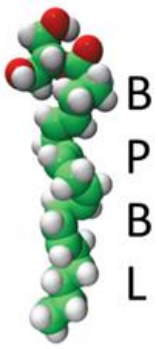
Université catholique de Louvain

Louvain Drug Research Institute

Bioanalysis and Pharmacology of Bioactive Lipids Research Group

Targeted and untargeted lipidomics studies: towards a better understanding of the role of lipids in inflammation

Julien Masquelier



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ABSTRACT

Lipids, and bioactive lipids in particular, are key players during inflammation. The study of their implications in the physiopathological processes linked to inflammation requires analytical strategies allowing for their quantification in biological matrices.

Thus this thesis explored two approaches used to quantify metabolites in biological systems (i) an untargeted approach and (ii) a targeted approach.

Using an HPLC-LTQ-Orbitrap XL we set-up an HPLC-ESI-MS method to analyze several phospholipids, lysophospholipids, (dihydro-) ceramides, galactosylceramides, (hydroxylated-) sulfatides, and fatty acids from the same biological sample. We then applied this method to the characterization of the effect of J774 macrophage activation on lipid levels.

The other topic of this work revolves around the study of lysophosphatidylinositols (LPI) in the context of inflammation. Indeed these lipids are produced, among other pathways, through the activity of the phospholipase A_2, known to be activated during inflammation. We started our investigations by setting up and validating an original HPLC-ESI-MS method for the quantification of lysophosphatidylinositols and applied it to the determination of their levels in mice tissues. Then, using this method we studied how macrophage activation in vitro and inflammation in vivo would affect lysophosphatidylinositol levels. To try to understand the causes of the variations in lipid levels, we also investigated the expression of the enzymes involved in lysophosphatidylinositol metabolism. The changes in lysophosphatidylinositol levels observed in macrophages prompted us to investigate whether lysophosphatidylinositols would be able to affect lipopolysaccharides-induced macrophage activation. Using J774 cells overexpressing the GPR55 receptor, we were also able to suggest that part of the effect was indeed mediated by GPR55 activation.

Taken together these studies illustrate how developing lipid quantification methods will advance our understanding of their role in inflammatory settings.

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Abbreviations list

(p)	plasmalogens
AA	Arachidonic Acid
AEA	Arachidonoylethanolamine, Anandamide
AGPAT8	1-acylglycerol-3-phosphate O-acyltransferase 8
APCI	Atmospheric Pressure Chemical ionization
APPI	Atmospheric Pressure PhotoIonization
ATX	Autotaxin
Cer	Ceramides
CL	Cardiolipin
DDHD1	DDHD Domain Containing 1
DHA	Docosahexaenoic Acid
DihydroCer	Dihydroceramides
DSS	Dextran Sulphate Sodium
EIC	Extracted-ion chromatogram
EPA	Eicosapentaenoic Acid
ESI	Electrospray
FA	Fatty acids
GalCer	Galactosylceramides
GlcCer	Glucosylceramides
GPI	Glycerophosphoinositol
LPA	Lysophosphatidic acids
LPC	Lysophosphatidylcholines
LPE	Lysophosphatidylethanolamines
LPG	Lysophosphatidylglycerols
LPI	Lysophosphatidylinositols
LPS	Lysophosphatidylserines
MALDI	Matrix Assisted Laser Desorption Ionization
MBOAT7	Membrane Bound O-Acyltransferase Domain Containing 7
PA	Phosphatidic acids
PA-PLA_1	Phosphatidic Acid-Preferring Phospholipase A_1
PC	Phosphatidylcholines
PCA	Principal Component Analysis
PE	Phosphatidylethanolamines
PG	Phosphatidylglycerols
PI	Phosphatidylinositols
PL	Phospholipids
PLA_2	Phospholipase A_2
PLS-DA	Partial Least Squares Discriminant Analysis
PS	Phosphatidylserines
S1P	Sphingosine-1-Phosphate
SAT	Subcutaneous Adipose Tissue
SM	Sphingomyelins
SP	Sphingolipids
Spo	Sphingosine

TEM	Thioglycolate-elicited Macrophages
TIC	Total Ion Chromatogram
TNBS	Trinitrobenzene Sulfonic Acid

INTRODUCTION

This introduction is divided into 4 parts :

Part 1. Lipids: from energy substrate to bioactive compounds

Part 1. Lipids: from energy substrate to bioactive compounds

I. Definition of lipids

Even if lipid analysts and biochemists tend to reach a strong understanding of what is covered by the term "lipids", there is currently no commonly accepted definition. Lipids are a wide group of naturally occurring molecules showing a broad diversity in biological functions and structures. They are commonly considered to be compounds insoluble in water and readily soluble in organic solvents such as hydrocarbons, benzene, or chloroform. However, a definition like this one is ambiguous and inaccurate because a lot of different compounds, such as many peptides and some very hydrophobic proteins, could be soluble in chloroform. Another reason is that several substances which are now widely considered as lipids, such as lipids with large hydrophilic domains (e.g. saccharolipids), are not easily soluble in organic solvents (they are actually very easily soluble in water). To establish a more specific definition of lipids than just based on the solubility some authors thought about definitions more related to the structure and the biosynthetic pathways. Christie suggested this definition: "Lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds".[1] This vision includes cholesterol and plant sterols as lipids and could include bile acids or tocopherols and allows to consider gangliosides as lipids despite the fact they are more soluble in water than in organic solvents. The fatty acids usually described contain even numbers of carbon atoms, commonly C_{14} to C_{24}, and may be saturated or unsaturated and contain a wide variety of substituents groups.

In 2005, Fahy et al. described lipids based on a more chemical aspect. They suggested that "lipids are 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.)" (**Figure 1**).[2]

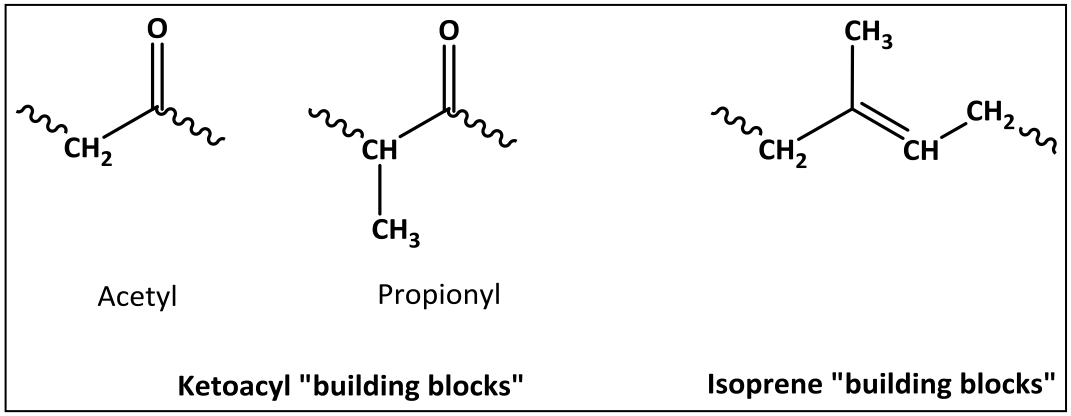


Figure 1. Lipid biochemical "building blocks".

II. Role of lipids

Today, it is well established that lipids could play a more important role than previously believed. The recent discoveries of their implication in a lot of biological processes or pathologies contribute to the general interest of the scientific community.

In a simplified view, lipids fulfil mainly three general functions: (i) energy storage, (ii) cell membranes constitution and (iii) signaling mediators.

(i) Triacylglycerols can be stored in several cells, but mainly in adipose tissue, and consists in the major form of energy storage in mammals. In the adipose tissue, the adipocytes are responsible for the continuous synthesis and breakdown of triacylglycerols. In adipocytes, droplets of lipids can merge to form a large globule, which may lead to the occupation of the entire cell volume.[3] Compared with the breakdown of carbohydrates and proteins, the complete oxidation of fatty acids is able to provide a higher caloric content (respectively 4 and 9 kcal per gram). For instance, migratory birds use stored energy of triacylglycerols to fuel their flights since they sometimes must fly very long distances without eating: the golden plover is able to fly more than 3000 km over the ocean without feeding, just using lipids as source of energy.[4]

Moreover, lipids can serve as a protective layer providing structural support helping to prevent injury to vital organs such as liver, kidneys or spleen.

(ii) A second very important role of lipids in cells is to form the lipid bilayer barrier of cells and organelles (e.g. plasma membrane of cells, intracellular membranes of organelles). In all

mammalian cells, the plasma membrane acts as physical barrier separating the intracellular components from the extracellular environment.[5] Phospholipids (glycerophospholipids), because they are amphipathic, are considered as the primary building blocks of membranes. However, several other lipids, such as sphingomyelin and sterols (with a high proportion of cholesterol in mammalian cell membranes), are present in the cell membranes (**Figure 2**). Cardiolipins, a subclass of glycerophospholipids, consisting of three glycerol groups and four acyl chains, are particularly abundant in the inner mitochondrial membrane.[6] Some other lipids, even if less present, could be functionally important. The eukaryotic cell membranes are defined by an irregular distribution of lipids, laterally as well as vertically.[7] Phosphatidylcholines, glycosphingolipids and sphingomyelins are mainly located on the exoplasmic leaflet while "aminophospholipids" such as phosphatidylethanolamines or phosphatidylserines are mostly enriched in the cytoplasmic leaflet. Phosphatidylinositols and phosphatidic acids, considered as minor phospholipids in cell membranes, are also mainly present in the cytoplasmic leaflet.

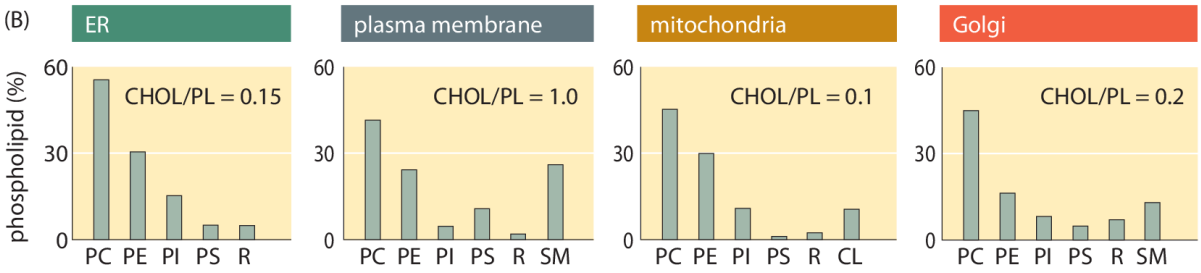


Figure 2. Lipid composition of different membranes in mammals. (Adapted from [8]) The lipid composition of membranes changes throughout the cell. The lipid compositional data are expressed as a percentage of the total phospholipid (PL). As a measure of sterol content, the molar ratio of cholesterol (CHOL) phospholipid is also included. For instance, the main lipids assembled in the endoplasmic reticulum (ER) are phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylinositols (PI) and phosphatidylserines (PS). In addition, ER synthesizes other lipids such as ceramides, galactosylceramides called remaining lipids (R). Sphingomyelins (SM) and Cardiolipins (CL) are also found as main lipid in Golgi or mitochondria, respectively.

Phosphatidylserines are also considered as quantitatively minor components of membranes since their proportions are comprised between 5 and 10 % of the total phospholipids in mammalian cells. However, even if phosphatidylserines are not very abundant, they are widely distributed in organelles, highlighting their fundamental structural role in biological cell membranes.[9] Indeed, the presence of these lipids and their asymmetric distribution in

the plasma membrane are very important for the cell and have been shown to play a major role in numerous physiological and pathological events.[10,11] Conservation of an adequate lipid asymmetry is necessary for the mechanical stability of the cell membrane as well as for the vesicular transport. In the same time changes in lipid asymmetry appear to be important for cell cycle progression, platelet coagulation or apoptosis.[12,13]

Also, the current view of biological lipid membranes is that they are constituted of microdomains (e.g. lipid rafts) of different lipid and protein composition (mainly sphingomyelins, cholesterol and glycosphingolipids) and that these microdomains serve to further compartmentalize cellular processes. These domains can be considered as a lateral phase separation of the lipids within one leaflet of the bilayer.[14] These very dynamic domains (e.g. localization and time) are crucial in cell signaling processes and constitute sorting platforms for targeted protein. For instance, an alteration of lipid composition in membranes could lead to abnormal lipid raft organization and a deregulation of lipid raft-dependent signaling which can be associated with cancers[15-17], neurodegenerative diseases[18], deregulation in immune system[19]…

(iii) Last but not least, the place of lipids in cell signaling has emerged demonstrating the crucial role of lipid mediators as vital players of cell processes.[20] Numerous biological activities for lipid components have been reported over the years. A wide variety of lipid structures, from fatty acids to phytosterols[21], appear to mediate cellular functions and resulted in the coining of the term “bioactive lipids”. This notion will be described later in this chapter.

