig. 3). At the bottom of these structures reside the adult stem cells that divide and differentiate into fully mature colonocytes while ascending along the crypt. Thanks to spatial resolution achieved during the IMS analyses (10 μm), it is possible to follow, pixel by pixel, the changes occurring in the lipidome along the colon crypt [27,42,43]. These analyses revealed how precisely a very specific set of lipids changes along the colon crypt (Fig. 2D, 3) [27,43]. Impressively, this variation fits a mathematical equation, that is, it is possible to establish the percentage of a particular molecular species merely by knowing the position of the colonocyte in the crypt, providing the best proof that the lipidome is regulated by much stricter molecular mechanisms than could initially have been expected. Furthermore, taking into account the fact that colon epithelium consists of a single cell monolayer and that we can clearly "see" its lipidome, these results would indicate that cell resolution level was reached during the analysis. This is critical nowadays, a time when a great deal of effort is being devoted to single-cell "multi-omics" [59]. Altogether this example shows that it might be worth revisiting IMS results from time to time as, on many occasions, the biological information they enclose might not be easily appreciated at first glance.

There is no doubt that MS techniques have enabled us to take a firm step forward in our global understanding of lipids, prompting the field towards rapid evolution. In particular, IMS images break definitively with the general belief of a certain ambiguity in the molecular mechanisms defining lipid composition, and certainly escape from the homogenous "head and tail" scheme commonly used to represent cell membranes. Furthermore, IMS provides novel and crucial information as to how lipid species are accurately distributed within tissues. However, it also leaves many unsolved questions, since with our current knowledge of lipid metabolism regulation it is still difficult to envision the cell machinery underlying this precision (sections 3.2, 3.3) [13].

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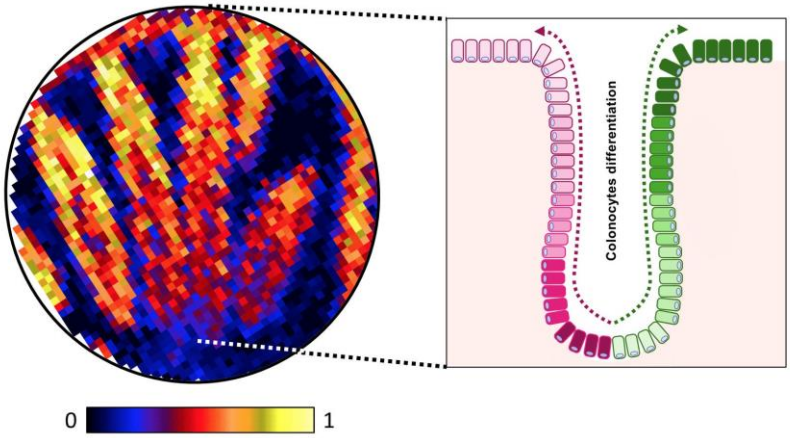

Figure. 3. Strict regulation of phospholipid metabolism unveiled using IMS techniques. The detailed analysis of the results obtained during the IMS analysis of human colon sections shows that the level of a subset of lipids, in particular phosphatidylinositol and phosphatidylethanolamine plasmalogens, change concomitantly with the differentiation process. On the left, one of the images obtained during the analysis in which it can be clearly observed how the signal intensity generated by a particular molecular species steadily increases, pixel by pixel, along the colon crypt; on the right, a diagram of the crypt representing the changes in lipid composition. Lipids containing mono-unsaturated fatty acids increase linearly from the bottom to the top of the crypt according to a first-degree equation (y=ax+b, R^2=0.95-0.98, in green), while those containing arachidonic acid decrease according to a logarithmic equation (y=-ln(x)+b, R^2=0.95-0.98, in pink) [27,43].

Given the obvious potential of these techniques, there is no surprise a large number of applications have been developed to exploit their possibilities, which clearly go beyond the mere characterization of a tissue. One clear example is the application of IMS to study drug metabolism and its impact on cell physiology. Thus, in addition to assessing the impact of a drug (cocaine, morphine, amphetamine [30,60]) or pollutants (bisphenol-S [61]) on tissue composition, IMS offers a label-free method to assess drug levels at therapeutic concentrations, which may be free or included in a carrier distribution [62,63]. Within this context, IMS in combination with other analytical techniques have been used to assess absorption profiles along the crypt-villus axis of compounds differing in permeability [64]. This is particularly important in this field, as the way and location where molecules are absorbed may determine the final pharmaceutical formulation, dosage, etc. Currently, efforts are focused on improving IMS techniques to be able to describe the lipidome at the single-cell level, to reach subcellular resolution, and even to generate three-dimensional lipidomes [65–67]. The latter has already been possible using high-resolution MALDI-IMS to analyze newly fertilized individual zebrafish. In this beautiful study, the authors showed a subcellular segregation amid the major lipid families within the fertilized egg [68]. Similarly, using SIMS (Secondary Ion Mass Spectrometry) – a different technique based on IMS – it was possible to differentiate lipid domains during cell-to-cell conjugation [67].

However, IMS shares the limitations common to all MS techniques based on direct analysis, that is, with no chromatographic pre-separation. Thus, none of these techniques are capable of differentiating between isobaric species (molecules species with iso-elemental composition but structurally different). In most of this equipment, ions can be further fragmented (MS/MS), and with good collision chambers some

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structural information, such as the position of double bonds, can be faithfully established. In any event, this is a rapidly evolving area and great efforts are being made to develop the necessary technology in order to analyze complex lipid samples with the same structural resolution as chromatographic-based MS [69,70]. Eventually, all these advances can be and are coupled to IMS, and some recent examples include: IMS coupled to gas-phase ion mobility spectrometry [71], and IMS coupled to CARS (coherent anti-Stokes Raman spectroscopy) microspectroscopy [72]. An additional aspect for concern is how good IMS is in terms of the limit of detection. In this sense, there are several factors to consider: the abundance and stability of the analyte, its ionization ability, and ion suppression events, which may diminish the ability to detect minor lipid species. There are different strategies to address the detection of minor or unstable species, which often involve some type of derivatization process, such as a reaction with metallic ions for prostaglandin detection [73]. Despite all these limitations, the information existing on lipidome distribution within tissues and its response to pathological insults is scarce, and therefore, IMS techniques provide a unique means to generate rather novel information.

3. Interpretation of lipidomic results

Regardless of the high-throughput technique used to establish the lipidome, the amount of data generated after the analysis of a sample is vast and complex. Then, these data can be analyzed using a diversity of bioinformatic approaches that will finally provide a list of specific lipid species. If the study aims to establish biomarkers, then these species would need to be validated in larger cohorts and, eventually, could be used in daily practice of healthcare facilities. However, if the aim is to understand the biological implications of those changes, then harder questions to address would arise: 1) What is the physiological impact of the changes described? and 2) What are the metabolic/signaling pathways underlying these changes? In the following sections, we tease out some of the reasons why there are no straight and simple answers to these questions.