III. Lipid classification

Defining lipid roles and functions remains a difficult challenge because of the diversity of several properties (physical and chemical) and because each lipid may be implicated in different cellular functions at various levels. Moreover, new technological advances in the analytical field have opened a new area of research in the field of biological sciences. More specifically in the lipid analysis field, the global analysis of an organism’s (cell, entire tissue, animal…) response to a perturbation at the “omics” level is progressively resulting in an increasingly complete understanding of the underlying mechanisms in complex systems. Since the lipidome represents a very huge number of compounds, with different chemical

and physical properties in different concentration ranges, it is essential to be able to properly classify these compounds.

Until recently, no universal classification system for endogenous lipids was commonly accepted. In 1943, Bloor in "biochemistry of fatty acids" proposed the following classification based on their chemical compositions:

(i) simple lipids (fat, oils and waxes)
(ii) compounds lipids (phospholipids and glycolipids)
(iii) derived lipids (substances derived from simple and compounds lipids by hydrolysis such as fatty acids).

This classification was obviously not complete and more recently lipids were divided into "simple" and "complex" groups. The lipid Library[a] or Cyberlipid[b] web tools separate them in simple lipids, being those yielding at most two types of distinct moieties upon hydrolysis (such as acylglycerols), and complex lipids yielding three and more products upon hydrolysis (such as glycerophospholipids).

In 2005, the International Lipid Classification and Nomenclature Committee with the partnership of the LIPID MAPS Consortium[c] developed and established a "comprehensive classification system for lipids" based on chemical and biochemical principles. This new classification system, compatible with data-basing and informatics requirements, has now been widely adopted and accepted internationally.[22] This has been recently updated to better take into account lipid components from non-mammalian sources (i.e. lipids from plants, bacteria and fungi). In this classification system, lipids are divided into 8 different categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols lipids, prenol lipids, saccharolipids and polyketides (**Figure 3**). Each category of lipids is divided in classes and subclasses of structures and a 12 digit number is provided for each lipid, to allow identifying each unique lipid molecule. This is very important and this LIPID ID, in addition to providing a systematic means of assigning a unique identification "code" to each lipid, will

[a] [Lipid Library website, http://lipidlirbary.aocs.org]
[b] [Cyberlipid Center website, http://cyberlipid.org]
[c] The LIPID MAPS consortium (LIPID Metabolites And Pathways Strategy), collaboration of twelve independent laboratories, was pivotal in the effort to classify and improve lipid nomenclature. This multi-institutional organization was created in 2003 with the goal of identification and quantification of all the major and minor lipid species in mammalian cells but also to quantitate the variations in these species in response to perturbation.

facilitate the addition, in the future, of new categories, classes, and subclasses. Moreover, to help researchers, an unambiguous naming scheme was defined since a large number of novel lipid classes, without systematic name, have been discovered during the last 30 years. Thus they proposed a clarification of the IUPAC nomenclature and a guideline for the systematic naming for newly discovered lipid classes. This new structured appellation facilitates the systematization of lipid study and enables the use of "catalogs" of lipids and their properties.

In this new classification, some key features were decided for clarifying the lipid nomenclature[2] and among these some are crucial for better identification:

- The stereospecific numbering (*sn*) method is used for description of glycerolipids and glycerophospholipids and the glycerol group is most of the time acylated or alkylated at the *sn*-1 and/or *sn*-2 position.
- Definition of sphinganine and sphing-4-enine (sphingosine) as core structures for the sphingolipid category
- The use of names such as cholestane, androstane and estrane for sterols
- For the fatty acids and acyl chains adherence to the names defined by the IUPAC recommendations
- The adoption of a condensed text nomenclature for the glycan portions of lipids
- The "d" and "t" designations used for sphingolipids refer to 1,3-dihydroxy and 1,3,4-trihydroxy long- chain bases, for "d" and "t" respectively.

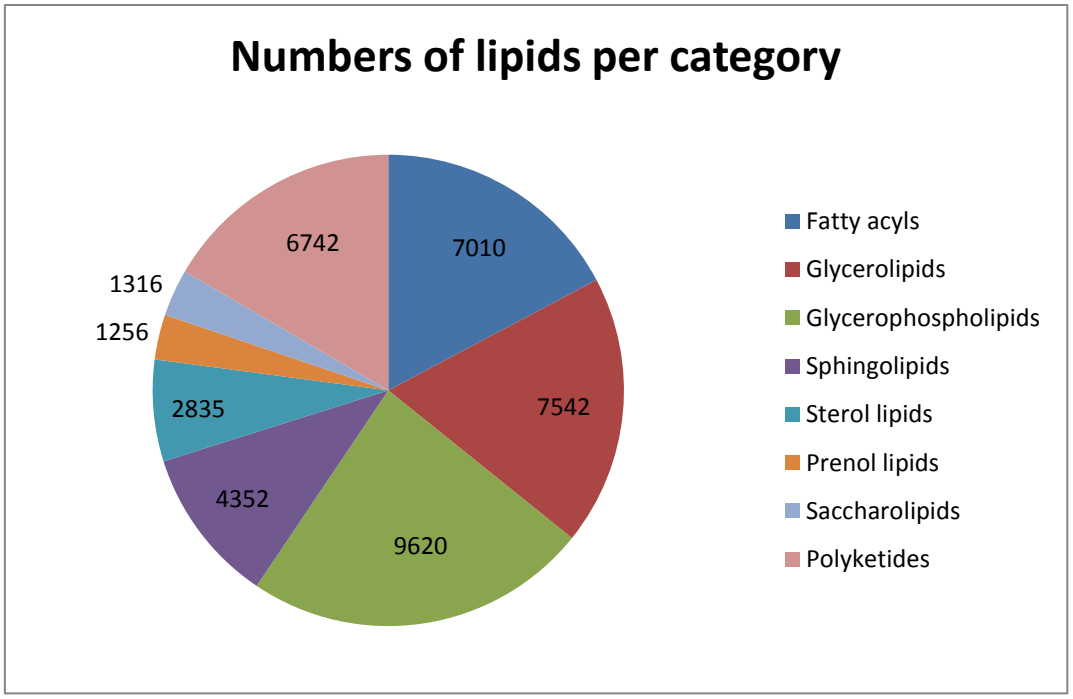


Figure 3. Lipidmaps classification of referenced lipids. The graph reports the number of individual lipids per family that are found in the lipidmaps database (as of 30/01/2017)

1. Fatty acyls (FA):

a) Free fatty acyls:

Free fatty acid represents the main lipid "building block" of complex lipids and for this reason they constitute one of the most fundamental categories of biological lipids. Fatty acids and derivatives are characterized by a repeating series of methylene groups that confer a hydrophobic character to these lipids. The simplest structure of this class, the subclass of straight-chain saturated fatty acids, contains a terminal carboxylic acid and is often represented by the most abundant saturated fatty acid in nature, the hexadecanoic acid (palmitic acid). Free fatty acids are constituted by an aliphatic chain which can be saturated or not. Fatty acids of plant, animal and microbial origins are generally constituted of an even number of carbon atoms linked in a linear chain. However some plants and bacteria are able to produce a larger structural diversity as it is possible to find either ramification in the chain either odd number of carbons.

Indeed, from the simple unbranched fatty acid, in all kingdoms of life, a very large number of variations on this basic structure occur, including heteroatoms (oxygen, halogen, nitrogen,

sulfur linked to the carbon chain), one or more double bonds, cycles as well as heterocyclic rings, thiols…

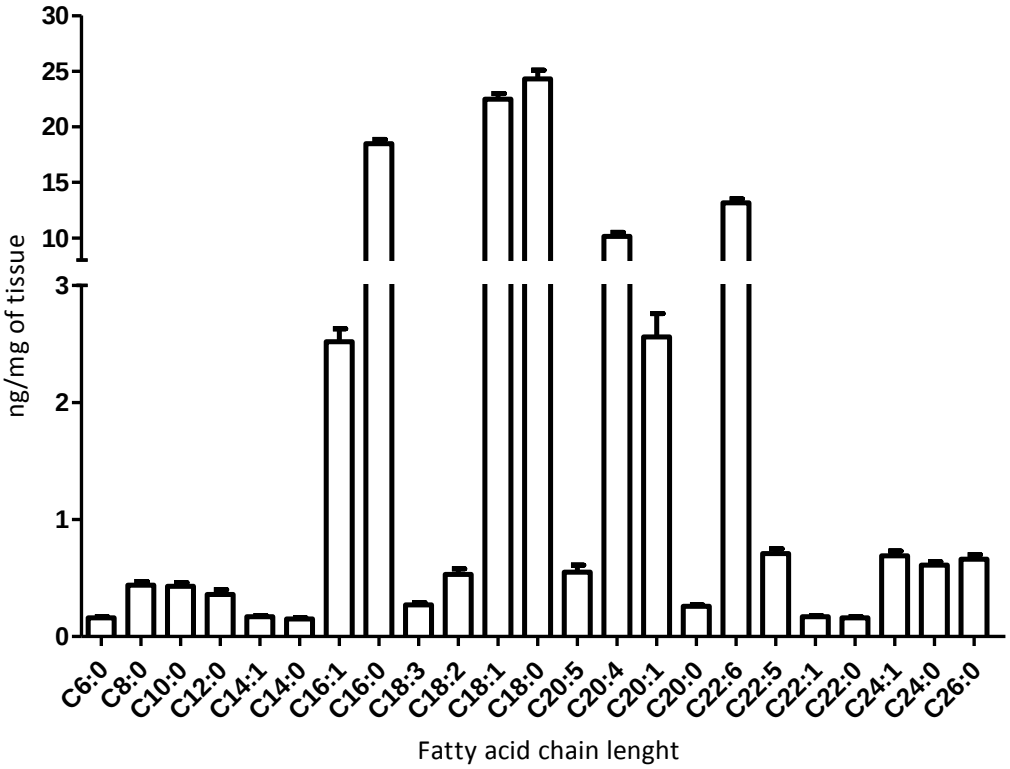


Figure 4. Proportion of 23 free fatty acids found in rat brain samples. Values shown are means±SD for the composition by each FFA concentration (ng/mg of brain rat) (Adapted from [23])

Palmitic acid, $CH_3(CH_2)_{14}COOH$, can also be designated as a 16:0 fatty acid, the first number describing the number of carbon atoms in the aliphatic chain while the second representing the number of insaturations (**Figure 5**). The most abundant saturated fatty acids in animal and plant tissues are straight-chain structures with 14, 16, 18, 20 or 22 carbon atoms (although shorter (e.g. 4) or longer (e.g. 36) fatty acids can be found) (**Figure 4**).[23] Nevertheless, only the 14 to 20 homologues are likely to be detected in substantial amounts in glycerolipids.

- Monoenoic fatty acids, i.e. straight-chain even numbered fatty acid containing one cis-double bond, are also well represented in the nature. The most abundant monoenoic fatty acid in most organisms is the oleic acid, $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ which can also be designed as "18:1". Even if the double bond can be in a variety of different positions (which could be specified in the systematic nomenclature in relation to the carboxyl group), some are poorly present.

As an example, while 18:1 fatty acid, cis-9-octadecenoic acid (oleic acid) is the most abundant, trans-11-octadecenoic acid (vaccenic acid) is mostly not detectable.

- Polyunsaturated fatty acids, also often abbreviated as PUFA, contain from two up to a maximum of six cis-double bonds separated by single methylene groups, and with the same terminal structure. Among them, linoleic acid (18:2) and arachidonic acid (20:4) are the most abundant. Linoleic acid is an essential fatty acid in animal diets, as it cannot be synthesized in animal tissues. Arachidonic acid is especially important as a component of membrane phospholipids or as a precursor of prostaglandins and other eicosanoids (e.g. thromboxane, leukotrienes…).