3.1. Assessing the potential impact of changes in the membrane lipidome

To understand how changes in the membrane lipidome can affect cell physiology, first of all it is important to remember how membranes are established and some of their chemical-physical characteristics. The plasma membrane is a ~4 nm thick phospholipid bilayer established because of the amphipathic nature of phospholipids and sphingomyelin (Figs. 4 and 5). Hence, in aqueous solutions, the presence in their structure of hydrophobic (fatty acid tails) and hydrophilic (head groups) domains induces the spontaneous self-association of the fatty acid tails. In this way, the total surface in contact with water is minimized, establishing a hydrophobic core, while polar head groups directly face the aqueous environments. This key feature allows biological membranes not only to segregate the intracellular milieu into sub-compartments (organelles), but also to provide the hydrophobic environment needed for the activity of transmembrane proteins.

Table 1 shows how specific lipid composition is in terms of lipid categories, depending on the tissue or subcellular organelle. However, this specificity goes beyond tissue identity, as each of the subcellular organelle membranes shows particularities at the compositional level [74]: whereas sphingomyelin is barely present in

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the endoplasmic reticulum (ER), cardiolipin is exclusively found in mitochondria [1,75]. In fact, there is a compositional gradient in lipid composition with a profound functional impact along the secretory pathway. Taking into account the fact that most lipids are synthesized in the ER, it is clear that there should be a wide variety of molecular mechanisms able to target and concentrate individual lipids at specific cellular locations [75]. A lack of location motifs, like the ones existing in proteins, complicates the study of how cells regulate the lipid composition within the complex network of membranes (ER, nucleus, plasma membrane, mitochondrion…). Regulation of the specific organelle lipidome can be carried out through at least three established processes: 1) cytosolic lipid transfer proteins [76], which are hydrophilic transporters with a hydrophobic compartment where lipid class monomers bind (e.g. ceramide transfer protein (CERT, [77–79]) or PC-specific transfer proteins [76,80]); 2) transport of lipid vesicles, independent of protein trafficking, which has been described at least for PC and PE [81–83]; and 3) membrane contact sites, which are multiprotein complexes enabling specific lipid transport, and they are particularly important for PS and PE transport between ER and mitochondria [84–86]. Even though these processes have been described as mechanisms of transporting newly synthesized lipids to the organelles, traffic in the opposite direction has also been described, which complicates the understanding of the net lipid contribution of each donor membrane to the receptor organelle [86]. In turn, the same cell organelle may show differences in composition according to its tissue origin. For example, it is common to find in the literature that cardiolipin fatty acid composition is rather homogeneous, remarkably enriched in linoleic acid (18:2n-6). However, this applies to heart and liver cardiolipin – wherein this fatty acid accounts for approximately 70% and 90% of total fatty acids, respectively – but not to brain cardiolipin, where it accounts for only 10% [87]. This implies that, somehow, cells have been able to develop different lipid signatures while subserving a common function.

Although it was initially assumed that lipids were randomly distributed within the membrane leaflet, and were incapable of adopting a coherent lateral structure, we now know that at least two additional organization levels can be established: 1) between the two leaflets, so-called transversal asymmetry; and 2) within each leaflet, i.e., formation of microdomains (lateral asymmetry). Transversal asymmetry refers to the differences in composition existing between the two leaflets, and modulates various bilayer properties, including membrane potential, surface charge, permeability, and shape, as well as stability [92]. In agreement with these critical functions, asymmetry is maintained by a complex enzymatic network, consisting of flippases (out-to-in), floppases (in-to-out), and scramblases (bidirectional), which mediate lipid translocation [93]. One of the most studied examples is phosphatidylserine translocation to the outer leaflet, a process that acts as a susceptibility signal and which is involved in processes as diverse as blood clotting [94], membrane trafficking [95], apoptosis [96], and cancer [97]. In most characterized eukaryotic cells, phosphatidylcholines and sphingolipids are found in the extracellular leaflet, whereas phosphatidylserines, phosphatidylethanolamines, phosphatidylinositols, and phosphoinositides are preferentially restricted to the cytosolic leaflet [98,99]. Yet the latter applies basically to the plasma membrane, as within any cell, there exists a vital gradient in transversal asymmetry along the secretory pathway, from the ER (symmetric and loosely packed) to the plasma membrane (asymmetric, thick, and rigid). This asymmetry is achieved by increasing the content in sterols, sphingolipids, and phosphatidylserine, which has a definitive impact not only on membrane thickness but also on membrane electrostatics [1,14,75]. The molecular mechanisms and signaling pathways regulating

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all these processes, involving many different specific lipid-protein interactions, are complex and the object of active research [100–102] .

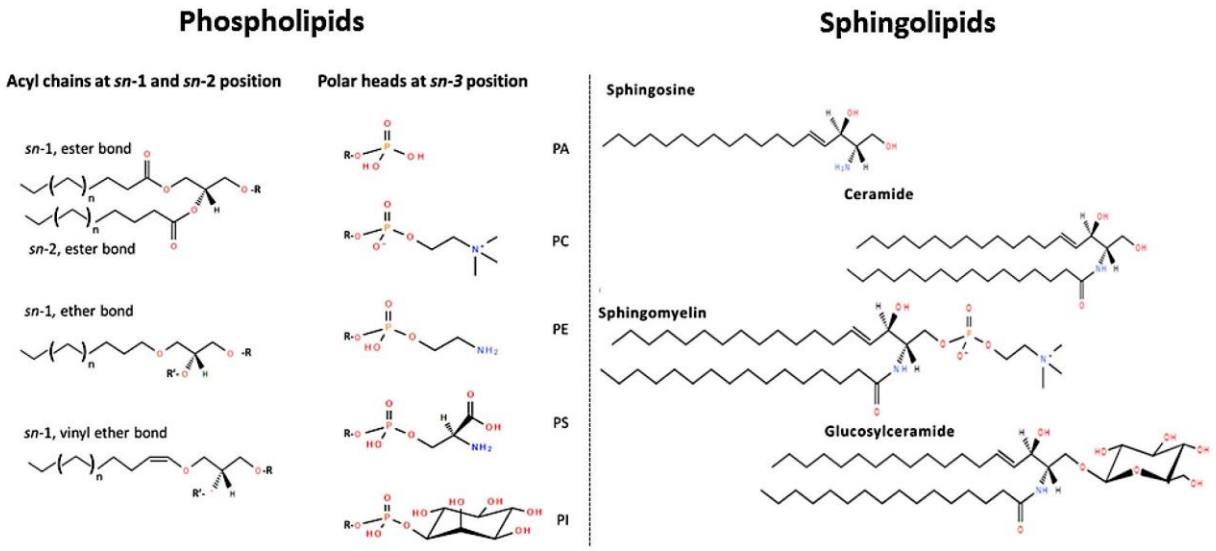

Figure 4. Structural elements of glycerophosphate- and sphingoid-based lipids. A) Glycerophospholipids. On the left, the different types of linkage established at sn-1 position between the fatty acid and the glycerophosphate backbone are shown. On the right, the most common polar heads found in mammalian cell membrane: phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI). Other less abundant or organelle specific lipid classes are phosphatidylglycerol (PG), cardiolipin (CL, exclusive of mitochondria), and bis(monoacylglycerol)phosphate (BMP, enriched in late endosomes). On the left, R: refers to polar head moiety; R': fatty acid moiety; on the right, R refers to glycerophosphate moiety. **B) Sphingolipids**. The most frequent sphingoid base is sphingosine, although there are also other structures such as sphinganines and 4-hydroxysphinganines. Depending on the polar head attached to the ceramide, different molecules are generated, such as sphingomyelins (phosphocholine), cerebrosides (glucose, galactose…), and gangliosides (oligosaccharides or sialic acid). In terms of the fatty acyl chain, in sphingolipids it is highly common to find saturated or monounsaturated fatty acids, which often contain a hydroxyl group at C2 position. Table S1 includes a summary about the nomenclature of fatty acid-containing lipid categories.

A final concept that needs introducing is the intrinsic molecular geometry of membrane lipids, which is mainly determined by the size of the head group and acyl chain composition (Fig. 5). Taking the size of the head group into account, phosphatidylcholine and phosphatidylserine adopt a cylindrical form; phosphatidylethanolamine and phosphatidic acid assume a conical molecular geometry because of the relatively small size of their polar head group; and lysophospholipids (lacking a fatty acid) and phosphoinositides (having an inositol ring) have the shape of an inverted cone. In addition, fatty acid composition does have a certain impact on membrane conformation as well. For instance, the presence of a double bond induces the straight chain to bend, thereby increasing the space it occupies. Hence, the combination of molecules with different geometry within the bilayer imposes membrane defects and a certain curvature stress, which is used for budding (vesicles), fission (during cell division), and fusion (e.g. during secretory pathway) events [103,104]. There are many examples demonstrating that cells do modify local lipid composition in order to regulate these processes. The best characterized might be the regulation of the

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phosphoinositide phosphorylation state in the context of synaptic vesicle exocytosis and recycling [105–107]. However, synaptic vesicle dynamics have also been shown to be regulated by sphingomyelin [108], plasmalogens [109], and PUFA [110] content, all highly enriched in neural structures. Interestingly, growing evidence indicates that lipid synthetic enzymes are able to sense membrane defects and use this cue to regulate their activity. A nice example of the latter is cytidine triphosphate:phosphocholine cytidylyltransferase (CCT; Fig. 6), the rate-limiting enzyme in phosphatidylcholine biosynthesis, which is able to adapt its activity according to the level of cylindrical or conical phospholipids present in the membrane [111,112].

Finally, lipid biophysicists were able to describe the separation of phases within model lipid bilayers, leading to the concept of membrane domains and to the possibility of their existence in living cell membranes [113]. Over the years, different types of membrane domains have been described, such as caveolae [114–116], clathrin-coated pit [116,117], ceramide platforms [118], and lipid rafts [119]. The latter, established in the outer leaflet, are defined as dynamic sterol-sphingolipid-enriched assemblies. Because of the specific lipid-lipid, protein-lipid, and protein-protein interactions occurring within lipid rafts, these domains have been proposed as signaling pathway regulating platforms [113]. Furthermore, it seems that, somehow, lipid rafts influence the organization of inner leaflet-associated proteins during signal transduction [120,121], suggesting the existence of inter-leaflet communication [92].

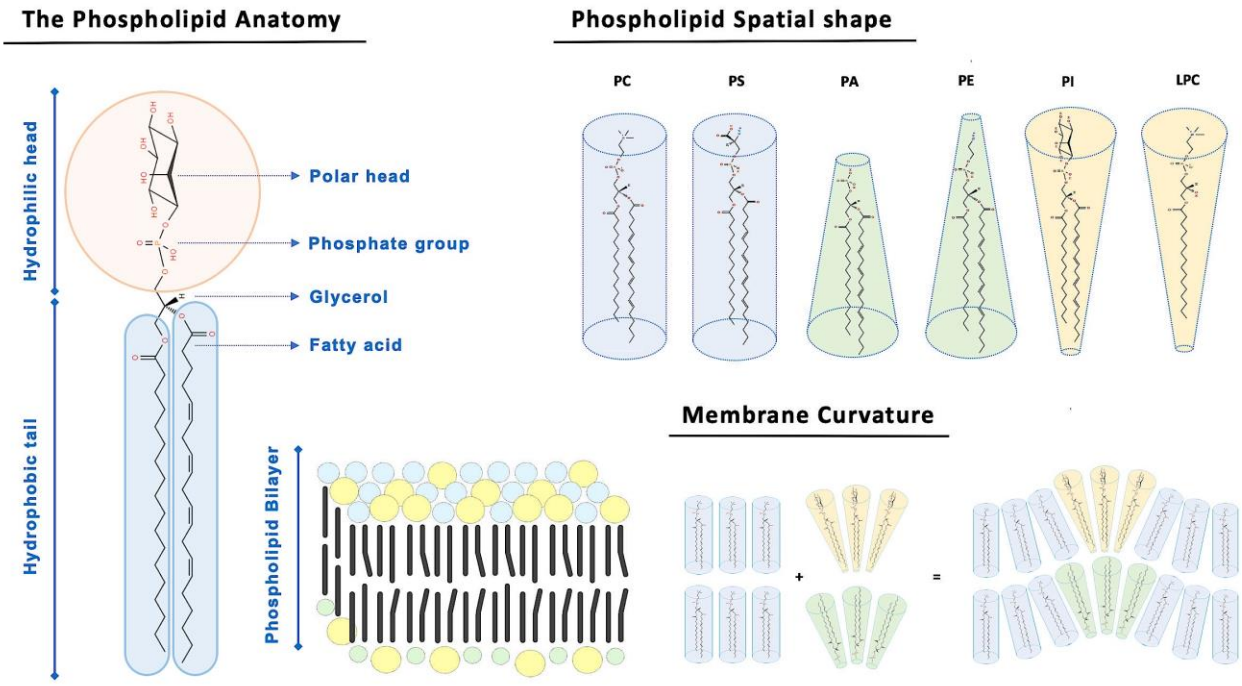

Figure 5. Impact of lipid composition on membrane biophysical properties. Membranes are established because of the amphipathic nature of their components, particularly phospholipids and sphingomyelin. Lipids are asymmetrically distributed between the two leaflets and, in turn, within each of the leaflets. Each phospholipid has a particular shape depending on the polar head and its fatty acid composition. Lipid composition determines many properties of the membrane, such as thickness, fluidity, and surface charge. Thus, membrane thickness can be modified by altering its composition. Thus, while short, unsaturated fatty acids increase fluidity, saturated fatty acids and sterols decrease it. Conversely, surface charge is determined by the presence or absence of anionic lipids,

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such as phosphatidylserine (positively charged) and phosphoinositides (negatively charged). Finally, changes in phospholipid intrinsic geometrical shape also impact membrane curvatures and regulate vesicle formation [75,122].

All together, a rather complex scenario is depicted in which, in addition to all the regulatory molecular mechanisms acting on lipid synthetic pathways, the membrane lipidome is also regulated between cell organelles, membrane leaflets, and even within each individual leaflet. This complexity makes it rather difficult to take all these elements into account in a single experiment. Although significant improvements have been made in this sense, such as the generation of asymmetric model membranes [92], it is still not possible to reproduce in vitro the complexity of the cell membrane at the composition level; therefore, fully evaluating the real impact of an altered lipidome on cell functioning is virtually impossible.