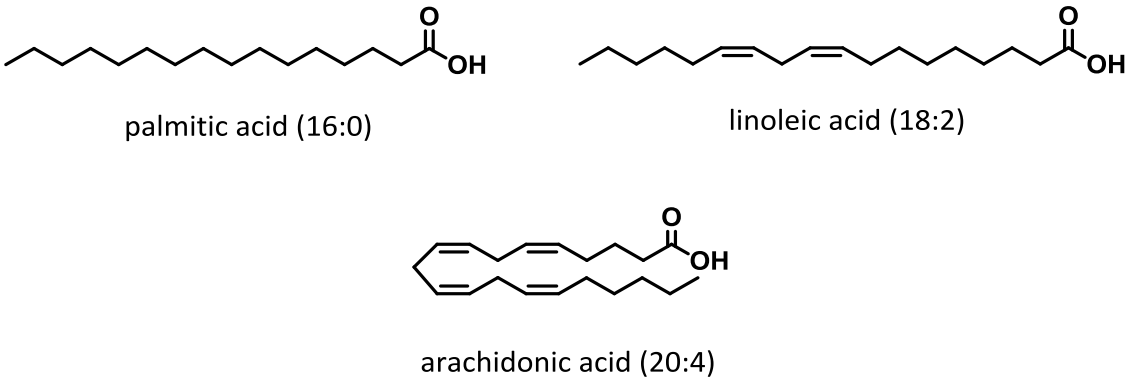


Figure 5: Typical examples of "simple" fatty acids.

b) Eicosanoids and docosanoids

Eicosanoids, are metabolites of fatty acids constituted of 20 carbons. This subclass includes the prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives, which are able to exert several effects at very low concentrations (**Figure 6**). Leukotrienes are mainly generated by cyclooxygenases (COX-1 and COX-2), lipoxygenases (LOX) or epoxygenases. In the synthesis of eicosanoids, the key precursor fatty acids are dihomo-γ-linolenic (20:3), arachidonic (20:4) and eicosapentaenoic (20:5) acids. More recently, "docosanoids", derived from fatty acids constituted of 22 carbons such as docosahexaenoic acid (22:6), have been described. These include important lipids in inflammation such as resolvins and protectins (see Part 2).

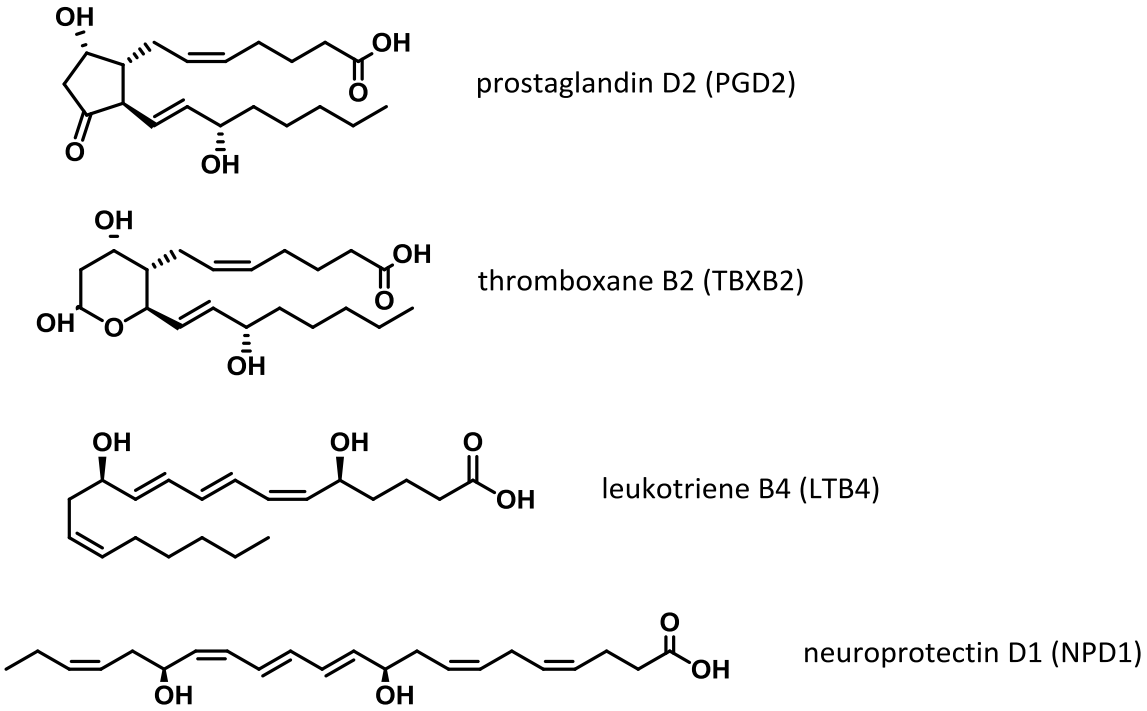


Figure 6. Representative structures of eicosanoids and docosanoids.

2. Glycerolipids (GL)

This class of acylglycerols is characterized by a common moiety of glycerol and is mainly constituted by mono-, di- or tri-substituted glycerols. Of note, due to their abundance, importance as membrane constituents and signaling molecules, glycerophospholipids were separated in a distinct class, even if the basal skeleton is common for both classes. Triacylgycerols (TAG), consist of a glycerol moiety with each hydroxyl group esterified with a fatty acid (**Figure 7**). To clarify their appellation, a stereospecific numbering system has been used to describe these forms and all the structures with this kind of glycerol moiety. In a Fischer projection of a natural L-glycerol derivative, the secondary hydroxyl group is shown to the left of C-2. The carbon atom above C-2 becomes C-1 and the last below C-3. When stereochemistry is defined, the prefix *sn* is placed before the stem name of the compound.

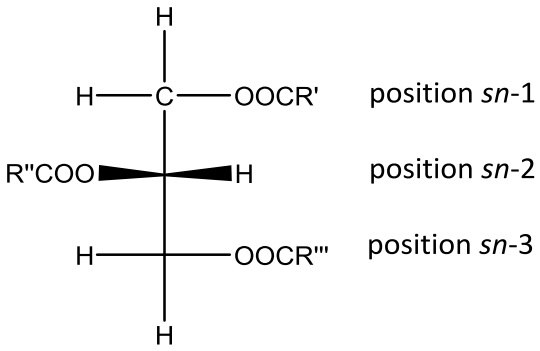


Figure 7. General structure of a triacyl-*sn*-glycerol.

Diacylglycerols (DAG) are key intermediates in the biosynthesis of triacylglycerols and other lipids, and they are crucial cellular messengers, mainly generated by phospholipase C upon hydrolysis of glycerophospholipids. Depending on the esterified hydroxyls (*sn*-1 et *sn*-2 or *sn*-1 et *sn*-3), the diacylglycerols generated are respectively 1,2-DAG or 1,3-DAG.

Monoacylglycerols (MAG), are mainly formed as intermediates or end-products of the enzymatic hydrolysis of triacylglycerols (**Figure 8**). DAG and MAG are less abundant than TAG, given the role of TAG as main form of storage of fatty acids in adipocytes.

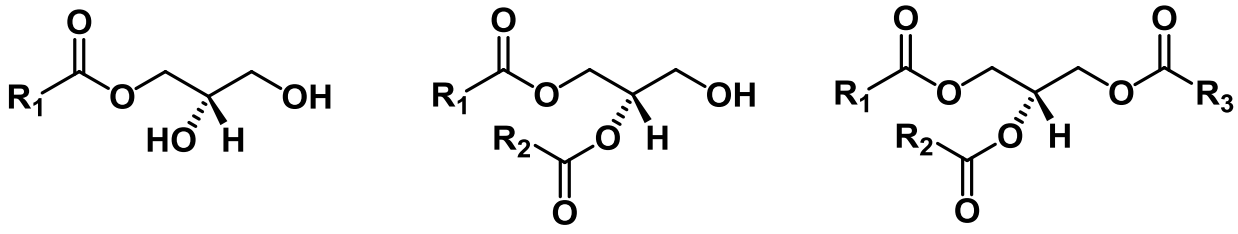


Figure 8. Structure of glycerolipids.

Glyceroglycans are considered as a subclass of glycerolipids and are characterized by the presence of sugar residues (one or more) attached to the glycerol via a glycosidic linkage. However, these lipids are not present in all systems.

3. <u>Glycerophospholipids (GP)</u>

Glycerophospholipids, also more simply called phospholipids, are glycerolipids with their *sn*-3 position linked to a phosphate, which can be or not substituted by a polar moiety.

Phospholipids are ubiquitous and are key components of the lipid bilayer of cells. The large variability in the polar moieties is responsible for the structural diversity of this class of lipids. Based on the nature of the polar "head group" at the *sn*-3 position of the glycerol backbone in eukaryotes, glycerophospholipids may be divided in several distinct subclasses. While the *sn*-3 position is often linked to a phosphate group, *sn*-1 and *sn*-2 positions are generally respectively linked to saturated and unsaturated fatty acids.

Several phospholipases are key enzymes in the metabolism of glycerophospholipids (**Figure 9**). Phospholipases A_1 (PLA_1) and A_2 (PLA_2) can hydrolyze esters links between the fatty acids and the glycerol backbone at respectively *sn*-1 and *sn*-2 positions. PLA_1- and PLA_2-mediated glycerophospholipid hydrolysis results in the corresponding lysophospholipids (i.e. glycerophospholipids lacking a fatty acid chain). Often, unsaturated fatty acids implicated in inflammatory processes (such as docosahexaenoic acid or arachidonic acid) are linked to the glycerol via an ester linkage in *sn*-2 position and the action of PLA_2 is mainly implicated in inflammation for this reason.[24] Hydrolysis of phospholipids via phospholipase C (PLC) leads to the formation of 1,2-DAG after cleavage of the phosphodiester linkage. The last phospholipase involved in glycerophospholipid metabolism is the phospholipase D (PLD) that generates phosphatidic acid and polar head.

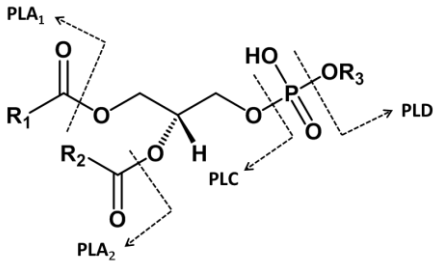


Figure 9. Action of phospholipases on glycerophospholipids.

Phosphatidic acid (PA), or 1,2-diacyl-*sn*-glycero-3-phosphate, is the simplest example of glycerophospholipids as its polar head is constituted of a unsubstituted phosphate (**Figure 10**). These lipids are usually found in trace amounts in tissues, but represent important metabolic compounds, as they are precursors of most of the other glycerophospholipids, and as signaling molecules. The hydrolysis of PA, via PLA_1 or PLA_2 activity, generates the corresponding lysophospholipid, lysophosphatidic acid (LPA), which are found in tissues at low levels but having a key biological role, influencing many biochemical processes.

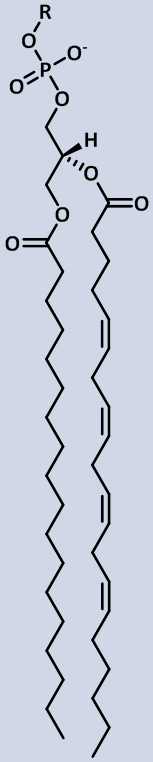
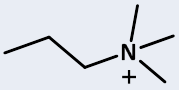
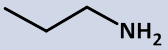
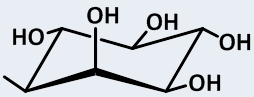
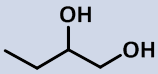
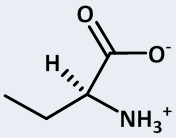
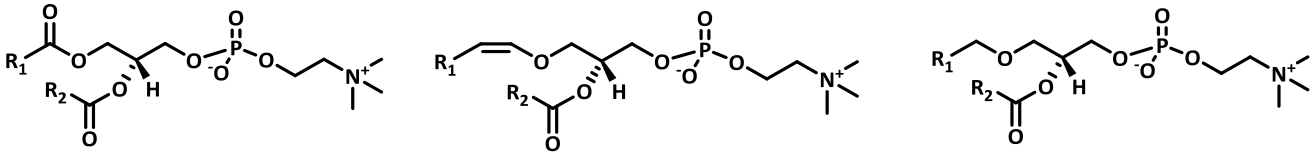
Basic phospholipid structure	Substituents (R)	Name
 18:0/20:4 phospholipid	 hydrogen	PA (phosphatidic acid)
	 choline	PC (phosphatidylcholine)
	 ethanolamine	PE (phosphatidylethanolamine)
	 inositol	PI (phosphatidylinositol)
	 glycerol	PG (phosphatidylglycerol)
	 serine	PS (phosphatidylserine)

Figure 10. Structures of glycerophospholipids.

Usually the most abundant phospholipid in membranes of animal tissues, and often a major lipid component of plant membranes, are phosphatidycholine (PC) or 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine (also called lecithin). Structurally speaking PC are built based on a PA linked to a choline polar head group via a phosphodiester link. In this subclass of glycerophospholipids, fatty acids can be linked to the glycerol in 3 different ways (**Figure 11**): (i) the classical diacyl PC which represents more than 96% in the rat brain, (ii) the alkenyl-acyl PC, via a vinyl ether link and (iii) the alkyl-acyl which is characterized by an ether link in *sn*-1 position. Together, the two last forms represent less than 4 %, in the rat brain.[25]



diacyl PC alkenyl-acyl PC alkyl-acyl PC

Figure 11. Structures of lateral chains of phosphatidylcholine.