As is well known, three roles are commonly ascribed to lipids: building blocks, signaling molecules, and energy molecules. So, traditionally, changes in phosphatidylcholine or phosphatidylethanolamine levels are rapidly associated to modifications in the biophysical properties of the membrane; while changes in arachidonic acid are usually linked to alterations in signaling pathways. However, we now know that when a phospholipid is hydrolyzed, any fatty acid released (saturated, mono- or polyunsaturated) is susceptible to being transformed into signaling molecules (e.g. eicosanoids, docosanoids, endocannabinoids…) or to binding many transporters (e.g. CD36 in palmitic acid mediated metastasis [123,124]) or cell receptors (e.g. PPAR, RXR nuclear receptors [125], Toll like receptor [126]), thereby affecting important signaling pathways. In addition, many receptors have also been described for the second product of hydrolysis, i.e., lysophosphatidyl moieties [127–129]. Moreover, there is no doubt of the impact on cell physiology of modifying the unsaturation index, cholesterol levels, or polar head composition of any biomembrane. As knowledge on lipids advances, the boundaries between these two fundamental roles are becoming less defined, particularly with regard to phospholipids and sphingolipids. Hence, we consider that currently all membrane lipid species should be envisioned as entities of a dual nature (structural/signaling) in terms of the roles they perform in the cell, and that no function should be considered without the other.

3.2. How were these changes achieved? Differential features of membrane lipid metabolism

As seen above, understanding the impact of lipid changes is no easy task. In this section, we explain why the answer to the question of "what are the molecular mechanisms underlying these changes?" is neither straightforward nor simple, and the reasons are intimately related to the intrinsic characteristics of lipid metabolism. The first aspect to take into account is that, generally speaking and compared to proteins, lipid species are not a stable end product. Although proteins may undergo certain post-translational modifications, the primary sequence is not altered, unless the protein is degraded or mutated. In lipids, the scenario found is very different. Thus, lipids are made up of highly interchangeable modules: in the case of phospholipids, the head group (including the phosphate group or not) and each of the esterified fatty acids; and in the case of sphingolipids, the head group (Fig. 4). These modules are subjected to remodeling reactions with kinetics that are much faster than is commonly assumed. It is worth stressing that most of these reactions are reversible

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and highly interconnected, so lipid metabolism may be pictured as communicating vessels with a rather high transfer rate. Unfortunately, the kinetics of these remodeling processes is one of the most difficult aspects to address during the study of lipid metabolism, and are therefore often underestimated (Fig. 6) [130]. A clear example of this interconnectivity between lipid classes was recently described in a model of the maturation process of intestinal stem cells. While studying the role of phospholipid fatty acid remodeling in this process, the authors observed that the cholesterol synthetic pathway was also greatly affected during intestinal stem cell differentiation [131].

A **second feature** to consider is the **concept of substrate specificity,** which is very different from other disciplines such that, generally speaking, lipid enzymes are rather promiscuous with regard to substrate preference. For instance, arachidonic acid (20:4n-6) was considered for decades to be the specific substrate of cyclooxygenase 1/2 and LOX, enzymes catalyzing prostaglandin and leukotriene synthesis, respectively [132]. Now we know that these enzymes are able to cycle PUFA other than arachidonic acid, including docosahexaenoic acid (22:6n-3) and eicosapentaenoic acid (20:5n-3), producing a wide range of anti-inflammatory compounds [133–135]. Thus, COX isoform inhibition affects the synthesis of many molecules with opposite effects involving a large number of signaling pathways. A second illustrative example is tafazzin, a mitochondrial acyltransferase that enriches cardiolipin with linoleoyl (18:2n-6) chains, even though it does not exhibit substrate specificity for particular acyl chains [136]. This is a common characteristic of many lipid enzymes and, quite often, in vitro studies do not fully support the functional specificity displayed in vivo, indicating that more elements should be taken into account so as to explain the precision in terms of lipid species distribution.

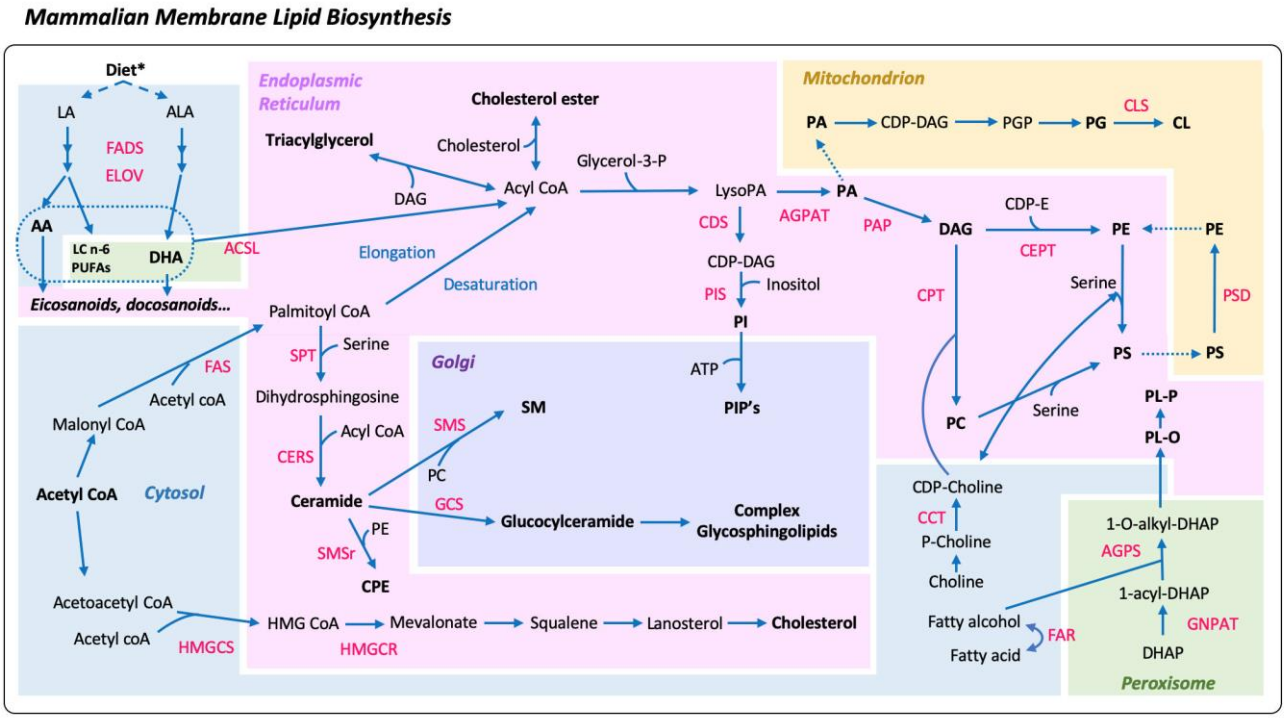

Figure 6. Principal pathways in membrane lipid biosynthesis in mammals. Phospholipids and triacylglycerides can be synthesized de novo from common diacylglycerol or phosphatidic acid precursors by the so-called 'Kennedy pathway' [137]. Briefly, phosphatidic acid is synthesized by the successive addition of two fatty acyl-CoAs (fatty acid activated form) to glycerol-3-phosphate, a product of

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glycolysis. Then, phosphatidic acid may be converted into CDP-diacylglycerol, which is used in the synthesis of phosphatidylinositol and cardiolipin, or dephosphorylated to diacylglycerol for the synthesis of phosphatidylcholine and phosphatidylethanolamine as well as triacylglycerol [138]. Phosphatidylethanolamine – choline and serine polar heads – may be interconverted by exchange, decarboxylation, and methylation reactions [139]. The equilibrium between phosphatidic acid (PA) and diacylglycerol (DAG) is a metabolic hub linking the synthesis of major phospholipids and triacylglycerides. However, the module with the highest modification rate is fatty acid moiety, and both acyltransferases (see section 3.3.3) and phospholipases are critical for this [140–143]. **AGPAT**, 1-acylglycerol-3-phosphate-O-acyltransferase; **AGPS**, alkylglycerone phosphate synthase; **ALA**: alpha-linolenic acid, **CCT**, CTP: phosphocholine cytidylyltransferase; **CDP-Choline**, cytidine diphosphate choline; **CDP-DAG**, cytidine diphosphate-diacylglycerol; **CDP-E**, cytidine diphosphate ethanolamine; **CDS**, cytidine diphosphate diacylglycerol synthase; **CEPT**, choline/ethanolamine phosphotransferase; **CERS**, ceramide synthase; **CL**, cardiolipin; **CLS**, cardiolipin synthase; **CPE**, ceramide phosphoethanolamine; **CPT**, cholinephosphotransferase; **DHAP,** dihydroxyacetone phosphate; **ELOVL** Fatty Acid Elongases; **FADS**: fatty acids desaturases; **FAR**, fatty acyl co-A reductase **FAS**, fatty acid synthase; **GCS**, glucosylceramide synthase; **GNPAT**, glyceronephosphate O-acyltransferase; **HMGCS**, hydroxymethylglutaryl coenzyme A (CoA) synthase; **HMGCR**, 3-hydroxy-3-methylglutaryl-CoA reductase; **LA**: linoleic acid; **PA**, phosphatidic acid; **PAP**, phosphatidic acid phosphatase; **P-Choline**, phosphocholine; **PG**, phosphatidylglycerol; **PGP**, phosphatidylglycerolphosphate; **PIP**, phosphoinositide; **PC**, phosphatidylcholine; **PE**, phosphatidylethanolamine; **PIS**, phosphatidylinositol synthase; **PS**, phosphatidylserine; **SMS**, sphingomyelin synthase; **SMSr**, sphingomyelin synthase-related enzyme; **SPT**, serine palmitoyltransferase. * Essential fatty acids; LA (n-6) and ALA (n-3), mammals cannot insert double bonds more proximal to the methyl end than the ninth carbon atom (D-9 desaturase), n-3 and n-6 fatty acids cannot be synthesized de novo, consequently these fatty acids have to be present in the diet [144]. For simplicity, lysophosphadylacyltransferases are not included. Adapted from [75].

The third characteristic to contemplate is that many lipid activities are redundant, i.e., there are many enzymes sharing the same activity despite the fact that they might be quite different at the structural level. Whereas in physiological conditions these activities may not overlap, it is quite common to find compensatory reactions in inhibitory scenarios, such as pharmacological inhibition or in gene knock out models, which often lead to either puzzled results or, even worse, to an apparently regular phenotype REF DAGT1/2[14]. An additional drawback is that knocking out some key lipid synthetic enzymes, particularly those involved in the de novo synthesis of phospholipid or sphingolipids, is often embryonically lethal (e.g., choline kinase [145], phosphatidylserine decarboxylase [139], sphingomyelin synthases [146], and fatty acid elongase 6 [147]). Last but not least, it should not be forgotten that the levels of each of these modules are, in turn, dependent on the activity of any of the lipid enzymes participating in their catabolic and de novo synthetic pathways, and maybe at any of the different regulatory levels (post/transcriptional, post/translational [125,148,149], epigenetic [150,151], or physical cues such as osmotic stress [152,153]).

At a practical level, the last important element to consider is the model used for studies on lipid metabolism. In in vitro studies, using either primary or commercial cell lines, the lipidome is very sensitive to the serum used to complement cell medium, cell confluence, physiological stage of cells, cell passage, and, obviously, precise cell type [154].

3.3. Investigating the molecular mechanisms underlying “PI 36:2” changes could lead to very different experimental designs

To understand the importance of correctly establishing the exact structure a lipid species, we could imagine that our MS analysis gave as a result a profound impact on PI 36:2 levels and that, unfortunately, it was not possible to obtain additional information regarding fatty acid moieties. In fact, this is a rather simplified scenario, as lipidomic analysis usually generates a relatively long list of lipids. In this particular

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example, it would be safe to consider that PI 36:2 encompasses at least two different molecules: PI 18:0/18:2n-6, and PI 18:1 n-9/18:1 n-9 (see Table S1 for different ways to abbreviate lipid molecular species [155]). Figure 7 summarizes the lipid enzymes that could, allegedly, be directly involved in the regulation of these two species levels. Whilewe could, in both cases, investigate whether de novo PI synthesis was affected, the origin of 18:2n-6 (an essential fatty acid) and of 18:1n-9 (product of 18:0 unsaturation) is radically different (Fig. 6). In the first case, we could investigate n-6 fatty acid metabolic pathways, in which members of fatty acid desaturases, fatty acid elongases, or COX enzyme families would be worth studying. Conversely, in the second case (18:1), an interesting enzyme to investigate would be SCD1. However, strictly speaking, the scenario is even more complicated because 18:1 and 18:2 have alternative isomers, which could be at the sn-1 rather than at the sn-2 position, and other fatty acid combinations such as 16:0/20:2 and 16:1/20:1 are also possible. In general, these species are much less abundant, but as analytical techniques improve, it is becoming more frequent to see them in lipidomic reports. Thus, in addition to obtaining the lipid structure as accurately as possible, the most important thing is to obtain complementary evidence, using different experiments, to support the initial hypothesis and always bearing in mind that, except for a few exceptions, a “black-and-white” situation is rather unusual when studying lipid metabolism. In the following subsections, a brief comment is made concerning some of the most relevant aspects of the enzyme activities participating in membrane lipid turnover which could be directly involved in the “first line” maintenance of these two particular lipids species: acyl-Co synthases (ACS, section 3.3.1), phospholipases (section 3.3.2), and acyltransferases (section 3.3.3), updating existing information. While phospholipases have been studied for many years, ACS and acyltransferase were activities always included in the diagrams but poorly studied. Readers may find many excellent reviews and books covering phospholipid metabolism extensively [2,7,14,75,88,156].