Phosphatidylethanolamines (PE), (also called cephalin), is the second most abundant phospholipid subclass in animal and plant tissues. In microorganisms, it can even be the major lipid class. As for PC, PE are also subdivided into three different groups depending of the nature of the link between the fatty acid and the *sn*-1 position of the glycerol. However, in contrast with PC, for PE, the alkenyl-acyl PE (also called plasmalogens) are abundant in the rat brain: while plasmalogen PE represent around 60-70%, "classical" diacyl PE account for only 30-40%.[25,26]

Phosphatidylinositols (PI) are anionic phospholipids present in membranes as minor components and are characterized by an inositol moiety as polar head. PI are mainly present at the cytosolic side of the membranes and can be phosphorylated (from 1 to 3 times) to give phosphoinositides (phosphoinositol monophosphate, bisphosphate and triphosphate, also known as PIP, PIP_2, PIP_3, respectively).

Phosphatidylserines (PS) are considered as weakly acid lipids, present in most tissues of animals, plants as well as microorganisms. Classically, PS are considered as a quantitatively minor components of the cell membranes, representing 5–10% of total phospholipids.[27] However, even if PS are less abundant, they are widely distributed in cellular organelles, playing a fundamental structural role in membranes including in apoptosis.[12,28]

Phosphatidylglycerols (PG) are present as « trace » constituents of most tissues, but are often considered as the main anionic phospholipids in bacterial membranes.[29] Note that PG are the biosynthetic precursor of cardiolipins, which are important constituent of mitochondrial lipids. PG were shown to be important lipids in the lungs, playing a crucial physiological role as surfactants.[30]

4. Sphingolipids (SP)

Sphingolipids constitute a large and complex class of compounds sharing a common structural characteristic: a sphingosine backbone (**Figure 12**). Similarly to glycerophospholipids, SP are considered as essential constituents of cell membranes. While some fatty acids (such as 16:0 and 18:0) are quite similar between GP and SP, polyunsaturated fatty acids (such as 20:4 and 22:6) are more frequently present in GP while long chain fatty acids (such as 24:0, 24:1 or 26:0) are more frequent in SP.

Depending of the distinctive additions on this sphingosine scaffold, several major compounds can be distinguished (**Figure 12**) and different subclasses can be identified. These include the simple derivative of the sphingosine (sphingosine-1-phosphate), its combination with an amide-linked fatty acid (ceramides), but also more complex sphingolipids, with “head groups” linked via phosphodiester linkages (phosphosphingolipids such as sphingomyelins) or via glycosidic bonds (glycosphingolipids such as cerebrosides or gangliosides).

As mentioned, ceramides (Cer) are constituted of a fatty acid coupled to the amine group of sphingosine by an amide bond. Cer are considered as the simplest SP but they are quite important as they are generally precursors of more complex SP. The FA contained in Cer are typically saturated or monounsaturated with carbon chain lengths ranging from 14 to 26 atoms. Dihydroceramides, based on a sphinganine scaffold (**Figure 12**), are also important in the metabolism of SP. Both Cer and dihydroceramides are essential lipids of cell membranes. Due to their physico-chemical properties they play a key structural role in lipid rafts stability.

Sphingomyelins (SM) are considered as “sphingophospho-lipids” because they consist of a ceramide unit linked on its primary alcohol to a phosphocholine group (**Figure 12**) .They are one of the most abundant lipid in animal tissues, as PC, and a key component of the myelin sheath. SM do not seem to be present in plants or microorganisms. This subclass is very important as precursor for a large number of metabolites. For instance, Jenkins et al. showed that ceramide production is more important from SM hydrolysis than de novo synthesis.[31] Glycosylceramides consist of a ceramide unit coupled on its primary alcohol to a glucose or galactose by a glycosidic bond. Glycosylceramides are present in brain and other tissues but are more abundant in the skin. Structurally speaking, the simplest

sphingoglycolipids are the monoglycosylceramides. Among those, the monogalactosylceramides (cerebroside, GalCer) are the most abundant, followed by the monoglucosylceramides (GlcCer). As SM, GalCer are very abundant in the myelin sheath.[32]

Sulfatides, i.e. sulfate esters of GalCer, with the sulfate group linked to position 3 of the galactosyl group are key components of brain lipids but also found (at lower levels) in other tissues. Similarly to GalCer and SM, they are abundant in myelin sheath, comprising about 5% of total lipids in myelin sheath.[33]

Other highly complex glycosylsphingolipids, such as oligoglycosylceramides (containing one or more sialic acid groups), can be found. Other combinations, more complex than a simple galactose, can also be linked to the ceramide resulting in gangliosides. Typically, glucose, galactose or sialic acid can be added to "simple" lactosylceramide (ceramide-glucose-galactose) resulting in gangliosides such as Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ, more commonly called GM1. They were first found in the ganglion cell of the central nervous system, but they are present, at different levels, in most animal tissues. Depending of the number of sialic acid groups, these complex glycosylsphingolipids are divided in mono-, di-, tri-, tetra- and penta-sialogangliosides.

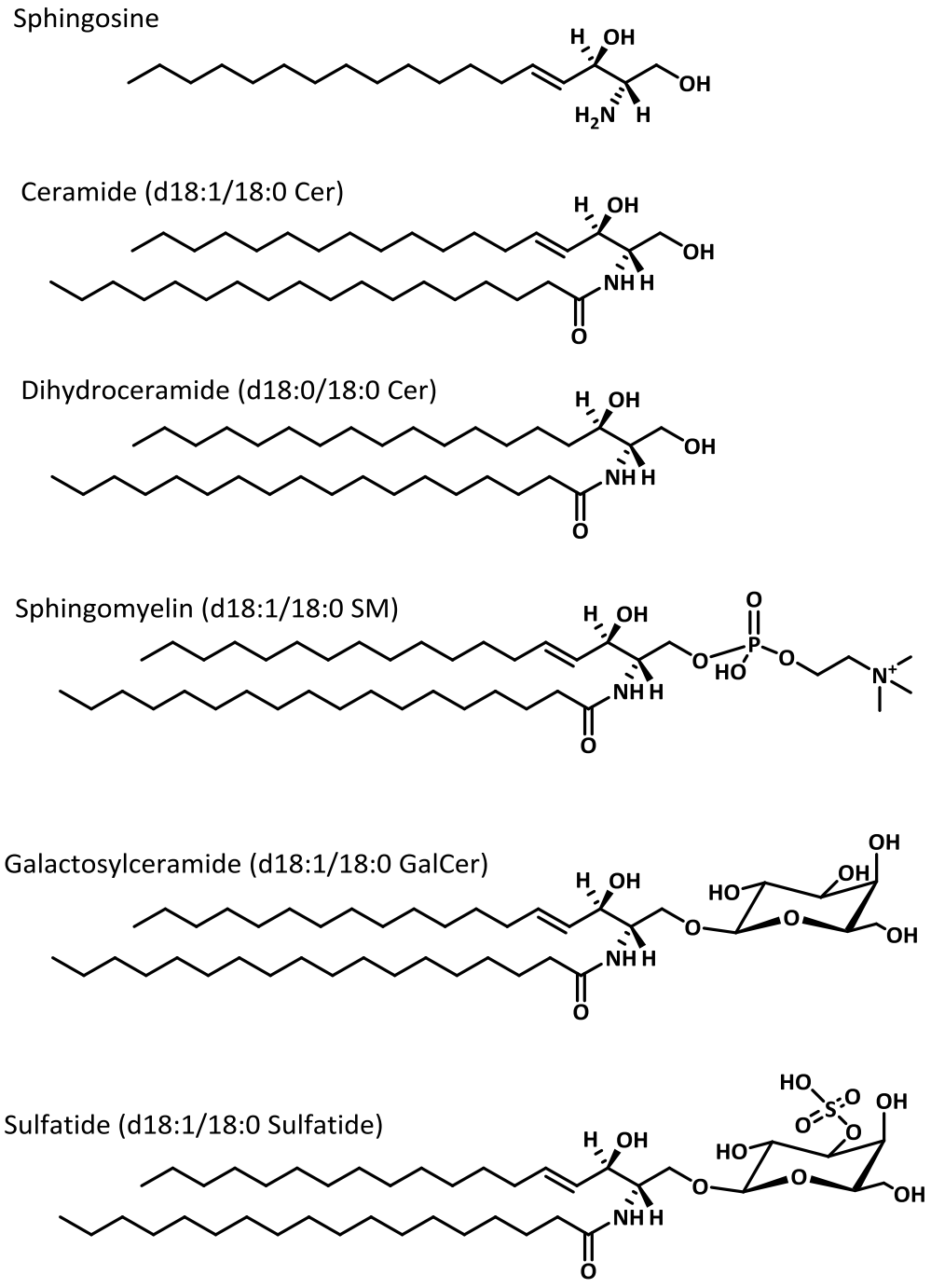


Figure 12. Structures of sphingolipids.

5. Sterols lipids (ST)

The class of sterols lipids, such as cholesterol and its derivatives, constitutes an important component of membrane lipids, together with sphingomyelins and glycerophospholipids as already described.[34] The sterol class is divided into subclasses primarily on the basis of

biological functions. The core structure of the majority of the sterol species, or from which the sterol species are derived, is constituted of seventeen carbon atoms, linked in four fused rings: three cyclohexane rings and one cyclopentane, respectively named rings A, B, C and D (**Figure 13**). Different functional groups can be attached to the ring D and the oxidation state of the rings can change to generate the wide variety of sterols lipids. Cholesterol and its derivatives represent a large part of the sterol lipids. Cholesterol is very abundant in plasma membranes, playing a role on the fluidity of the membranes. At physiological temperature, it is involved in the rigidity of the membrane and forms, together with sphingolipids, the lipid rafts.[35,36] Cholesterol is also a very important precursor in the biosynthesis of other sterol lipids such as steroid hormones, oxysterols, and bile acids. In fungi, ergosterol is a crucial sterol lipid found as the major constituent of plasma membranes and thought to be the equivalent of cholesterol in these organisms.

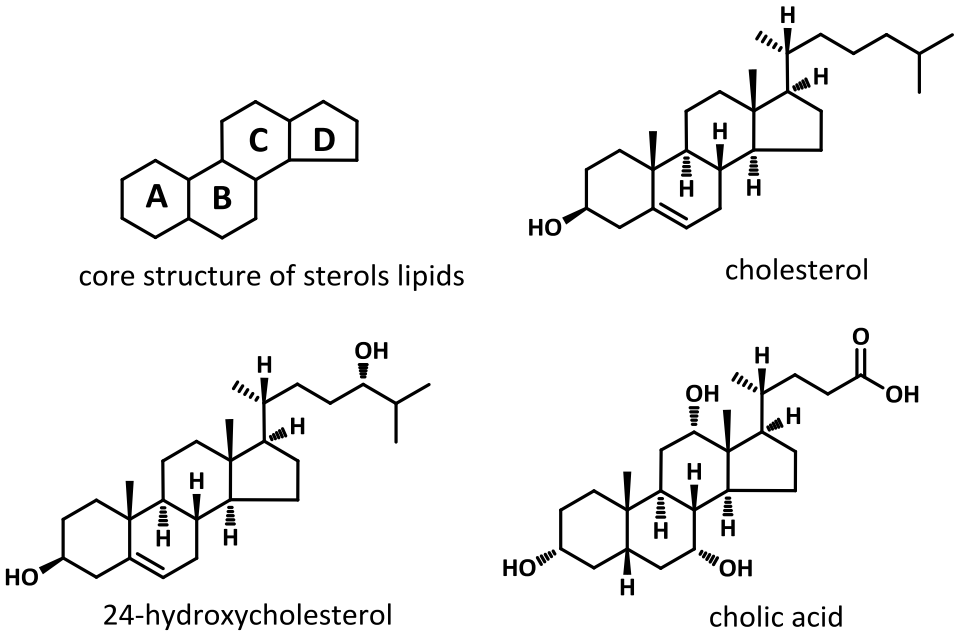


Figure 13. Structure of sterol lipids.

6. Prenol lipids (PR)

Prenol lipids are synthesized from isoprene units, which is a common 5-carbon organic compound (**Figure 1**). Some simple isoprenoids are synthesized by the successive addition of

C5 isoprene units (e.g. β-carotene) while for others the biosynthesis is more complex (e.g. quinones, polyprenols…). Among these polyisoprenic prenol lipids, fat-soluble vitamins such as vitamin A, E or K (**Figure 14**) are very important and deficiencies have been linked with increased risks of several pathologies such as cancer, diabetes…[37]

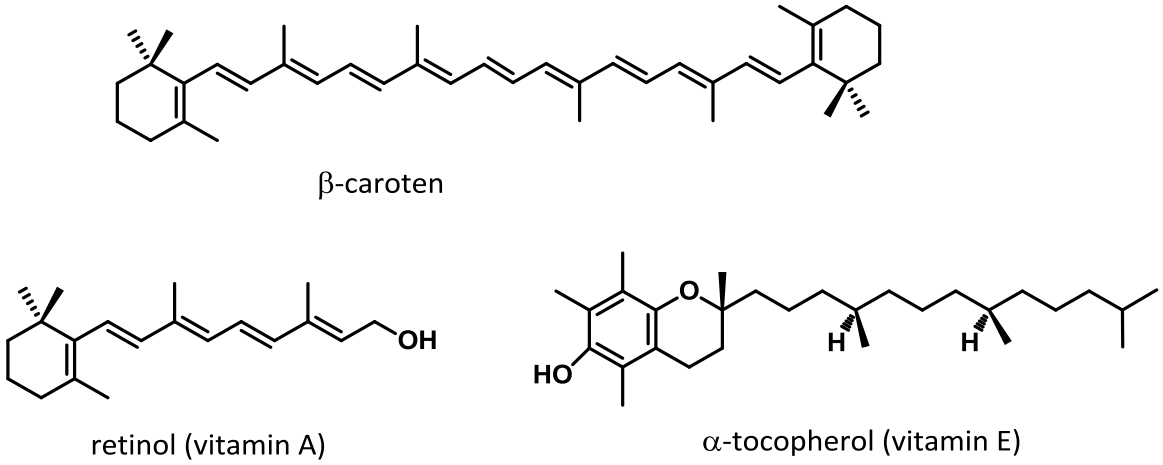
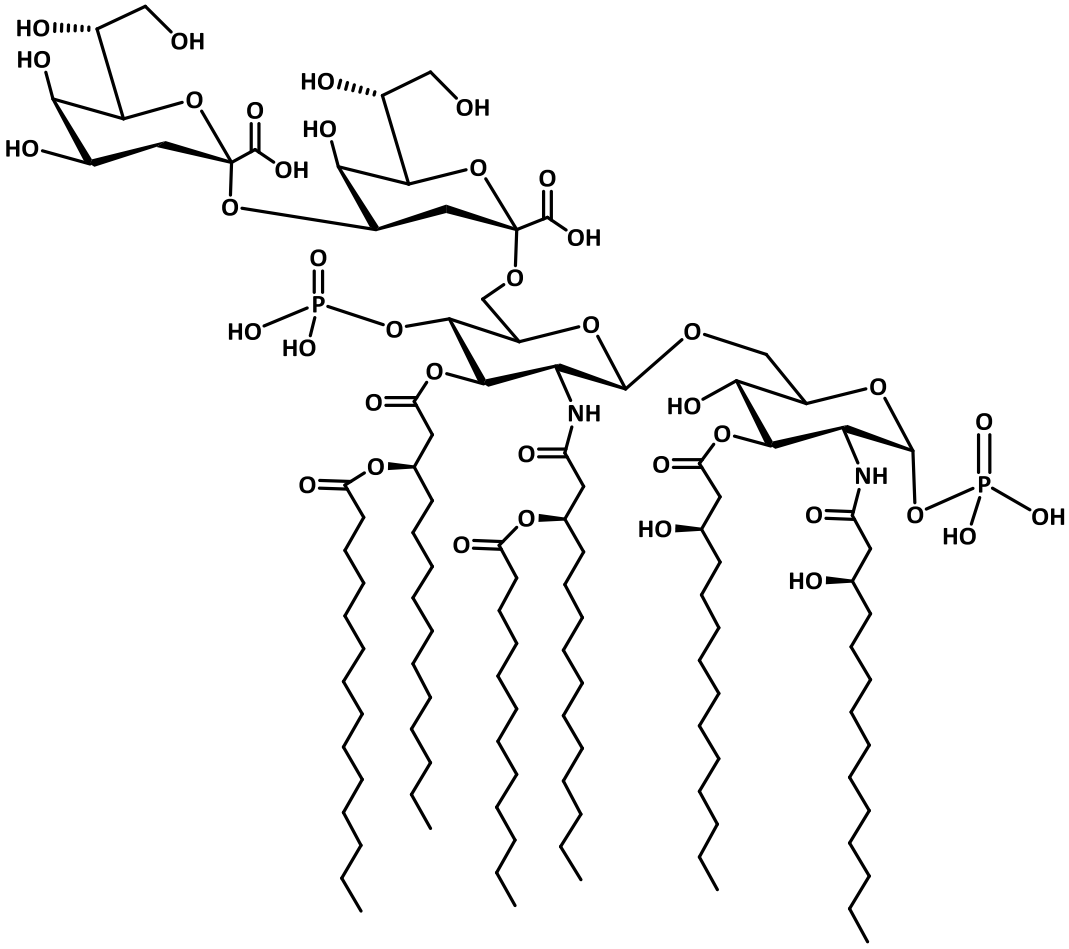


Figure 14. Structure of prenol lipids.

7. Saccharolipids (SL)

Saccharolipids can be described as compounds in which fatty acids are directly associated to a large sugar scaffold (**Figure 15**). This link forms structures compatible with membrane bilayers. Indeed, in saccharolipids, a sugar mimics the glycerol backbone in glycerolipids and glycerophospholipids. SL are generally observed in plants and microorganisms. A well-known saccharolipid is Kdo_2 lipid A which is the essential component of lipopolysaccharides in most Gram-negative bacteria and a well-known toll-like receptor 4 ligand.[38,39]



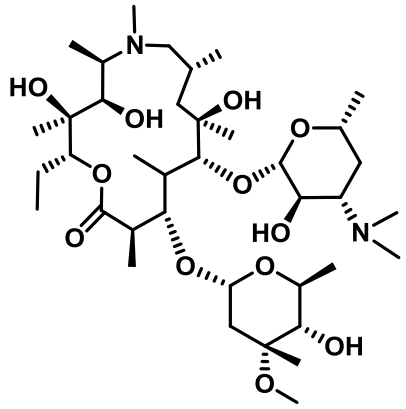
Kdo2-Lipid A

Figure 15. Structure of saccharolipids.

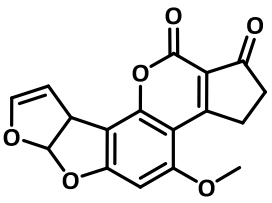
8. Polyketides (PK)

Polyketides comprise a very large group of metabolites and natural products from animals, plants and microbial sources with a great structural diversity (**Figure 16**). They usually come from the condensation of acetyl or malonyl units (**Figure 1**) by polyketide synthase and they represent the lipid class with the highest heterogeneity.

Many commonly used anti-microbial or anti-parasitic compounds are polyketides and polyketides derivatives (e.g., tetracyclins, azithromycin etc) but other compounds could also be considered as mycotoxins (e.g. aflatoxin B1).[40] Polyketides backbones are generally modified by methylation, hydroxylation, glycosylation or oxidation.



azithromycin



aflatoxin B1

Figure 16. Structure of polyketides.

The key challenges for the coming years will be to determine the functions of all the lipid species already described, but also the one yet to be discovered.

IV. Bioactive lipids

As previously mentioned, lipids are not only confined to membrane structure or used for energy metabolism but can act as *bona fide* mediators. This progressively led to the concept of bioactive lipids. In 2008, this concept of bioactive lipids was described by Hannun et al. as "changes in lipid levels that result in functional consequences".[41] This is a quite large definition that could be limited by saying that "*bioactive lipids are lipids whose changes in levels result in functional consequences mediated by interaction with molecular targets*". This lipid signaling can involve activation of receptors such as G protein-coupled receptors, nuclear receptors, but also ion channels. Moreover, lipids from several different lipid categories have been identified as cellular messengers or signaling molecules in numerous physiological processes.

Over the years, bioactive lipids (some examples are presented in **Figure 17**) have been shown to play key roles in numerous physiopathological situations, from regulation of immune response and inflammation to cell proliferation and cancer to name a few.[42-49]

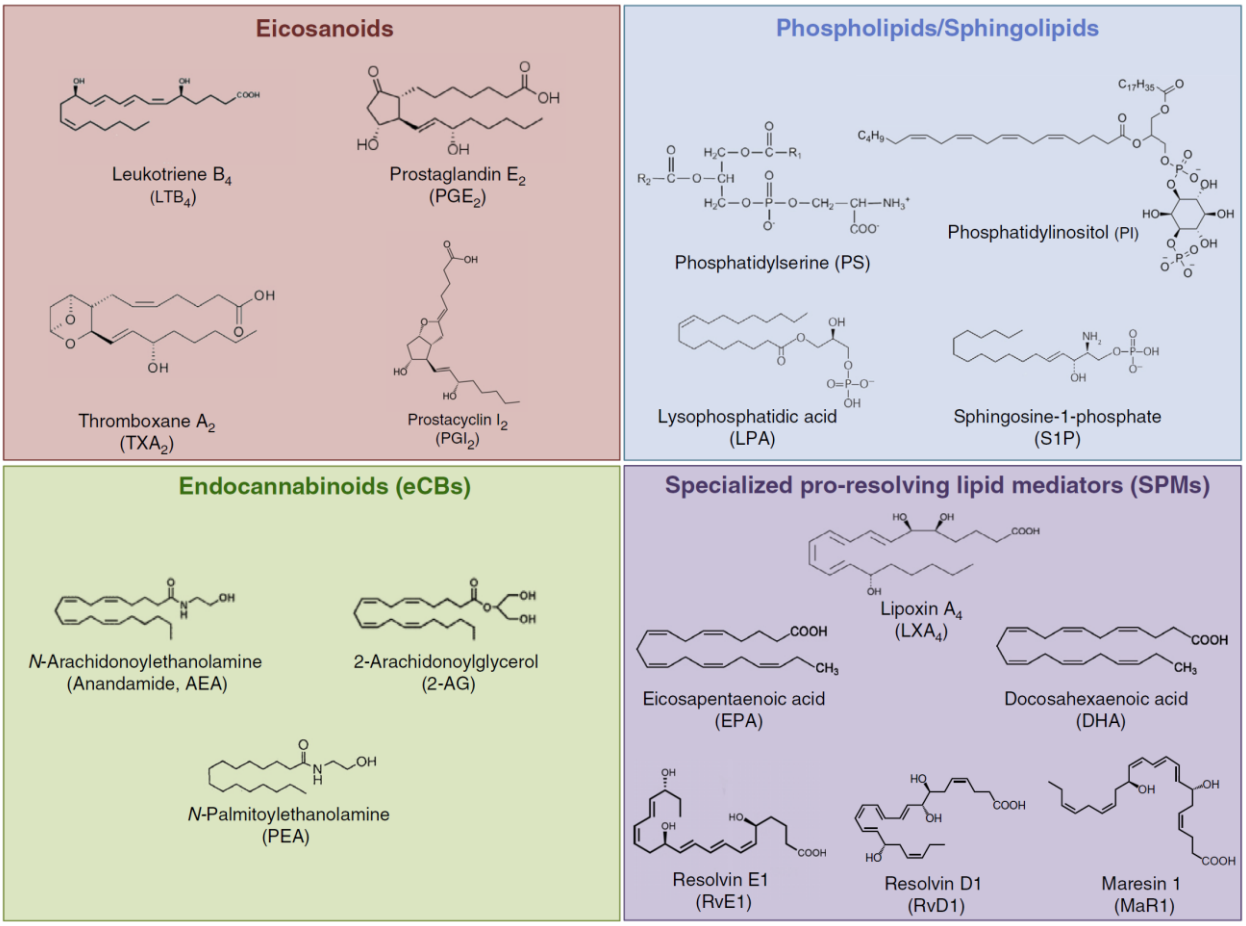


Figure 17. Structures of some representative bioactive lipids. (Adapted from [43])

Because of the large number of bioactive lipids, a full description of all the bioactive lipids is not possible, and out of the scope of this work. Thus we will here briefly illustrate this concept of bioactive lipid signaling by summarizing three typical examples. These examples were chosen because we participated in their quantification and study during this work.

(a) sphingosine-1-phosphate

(b) endocannabinoids and related compounds

(c) oxysterols

(a) Sphingosine-1-phosphate, S1P, is a sphingolipid derived from ceramide and perhaps represents the simplest example of bioactive lipid with a pattern of lipid signaling composed of (i) a bioactive lipid, (ii) a regulated enzyme and (iii) specific downstream targets (**Figure 18**).[41] The expression of cytokines or growth factors can lead to the activation by phosphorylation of sphingosine kinase resulting in formation of S1P from sphingosine. This bioactive lipid, intracellularly generated, binds and activates at least five GPCRs - termed S1PR1-5 - initiating intracellular calcium signaling.[50,51] Among other functions the role of S1P and S1P receptors signaling are well characterized in immune cells trafficking and activation of the innate and adaptative immune systems.[52] S1P has also emerged as a potent bioactive lipid mediator, associated to several cellular processes such as cell proliferation, apoptosis and several diseases (e.g. multiple sclerosis).