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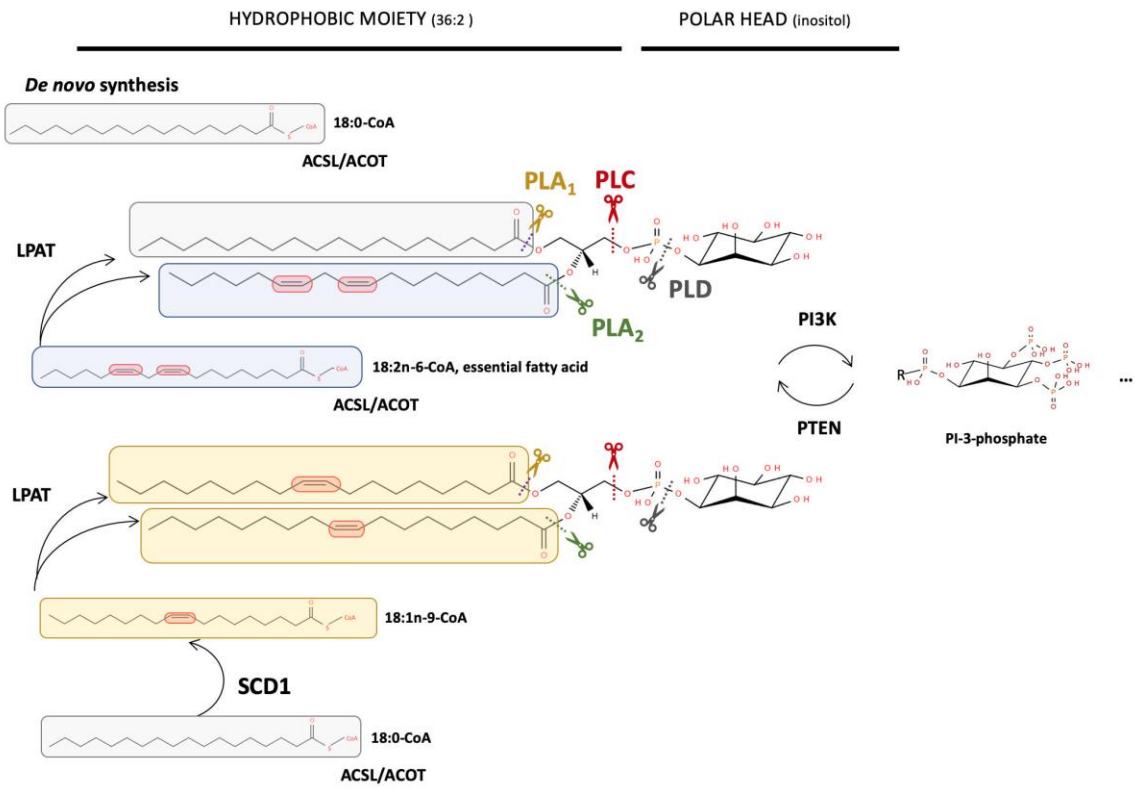

Figure. 7. Importance of accurately establishing the precise structure of a lipid species. This diagram summarizes the lipid enzymes that would be directly involved in the regulation of these two species, namely, PI 18:0/18:1 and PI 18:0/18:2. In turn, each of these enzymes may have different isoforms whose expression might be cell type- or substrate specific. Finally, like any other protein, they are the target of gene expression programs, which determine lipid species levels in the end, which on most occasions have not yet been identified. **ACSL**: long chain acyl-CoA synthase; **ACOT**: Acyl-CoA thioesterases; **LPAT:** lysophosphatidyl acyl transferase, **PI3K:** phosphatidyl inositol 3-kinase, **PLA1:** phospholipase A 1, **PLA2:** phospholipase A2, **PLC:** phospholipase C, **PLD:** phospholipase D, **PTEN:** phosphatidylinositol-3,4,5-triphosphate 3-phosphatase, **SCD1:** stearoyl-CoA desaturase-1, involved in the conversion of saturated into monounsaturated fatty acid.

3.3.1. Acyl-Coa synthetase, effectively "trapping" fatty acids within cells

Fatty acids may participate in reactions as different as protein acylation (myristoylation or palmitoylation), membrane phospholipid biosynthesis, energy storage, oxidation for energy production, and synthesis of signaling lipids. In nearly all situations, fatty acids need to be activated prior to being shuttled into the metabolic circuit. Interestingly, the only noticeable exemption in which ACSL seems not to be needed is in the eicosanoid synthetic pathway [157]. Activation, catalyzed by acyl-CoA synthetases (ACS), consists of a two-step ATP-dependent irreversible reaction that establishes a thioester linkage between a fatty acid and coenzyme A. ACS include a large family of enzymes consisting of 26 members, all of which have nucleotide (AMP/ATP) and fatty acid binding motifs. They are highly heterogeneous and for this reason are usually classified depending on the length of the activated fatty acid. Thus, short chain acyl-CoA synthases (ACSS) (<12 carbons) play a key role in lipogenesis by synthesizing acetyl-CoA from acetate [158]; while long-chain acyl-CoA synthetases (ACSL), acting on long-chain (16-22 carbons) and very-long-chain (>22 carbons) fatty acids are mostly involved in the synthesis of complex lipids like phospholipids, cholesteryl esters, ceramides,

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triacylglycerides, and fatty acid remodeling [159]. Further, acyl-CoAs may also be substrates for ω-oxidation in the endoplasmic reticulum, and for β-oxidation in peroxisomes and mitochondria. Conversely, despite the high-energy cost of acyl-CoA synthesis, numerous acyl-CoA thioesterases are able to reverse this reaction. Hence, activation and deactivation of synthetases and thioesterases appear to direct the metabolic fate of fatty acids by channeling the substrate towards or away from downstream enzymes [160,161].

Currently, thirteen ACS isoforms are classified as ACSL, each of which has differences in terms of both tissue expression levels and subcellular locations (Table S2). These proteins are coded by 5 genes, annotated as members 1, 3, 4, 5, and 6 (ACSL1 to ACSL6), which in turn are represented by many spliced transcript variants [162]. Initially, it was considered that the function of each of the isoforms could somehow be related to their subcellular location; however, current evidence shows several isoforms found on the same organelle membranes as well as the same isoform present on multiple organelles [162]. Despite the overlap in terms of substrate, ACSL isoforms (including splice variants) do show certain differences in terms of enzyme kinetics and substrate preference. In Table S2, we summarize the advances that have occurred in this topic over the last five years.

3.3.2. Phospholipases: “master generators” of bioactive lipids

The term phospholipase refers to enzymes hydrolyzing an ester or a phosphodiester bond present in a phospholipid. Depending on their site of action they are classified in the following superfamilies: phospholipase A1 (PLA1) and phospholipase A2 (PLA2) cleave the fatty acid at the sn-1 and the sn-2 position, respectively; phospholipase B (PLB) cleaves fatty acids at both sites; phospholipase C (PLC) cleaves the glycerophosphate bond; and phospholipase D (PLD) hydrolyzes the polar moiety (Fig. 7). Phospholipases are soluble proteins, preventing uncontrolled membrane degradation and acting only upon functional coupling [156]. As previously mentioned, while participating actively in membrane remodeling, all these enzymes release a wide collection of highly active lipid signaling mediators such as lysophospholipids and fatty acid precursors of many bioactive lipids involved in cell signaling, phospholipid remodeling, and membrane perturbation [163–165]. Readers may find extensive literature on this topic, including a series of recently published thematic reviews [166–171]. Herein, the most relevant aspects of each of the superfamilies are briefly commented.