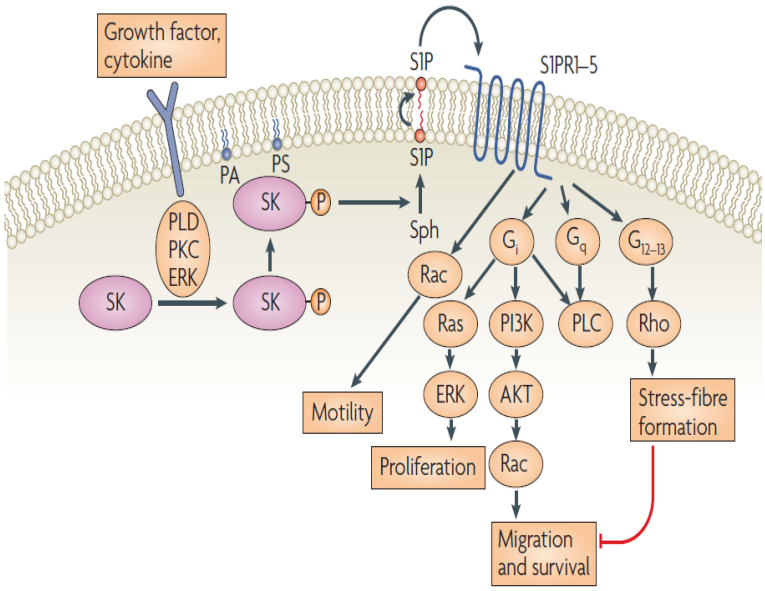


Figure 18. Mechanism of action of S-1-P. (Adapted from [41])

(b) Endocannabinoids and related compounds are also good candidates to illustrate the concept of bioactive lipids. This family of lipids is mainly represented by 2-arachidonoylglycerol (2-AG) and *N*-acylethanolamines such as *N*-arachidonoylethanolamine (commonly known as anandamide (AEA)), *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA). These lipid mediators

are produced from membrane precursors via specific enzymes. *N*-acylethanolamines are produced either directly through the hydrolysis of *N*-acylphosphatidylethanolamines (NAPE) by a phospholipase D (NAPE-PLD) or indirectly via the sequential action of several phospholipases. 2-AG is mainly the result of the hydrolysis of diacylglycerols by diacylglycerol lipases (DAGLα and β). The activity of these bioactive lipids is dependent on their endogenous levels and is mediated by several receptors as illustrated in **Figure 19**. The signaling of these bioactive lipids is mainly terminated upon hydrolysis by several lipases. Thus, *N*-acylethanolamines can be hydrolyzed by the serine hydrolase fatty acid amine hydrolase (FAAH) and by the cysteine amidase, *N*-acylethanolamine hydrolyzing acid amidase (NAAA). On the other hand, 2-AG is hydrolyzed by two different serine hydrolases, α/β hydrolase domain 6 (ABHD6) and monoacylglycerol lipase (MAGL).[53]

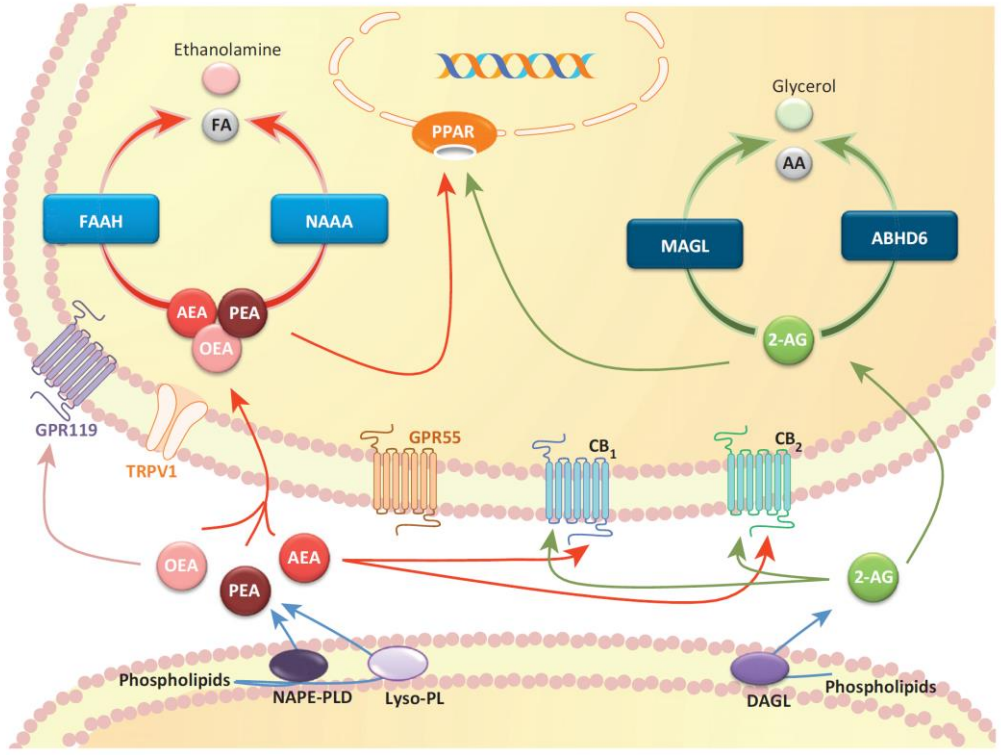


Figure 19. Endocannabinoids in a nutshell. (Adapted from [54]) Several receptors are implicated in the endocannabinoids effects such as the CB1 and CB2 cannabinoid receptors for AEA and 2-AG or GPR119 for OEA, peroxisome proliferator-activated receptor (PPAR) for AEA, OEA, PEA and 2-AG or channel receptor TRPV1 for AEA.

Endocannabinoids, but also related compounds such as PEA or OEA, are ubiquitously produced and are used as a homeostatic system controlling several physiopathological states

that maintain human health.[55] These lipids have already been shown as having numerous effects including in controlling the immune system[56], inflammation[54,57], food intake[58]…

(c) Oxysterols, which are 27-carbons cholesterol-related structures, are now considered per se as bioactive lipids.[59] They are different from cholesterol since their structures include one or several additional oxygenated function(s). Besides being an intermediate between cholesterol and bile acids, they are able to regulate several processes by binding to specific receptors. Oxysterols can be produced either by enzymatic (mainly involving the CYP450 enzymes super-family) or through non enzymatic pathways (**Figure 20**).[60,61] A large number of enzymes is involved in oxysterols' metabolism including oxydoreductases, hydrolases and transferases. As for many bioactive lipid families, oxysterols can act through several receptors such as GPCR (e.g. Epstein-Barr virus-induced gene 2 (EBI2)) or nuclear receptors (such as liver X receptor (LXR)) and via regulatory transporters or proteins. The implication of oxysterols has already been shown in the regulation of numerous physiopathological processes such as immune functions[62], brain homeostasis[63], inflammatory processes[64], obesity[65,66]or Alzheimer disease[67,68].

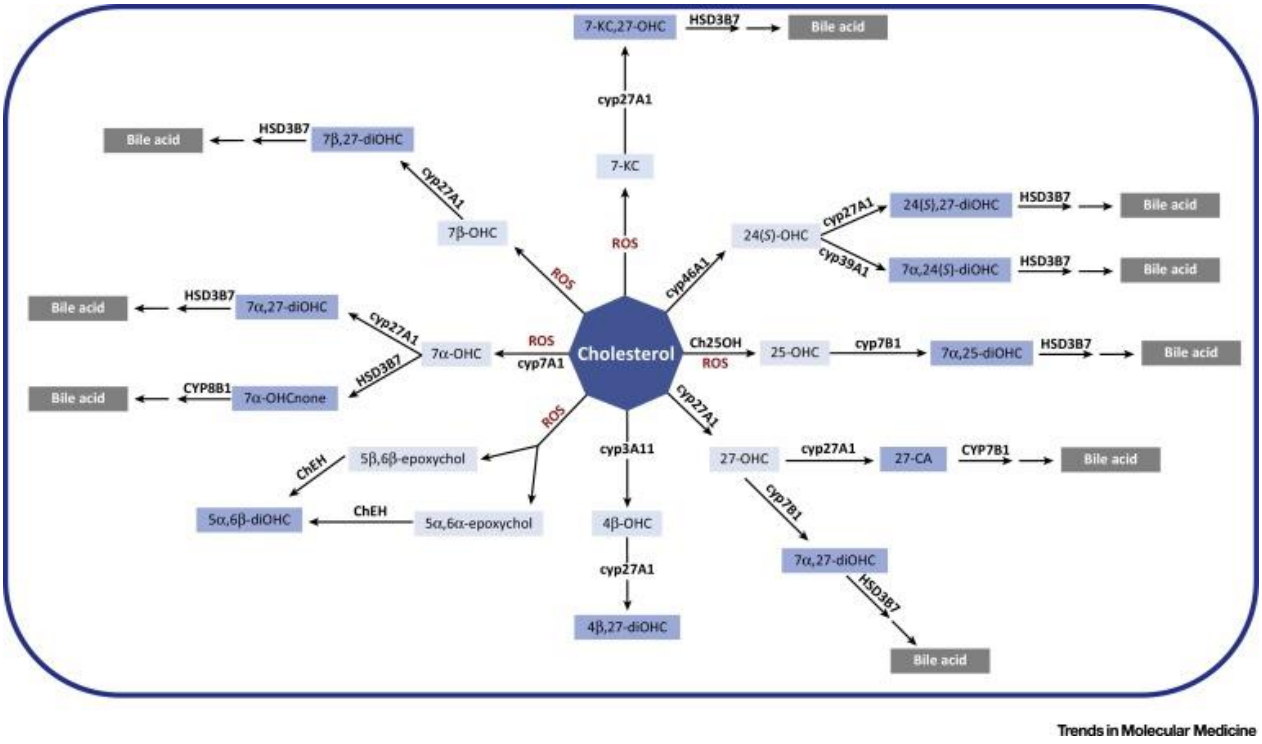


Figure 20. Summarized view of oxysterol metabolism in mouse liver. (Adapted from [59])

The elements outlined in this chapter clearly show that a full understanding of any given (patho)physiological processes can only be reached if taking into account lipid signaling. Moreover, we are almost at a point where we should assume that lipids with regulated levels are bioactive until the contrary is proved. The complexity of lipid metabolism and the metabolic interconversion of a lot of bioactive lipids provide to the cell a large array of possibilities for generating signals and responses. Therefore, given this diversity, a lot of work is still necessary to fully unravel all the pathways, to study all the roles, and to describe the underlying mechanisms by which a given bioactive lipid is responsible for an effect.

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Part 2. Lipids and inflammation

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I. Inflammation

Inflammation is considered as a pathophysiological response against an infection or tissue damage: it is a crucial component of the host response to injuries or infections as its main goal is to restore homeostasis. After harmful stimuli (pathogens, irritants, dead cells…), a tightly regulated response is initiated. This inflammatory response includes multiple interactions between and among several cell types. The classical symptoms associated with these responses are local reactions described as the cardinal signs of inflammation including heat, redness, swelling, pain and loss of function (**Figure 1**).

The initiation of the inflammatory process can arise from numerous different stimuli. Among them, two distinct molecular specificities of the innate immune response are described: pathogen-associated molecular patterns (or PAMPs) and damage-associated molecular patterns (or DAMPs). On the one hand, PAMPs are often derived from microorganisms, they are structurally conserved among microbial species, and are identified by pattern recognition receptors (PRR) present on cells of the innate immune system, as well as on many epithelial cells. On the other hand, DAMPs are endogenous molecules released by damaged cells. They are implicated in response to ischemia, trauma and tissue damage. Similarly to PAMPs they are recognized by PRR. The most frequently described PAMPs are bacterial lipopolysaccharides (LPS) (endotoxins found on the cell membranes of gram-negative bacteria), flagellin (the main component of bacterial flagellum), lipoteichoic acid (a major constituent of the cell wall of gram-positive bacteria) or nucleic acids. DAMPs include intracellular proteins such as HMGB1 (high-mobility group box 1) but also non-protein compounds as uric acid.[1] Moreover, some PAMP–DAMP partnerships have been shown: PAMPs can interact with DAMPs or DAMPs can act as PAMP transporters for instance.[2] During this work, we used LPS to induce an immune response *in vivo* or to activate macrophages *in vitro*. The PRR for LPS is Toll-like receptor 4 (TLR4), a transmembrane protein whose activation drives to an intracellular signaling pathway leading to transcription of pro-inflammatory genes under the control of the transcription factor NF-κB and inflammatory cytokine production, responsible for the activation of the innate immune

system.[3] TLR4 is expressed in numerous cells such as endothelial cells, cardiac myocytes and cells of the central nervous system.[4] TLR4 activation, most often via MyD88 (myeloid differentiation primary response gene (88))-dependent pathways, will lead to the activation of the MAPK cascade and the transcription of pro-inflammatory cytokines genes.[5,6]

The response to acute inflammation is protective and is an organized process allowing the repair of injured tissues, but also the elimination of invading organisms leading to complete resolution to restore the homeostatic state. During all this process, the balance between anti- and pro-inflammatory mediators governs the persistence and the severity of the response and eventually the resolution.[7] Insufficient inflammation can provoke persistent infection of pathogens while excessive inflammation can lead to many chronic diseases including vascular diseases, metabolic syndrome, neurological diseases and many others. Indeed, because inflammation is developed as a favorable strategy for the host in reaction to a potential danger, the inflammatory response is commonly completed once this potential danger is eliminated. Usually, the return to the homeostasis proceeds rapidly but if the resolution of the inflammatory state is unsuccessful for any reason, the acute inflammation can convert into a more chronic state.

The process of inflammation can be divided into different phases including (i) the early phase of the inflammatory response after recognition by mast cells and macrophages of a damaging stimulus, (ii) the vascular phase leading to vascular modifications such as increased permeability and vasodilatation promoting the recruitment of immune cells.[8] The activation of these resident immune cells results in the release of pro-inflammatory mediators including cytokines, lipid mediators - such as prostaglandins - but also bioactive amines such as serotonin or histamine. Under the influence of these mediators, the endothelial cells are activated promoting the extravasation of monocytes and polymorphonuclear granulocytes (such as neutrophils) from blood vessels to the site of tissue injury. Monocytes are further differentiated *in situ* into macrophages. Polymorphonuclear neutrophils (PMN) are the most abundant white blood cells and they are able to very rapidly converge to the site of infection. First of all, they are able to ingest microorganism and particles by phagocytosis and are also able to release reactive oxygen species (ROS) leading to the decrease of harmful stimuli. However, PMN are also very rapidly (within hours) starting the resolution process.[9] Indeed, they stimulate the switch of

arachidonic acid metabolism from pro-inflammatory lipid mediators to resolutive mediators. Leukotrienes and prostaglandins are responsible for the amplification of the acute inflammation whereas lipoxins have only pro-resolution activities.[10] This switch in arachidonic acid-derived metabolites leads to the inhibition of neutrophil recruitment and neutrophil apoptosis and it is crucial for the evolution from inflammation to resolution. Actually lipoxins are responsible for the inhibition of the recruitment of neutrophils, but also for the promotion of the recruitment of monocytes which eliminate debris and dead cells before initiating the tissue remodeling.[11] Pro-inflammatory macrophages can secrete ROS, but also release different cytokines such as the necrosis factor-α (TNF-α) to eliminate the pathogens or the source of tissue injury. Similarly to the PMN, in the context of host defense, pro-inflammatory macrophages are able to switch during the inflammation process, into alternatively activated macrophages secreting anti-inflammatory cytokines leading to basal tissue homeostasis.

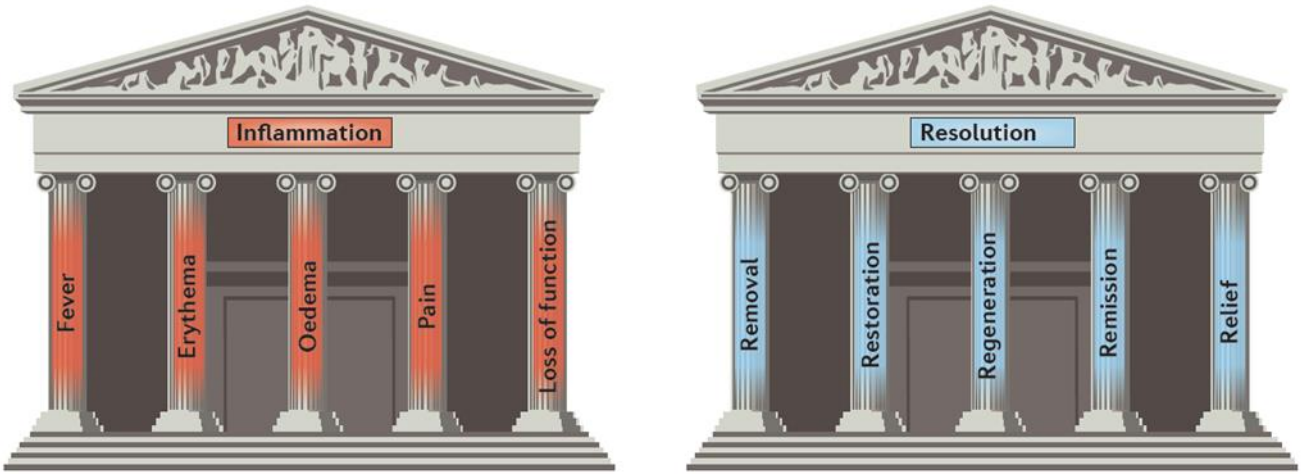

Figure 1. Cardinal signs of inflammation and its resolution. (Adapted from [12]) The five pillars of inflammation refer to the heat/fever (*calor*), pain (*dolor*), redness/erythema (*rubor*), swelling/oedema (*tumor*) and loss of function (*function laesa*) that are the main clinical symptoms of inflammation. Nowadays resolution is recognized as the last active step in the whole process of inflammation. For resolution too we can distinguish five pillars including remission of fever, restoration of perfusion and vascular integrity, removal of microbes, dead cells and other debris, relief of pain and regeneration of tissue.

A favorable acute inflammatory response consists in the complete eradication of the threat (infectious agents, injury…) followed by a resolution/repair phase, mediated by macrophages, including clearance of pathogens and pro-inflammatory debris, removing of

neutrophils by macrophages, recruitment of regulatory T cells as well as repair of tissue structure and function.

II. Resolution, a key event to end acute inflammation

Following macrophage and mast cell activation and the release of pro-inflammatory cytokines, chemokines, and vasoactive amines, neutrophils are attracted by chemokines, and exert potent antibacterial killing mechanisms after migration through the endothelium. At this stage, the balance between pro- and anti-inflammatory mediators should be equilibrated to avoid a failed outcome such as chronic inflammation (**Figure 2**). Indeed, this inflammatory reaction must be quickly resolved, to avoid more severe tissue damage. If the immune system remains overstimulated, with an excessive response to the initial threat or if the infectious agent is not completely eliminated, chronic inflammation and linked diseases can follow acute inflammation.

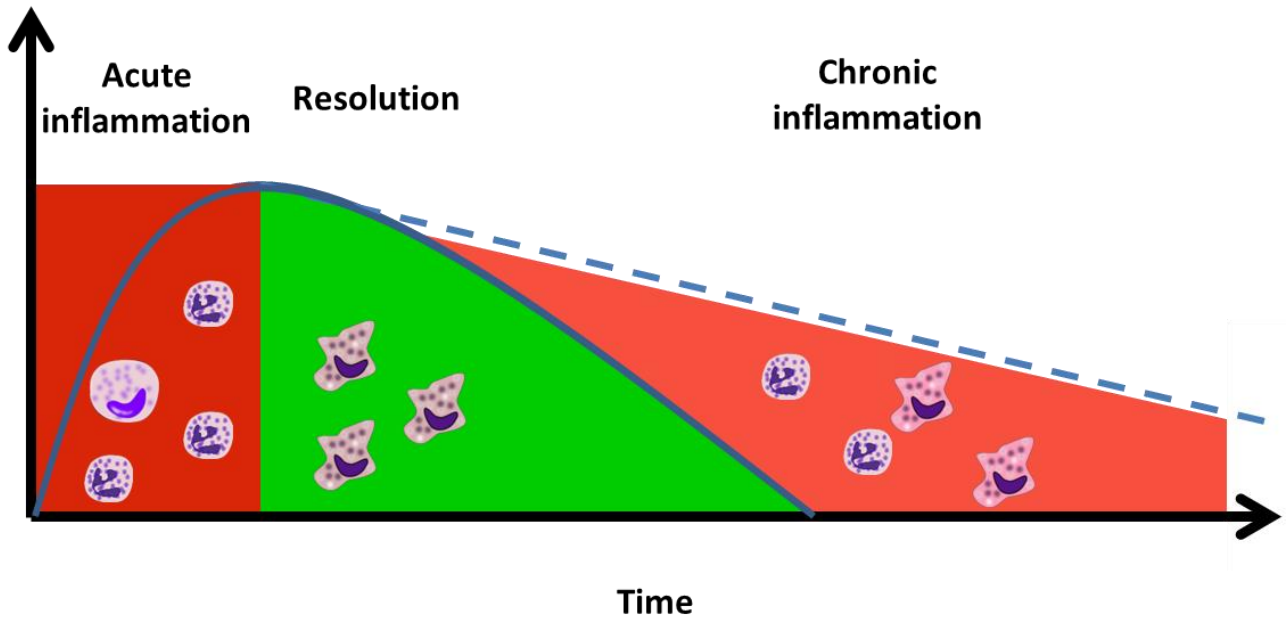

Figure 2. Evolution of acute inflammation. Depending on the mediators implicated in the resolution and on the balance between pro- and anti-inflammatory mediators the pathological consequences of an acute inflammation can be drastically different.

Even if in the past it was supposed that the resolution of inflammation was a "passive process" with a decrease of the chemokine gradients over the time leading to a reduced recruitment of the leukocytes to the site of injury, it is now well established that the

resolution of inflammation is a fully elaborated active process to avoid chronic inflammation. The resolution of inflammation is thus a complex process including a number of different parameters needed to reach basal homeostasis such as the cessation of the neutrophils tissue infiltration and the following induction of apoptosis in these cells and their efferocytosis by macrophages, the normalization of chemokines gradient and cytokines or the differentiation of macrophages to “regulatory” macrophages with an anti-inflammatory activity.

Moreover, several pro-resolving mediators and among them “specialized pro-resolving lipid mediators” such as lipoxins, resolvins, protectins or maresins but also proteins such as annexin A1 or gaseous mediators are implicated in the process of resolution.[13-15] The effects of these pro-resolution mediators are broad and mediated by the nature of their storage and production: for instance, annexin A1 is a “prestored” protein released by cells during the resolution process while lipoxins are pro-resolving lipids produced on-demand via transcellular biosynthesis.

PMN are important players in resolution. For instance, in models of neutrophils depletion, wound healing has been shown to be delayed and in ulcerative colitis an exacerbation of the disease was found.[16,17] Because neutrophils are primarily involved in the inflammatory reaction, the inhibition of this neutrophil influx is required to avoid the continuity of the inflammatory state. To reach this depletion of neutrophils, chemokine and cytokines should be regulated and pro-inflammatory mediators switched into pro-resolution mediators. After the necessary recruitment of inflammatory cells, mainly neutrophils, a crucial step of resolution is ensuring the clearance from the site of injury. These developed clearance mechanisms are necessary to prevent an improper activation which could lead to a host tissue injury. These global processes being responsible of the removal of neutrophils include local efferocytosis, apoptosis and retrograde chemoattraction to the vasculature. For instance, efferocytosis of apoptotic neutrophils is considered as a pro-resolving event and acts to the prevention of neutrophil lysis with a linked release of inflammatory and cytotoxic components.[18]

Macrophages are also key players in inflammatory responses and in the resolution of inflammation, having numerous implications in the pathophysiology of several chronic

diseases. The behavior of phagocytes and their phenotype is dependent of specific stimuli coming from the extracellular medium. Two main types of macrophages coexist: resident macrophages and inflammation activated-macrophages. Resident macrophages such as Kupffer cells and alveolar macrophages are considered as mature macrophages, located in precise areas in the body performing immune surveillance functions. They maintain homeostasis recruiting immune cells in response to inflammatory stimuli and phagocytosing dead cells and cellular debris. Monocytes can be recruited from the circulation following infection or tissue injury and differentiate in macrophages, having a crucial role in host defense.[19]

The efferocytosis, which prevents the release of intracellular content of neutrophils, is also a crucial function of macrophages and induces their phenotypic switch from a "pro-inflammatory" M1 phenotype to an "anti-inflammatory" M2 phenotype.[20] During the resolution phase, anti-inflammatory macrophages change their metabolism of lipids from a pro-inflammatory pattern (generating prostaglandins and leukotrienes for instance) to a pro-resolving pattern producing lipoxins, resolvins or protectins, as they switch from the M1 to the M2 phenotype. M2 macrophages are also able to produce anti-inflammatory cytokines such as interleukin 10 (IL-10), inhibiting pro-inflammatory cytokine production (such as IL-1β).

Thus as mentioned, the active resolution of inflammation is a key process to avoid chronic inflammation, a state occurring when acute inflammatory response mechanisms have been unsuccessful in the elimination of tissue injuries, leading to excess tissue damages, dysregulation of tissue healing and having implication in several disease states, including obesity, cancers, multiple sclerosis or inflammatory bower diseases. In this process lipid meditators play a key role.