PLA1 exclusively catalyzes the hydrolysis of fatty acids at the sn-1 position [172]. In mammals, nine isoforms have been described but no crystal structures for any true PLA1 are available as yet [173,174]. Extracellular PLA1 enzymes, also called pancreatic lipase family, consist of six isoforms that may hydrolyze triacylglycerides (TG), phospholipids, or both. However, three of these isoforms show unique substrate preference toward specific phospholipids, such as phosphatidylserine and phosphatidic acid, but not triacylglycerides [175], although little is known about the underlying reasons accounting for this strict substrate specificity. The main function of extracellular PLA1 is to participate in fat digestion in the gut, but also in the generation of bioactive lysophospholipids such as lysophosphatidylserine and lysophosphatidic acid [175]. Conversely, three intracellular PLA1 enzymes have been described (iPLA1α, iPLA1β, and iPLA1γ).

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Intracellular PLA1s are evolutionarily well conserved in a wide range of organisms and are involved in Golgi complex regulation and vesicular transport (in particular iPLA1γ, together with other PLA2s) [176].

PLA2. Currently, over 30 enzymes with this activity have been described in mammals, classified according to their localization (extracellular vs. intracellular), sequence homology, molecular weight, disulfide bonding patterns, and biochemical characteristics [177,178]. The traditional classification in families includes: secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), Ca^{2+}-independent PLA2 (iPLA2), PAF-acyl hydrolases (PAF, platelet activating factor), lysosomal PLA2, and adipose-specific PLA2 (AdPLA). The main features of these families may be found summarized in Table S3. Research in this field has continued to expand with the discovery of important new functions for many of these enzymes [168,179,180].

PLB is an integral membrane enzyme that is able to hydrolyze both the sn-1 and sn-2 acyl ester bonds of glycerophospholipids; that is, it displays Ca^{2+}-independent PLA1, PLA2, and lysophospholipase activities [181]. For a long time PLB activity was mostly associated to the digestion of dietary lipids [182], but its expression has been reported in several mammalian tissues (intestine, epididymis, and testes) [183]. However, the physiological functions of mammalian PLB and its potential role in phospholipid remodeling remain understudied.

PLC, in particular the isoform specific for phosphatidylinositols (PI-PLC), has been present in textbooks for decades as one of the key enzymes in the regulation of intracellular Ca^{2+} reservoirs via G-protein coupled receptors. These enzymes hydrolyze the phosphodiester bond at the sn-3 position of phosphoinositides, yielding inositol phosphates and diacylglycerol. To date, thirteen PLC human isoforms have been described and classified into six families (PLC$\beta_{(1\text{-}4)}$, $\gamma_{(1\text{-}2)}$, $\delta_{(1,3,4)}$, ε, ζ, and $\eta_{(1,2)}$) based on their structures and regulatory mechanisms of activation [184]. Although in its native cellular environment PI-PLC seems to act primarily on PIP2[4',5'], in vitro, this family of enzymes can use PI, PIP[4'], and PIP2[4',5'] as substrates but not 3-phosphorylated inositides [105]. PIP2[4',5'] hydrolysis by PI-PLC generates two second messengers: inositol 1,4,5-trisphosphate, a universal Ca^{2+}-mobilizing second messenger; and diacylglycerol, an activator of several types of effector proteins including protein kinase C isoforms [171]. Consistent with the importance of Ca^{2+} in cell physiology, PI-PLC has been associated with brain disorders [185], impaired T-cell migration [186], leukemias [187], and carcinogenesis [171]. Interestingly, PI-PLC has been involved in β-catenin independent Wnt signaling pathways (non-canonical pathway) as it is one of the targets of the Wnt/Frizzled receptor [188].

The mammalian PLC specific for phosphatidylcholine (PC- PLC) has not been cloned yet. However, two PC-PLC isoforms have been reported in natural killer cells, located in the outer leaflet of the plasma membrane and within cytoplasmic compartments [189]. Furthermore, several studies suggest the involvement of PC-PLC in differentiation [190], apoptosis [191], and carcinogenesis [192].

PLD. The classic PLD isoforms, PLD1 and PLD2, are expressed in nearly all mammalian tissues. PLD1 and PLD2 are both robustly expressed in heart, brain, and spleen while PLD1 exhibits low expression in peripheral blood leukocytes, and PLD2 in liver and skeletal muscle. At the cellular level, PLD1 preferentially localizes to the perinuclear area and PLD2 to the plasma membrane [170]. So far, no enzymatic activities have been discovered for the related proteins PLD3, PLD4, and PLD5, while PLD6 can hydrolyze cardiolipin on the

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outer surface of the mitochondria [167,193]. Updated information regarding all PLD isoforms, their structure, and regulation may be found in this recent review [167].

The mammalian PLD superfamily is best known for the hydrolysis of phosphatidylcholine generating choline and phosphatidic acid. In addition to being a critical metabolic hub (Fig. 6), phosphatidic acid is a potent second messenger that has both mTOR1 and mTOR2 as direct targets, integrating the growth and nutritional signals controlling cell proliferation [194]. Given the importance of these signaling pathways, PLD activity is tightly regulated by post-translational modification, inhibitory factors, and a large number of factors including small GTPases, PKC, and phosphoinositides [167,170]. PLD also participates in a variety of physiological cellular functions, such as cell migration, vesicle trafficking, cytoskeleton remodeling morphogenesis [195], and very recently, PLD2 was demonstrated to participate in the biogenesis of extracellular vesicles/exosomes [170]. Altered PLD activity has consistently been associated to many pathophysiological processes such as cancer [196,197], fibrosis [167,198], thrombotic disease [193], Alzheimer's disease [199], and immune function [200].

3.3.3. Acyltransferases: always there, but only now systematically studied

Most of the efforts to study the biological role of lipid diversity have traditionally focused on the nature of lipid head groups. However, specific acyl chain distribution does not only define cell types or organelles. Interconversion between lipid species by transferring fatty acids is indeed a continuous and ubiquitous event in lipid metabolism, which needs regulating as changes in acyl chain composition can affect the function of that particular species and even lead to disease states. Hence, rapid changes in lipid composition are necessary to perform processes like fission, fusion [201], and complex protein formation at any cell level [202,203]. This process is also present in our daily life whenever we take a fatty acid-enriched capsule, or in some organisms when there are changes in ambient temperature, such as yeast [204,205] and fish [206–208]. Acylation-reacylation reactions were described back in the 1960's and were coined as "Lands cycle", so most of the work on this topic was carried out fifty years ago. Unexpectedly, despite the fact that these activities have been included or assumed in many metabolic charts, the systematic characterization of the activities catalyzing these reactions was restarted only two decades ago [142].

It is worth mentioning that phospholipid species are mostly maintained by fatty acid remodeling, rather than by de novo synthesis. Thus, the second activity needed during fatty acid remodeling involves the action of acyltransferases (and transacylases) that catalyze fatty acid transfer between an acyl donor and acceptor [209–214]. These enzymes are involved not only in fatty acid incorporation into phospholipids but also into triacylglycerides and cholesteryl esters. While acyltransferases employ activated fatty acids such as acyl-coenzyme A (CoA) as an acyl donor, transacylases employ fatty acids esterified in phospholipids [142]. In principle, fatty acids at both positions (i.e. sn-1 and sn-2) are susceptible to being trans/acylated, however the highest turnover occurs at the sn-2 position by the action of PLA2 and lysophospholipid acyltransferase enzymes (LPLAT) (Figure 7) [215]. All acyltransferases have affinities for different polar heads and acyl groups. To date, five enzymes with lysophosphatidylcholine acyltransferase (LPCAT) activity have been identified: LPCAT1, LPCAT2, LPEAT2, LPCAT3, and LPCAT4. Readers may find the latest advances on these enzymes and

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more detailed information summarized in Table S4. In our opinion, these enzymes will be critical in order to understand how specificity in terms of lipid composition, such as the ones described by IMS, can be achieved.

4. Current and new approaches to investigate lipid metabolism

Despite the difficulties described so far, membrane lipid metabolism is slowly becoming a hot topic. Proof of this is the fact that very interesting studies have been published in the last few years, combining data on lipid, mRNA, and protein composition. Thus, Sunaga et al. found low levels of Elovl6 (fatty acid elongase 6), the rate-limiting enzyme in the elongation of saturated and monounsaturated fatty acids, in patients suffering idiopathic pulmonary fibrosis [216]. Furthermore, using an Elovl6$^{-/-}$ mice model, the researchers were able to provide several lines of evidence indicating that the lack of Elovl6 has a causative role in lung fibrosis. Interestingly, linoleic acid (LA, 18:2n-6) levels dropped in Elovl6$^{-/-}$, which was unexpected as it was not one of the direct products of this enzyme. The study is rather complete and, in addition to analyzing fatty acid composition and mRNA levels of fatty acid transporters and desaturates, the authors evaluated the role of essential fatty acid uptake on ROS generation and cell apoptosis. Interestingly, an enzyme closely related to LA metabolism, delta-6 desaturase, was found to be key in maladaptive cardiac remodeling and contractile dysfunction associated to chronic pressure overload. Thus, the analysis of heart biopsies obtained from patients with dilated cardiomyopathy showed that the PUFA to fatty acid precursor ratio was elevated, suggesting D6D hyperactivity. Using a rat model of pressure overload, the authors were able to inhibit the enzyme in vivo and reverse the fatty acid patterns. D6D inhibition also had an impact on eicosanoid metabolism, and lipid peroxidation, restored cardiolipin composition (particularly enriched in LA), and led to the activation of receptor kinase 1/2. Hence, for the first time this study established a relationship between phospholipid remodeling and heart contractile dysfunction [217]. Interestingly, both articles mention unexpected observations regarding lipid composition in their models of study.

The following two examples were developed in the context of intestinal cells. Wang et al. showed a link between phospholipid remodeling and cholesterol metabolism, with the latter acting as a mitogen for intestinal stem cells (ISC) [131]. Using crypt organoids, the authors demonstrated that LPCAT3 inhibition increased membrane saturation and cholesterol biosynthesis, triggering ISC hyperproliferation. Conversely, inhibition of cholesterol biosynthesis restored normal proliferative patterns. Hence, the disruption of LPCAT3-dependent phospholipid remodeling and cholesterol homeostasis dramatically enhances tumor formation in APC^{min} mice (Min, multiple intestinal neoplasia). Interestingly, using mouse derived organoid models and human colon cancer cell lines, Schewe et. al showed that during homeostasis, the group IIA and group X sPLA2s inhibit Wnt signaling through Yap1 activation. However, during inflammation, these sPLA2s are secreted into the intestinal lumen, and the synthesis of prostaglandin is promoted, leading to the activation of Wnt signaling. Finally, genetic ablation of both sPLA2 subtypes improved recovery from inflammation but increased colon cancer susceptibility, suggesting significant compensating functions as genetic modifiers of inflammation and colon cancer [218].

Nevertheless, the field is experiencing a new revolution due to the possibility of analyzing biological samples using “multi-omics” approaches simultaneously (genomics, transcriptomics, proteomics,

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lipidomics…), offering a powerful and unique opportunity to study cell metabolism in a more integrative way. It is worth stressing that, parallel to the development of new methodology and equipment, the generation of software that is able to deal with the astonishing amount of data generated and capable of extracting meaningful information will be key in the near future. Some studies have already started this type of approach, providing new insights into lipid metabolism. Thus, in a recent study, a systems genetics resource incorporating quantitative lipidomics and proteomics was generated [219]. The latter was used to interrogate lipid regulatory networks in 107 genetically distinct mouse strains. Doing so, the authors were able to bring to the surface new pathways that are critical in lipid metabolism, as well as the potential sub-cellular localization and function of unique lipid regulatory proteins [219]. In a second very interesting study, the model of study used immune cells in the context of TLR-induced inflammatory response [130]. The combination of lipidomics with genetic perturbations made it possible to conclude that the coregulation between membrane lipid species, mostly between sphingolipids and phospholipids, is organized in a circular network similar to those displayed in the citric acid cycle and the urea cycle. Importantly, this coregulation is sensitive to the pathophysiological state of the immune cell. Thus, the authors were able to identify sphingolipids as a key lipid category in TLR trafficking, signaling, and cytokine release. These results have an enormous translational impact as the network-wide functional lipid annotation could predict TLR responses in patients' cells [130].

5. Conclusions

Despite this huge potential in generating new knowledge, membrane lipid metabolism is often perceived as something tedious to study. Some reasons are related to the intermingled characteristics of lipid metabolism that often complicate studies at the molecular level. Some are more historical. Hence, despite the analytical efforts made to show how specific lipid composition is, the lipid component of cell membranes is still imagined as an anarchic mixture of a limited number of lipids. Well, if "seeing is believing", then IMS is currently providing the final evidence that should convince any researcher that each lipid species is carefully selected and precisely regulated by the cell in order to maintain its physiology. How is this achieved? Here is where a large gap in knowledge is found. Even though, in principle, it should be possible to withdraw copious biochemical information from both MS and IMS experiments, the link is not always so straightforward, and the reasons are intimately related to the characteristics of the lipid biosynthetic pathways described herein. Being aware of them is key to understanding the challenges of studying the molecular mechanisms controlling the lipidome at any regulatory level. Furthermore, as gene expression methodology is becoming more available, there is also a rise in the number of publications showing altered expression in rather unexplored membrane lipid isoenzymes, on which information is scant. Altogether, the evidence coming from very different disciplines is leading us to a scenario wherein all the peculiarities and specificities of membrane lipid metabolism should be taken into account. While this is no easy task, it is undoubtedly most challenging, and a very multidisciplinary approach is strongly required.

Supplemental Material

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The Supplemental Material contains a summary on membrane phospholipid and sphingolipid metabolism and Table S1, S2, S3 and S4.

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