III. Specialized pro-resolving mediators

Specialized pro-resolving mediators (SPM) are lipids derived from several fatty acids playing an important role in the resolution of tissue inflammation. These lipid mediators, being part of the pro-resolving molecules, promote host defense restoring tissue homeostasis after infectious stimuli or injury. Among these compounds, lipoxins, resolvins, protectins and

more recently identified maresins are now considered as potent players in the regulation of acute inflammation.[21,22]

Several lipid mediators enzymatically synthesized from fatty acids play a key role in the process of inflammatory response (**Figure 3**). For instance, polyunsaturated fatty acids (PUFA) as arachidonic acid (AA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are well described as precursors of inflammation modulators.[23] Among these, eicosanoids are crucial players in inflammation: prostaglandin E2 (PGE_2) and leukotriene B_4 (LTB_4) are implicated in the first steps of the process of inflammation in the promotion of early vascular permeability and in the stimulation of leukocyte chemotaxis, respectively. They are very rapidly synthesized –within minutes of acute inflammation– by leukocytes from arachidonic acid, via cyclooxygenase or lipoxygenase. However, after a certain time -within hours- the resolution of inflammation is accompanied by an active switch in the arachidonic acid-produced mediators and it is converted into pro-resolving mediators such as lipoxins.[24] In the same time, the importance of essential omega-3 polyunsaturated fatty acids, such as EPA and DHA was also described since their bioactive metabolites(resolvins, protectins and maresins) are also well implicated in the resolution of inflammation.[25]

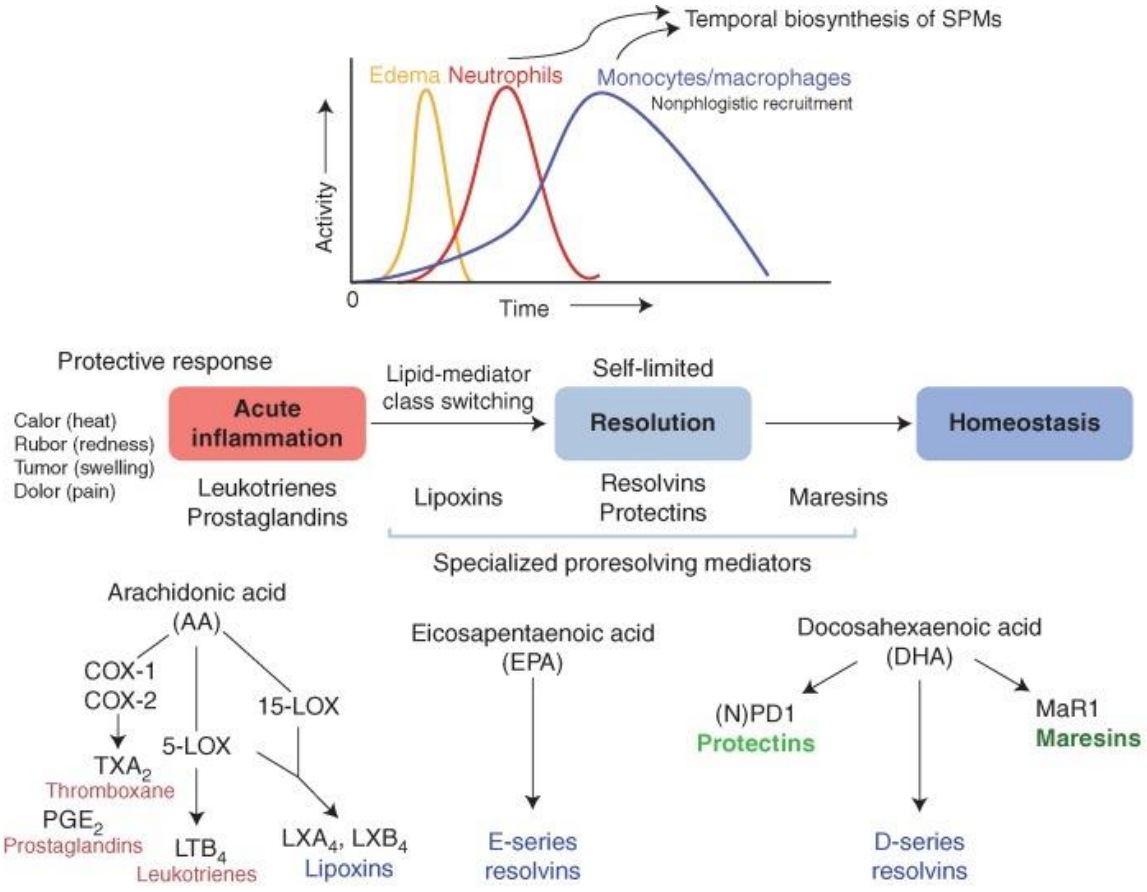

Figure 3. Lipid mediators synthesis. (Adapted from [26]) During the resolution of inflammation, specialized pro-resolving mediators (SPM) are synthesized from polyunsaturated fatty acids to restore previous homeostasis. Of note the time scale is dependent on the time of inflammatory process.

Lipoxins (LX) are generated from arachidonic acid by lipoxygenases (LOX) such as 5-LOX, 15-LOX or 12-LOX via formation of 15-hydroxyeicosatetraenoic acid as intermediate. After the initial formation of leukotrienes, simultaneously to the increase of PMN infiltration, PGE_2 and PGD_2 are firstly increased. Then, the general profile of lipid mediators is switched from these pro-inflammatory eicosanoids to lipoxins under the stimulation of 15-LOX by PGE_2 and PGD_2. LX are implicated in the regulation of leukocyte trafficking and administration of LXA_4 was shown to decrease PMN transmigration, pro-inflammatory cytokine production and PMN infiltration to inflamed sites. LX are also involved in the stimulation of IL-10 production in macrophages and in the improvement of the resolution by stimulating neutrophils phagocytosis by macrophages. Moreover, LX also promote the resolution by postponing the apoptosis of macrophages since they are required to phagocyte dead cells and pathogens at

the site of infection: the increase in survival of macrophages helps the "cleaning" of the inflammation site.[27]

Resolvins (RV) can be synthesized from either DHA or EPA and are therefore divided into two different categories as members of D-series or E-series generated from DHA and EPA, respectively. Both series are generated by 5-LOX via 18-hydroxyperoxyeicosapentaenoic acid and 17-hydroxydecosahexaenoic acid from EPA and DHA, respectively. The E-series comprise two bioactive mediators, RvE1 and RvE2 and D-series comprise four compounds, RvD1, RvD2, RvD3 and RvD4. Resolvins are involved in the inhibition of neutrophils accumulation in sites of inflammation: via the enhancement of their clearance but also via the blockade of their transendothelial migration. They have also been described as stimulants of macrophages for the ingestion of apoptotic neutrophils.[28]

DHA also serves as a precursor for the biosynthesis of protectin D1 (PD1), also termed neuroprotectin D1 when produced by neural tissues.[29] PD1 is produced from DHA by 15-LOX and this 10,17S-docosatriene is reported to be protective in several experimental models such as Parkinson's or Alzheimer's disesase.[30,31] Moreover, PD1 has resolutive effect by inhibiting neutrophil migration and toll-like receptor-mediated activation and by decreasing pro-inflammatory lipid mediator levels.

More recently, in 2009, a new lipid mediator biosynthetic pathway was identified, involving enzymatic conversion of DHA by macrophages during the resolution of inflammation. This new family was called maresins for "macrophage mediators in resolving inflammation". Maresin 1 (MaR1) is a potent pro-resolution compound, with implication in the limitation of neutrophil infiltration as well as the enhancement of macrophage uptake of apoptotic PMN.[32]

Many recent studies have shown the importance of pro-inflammatory and pro-resolutive lipids in the induction and resolution of inflammation through their action on leukocyte trafficking, efferocytosis and leukocyte clearance for instance. They should therefore be considered as key elements in the resolution process.

IV. Lipid analysis and inflammation

All the lipids contained in a biological system, known as the lipidome, can be analyzed in a same single run using lipidomics analysis, an emerging "omics" science. The qualitative and quantitative analysis of a majority of cellular lipids is now possible with recent advances in mass spectrometry (See Part 3 and more detailed discussion about lipid analysis). However, even if the key role of several lipids in cells and tissues has already been demonstrated in several studies showing the implication of the lipid metabolic pathways in numerous diseases, the complex composition and complicate functions of the lipidome is not yet well understood.

Using global analysis methods allows obtaining a more complete data set of lipid the levels of which are altered upon inflammatory process. This could help identifying lipids playing important roles during the inflammatory process.

For instance, Dennis et al. characterized lipidomics responses upon macrophage activation using a TLR4 ligand (Kdo). The global lipidome was studied and multiple course-times were investigated. Via lipid analysis by dynamic quantitative mass spectrometry, they showed immediate responses in fatty acid metabolism (illustrated by increased eicosanoid synthesis) leading to prostaglandins production, implicated in the beginning of the inflammation, but also delayed responses characterized by sphingolipid and sterol biosynthesis (**Figure 4**).

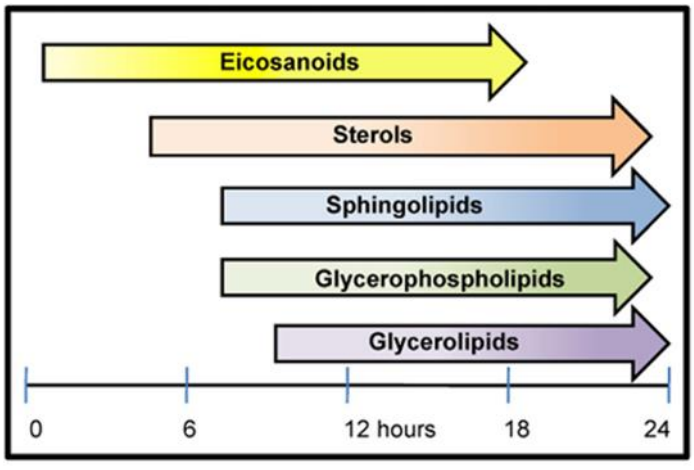

Figure 4. Temporal changes in lipid categories in response to an inflammatory stimulus. (Adapted from [35]) Temporal profile of quantitative modifications for some lipid species over a 24h period upon TLR4 agonist stimulus: the arrows show the time periods during which statistically significant modifications were observed.

Moreover, lipid remodeling of glycerophospholipids, prenols and glycerolipids indicate that macrophage activation by inflammatory mediators leads to alteration in several mammalian lipid categories. In this study, they extend the general knowledge of the macrophage lipidome and its response to an inflammatory stimulus by monitoring quantitatively the modifications in the main lipids during the response of murine macrophages (**Figure 5**).

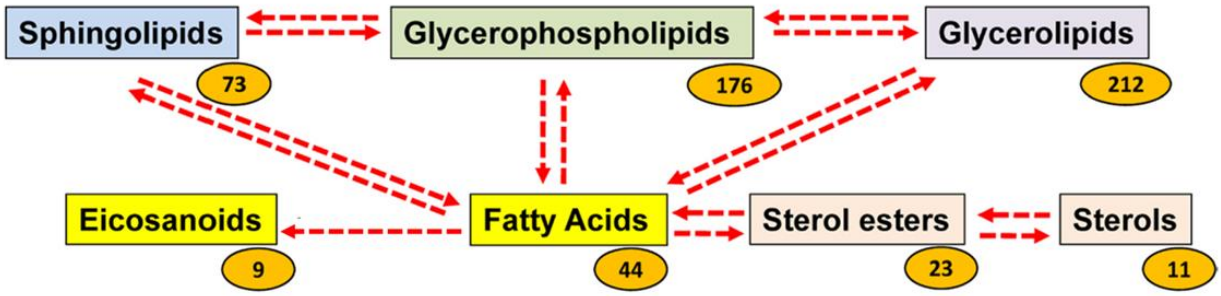

Figure 5. Macrophage lipid metabolism. (Adapted from [35]) Values in orange circles represent the number of lipids within each lipid category that were quantified by LC-MS in macrophages in response to stimulus.

Using these protocols dedicated to lipid analysis by mass spectrometry, several novel lipids associated with macrophage cells were identified: uncharacterized species of phospholipids such as PC and PE containing very long polyunsaturated fatty acids but also ether-linked phosphatidylinositols and phosphatidylserines were detected in mouse macrophages.[36]

As summarized here, inflammation is a complex process which is needed to ensure a positive end following tissue damage or infection. The resolution of inflammation is a complex active phase needed to avoid a dysregulation of the balance between pro- and anti-inflammatory mediators leading to chronic inflammation and related diseases. Lipids are potent signaling molecules and they are involved in the regulation of a multitude of cellular responses, especially in the context of inflammation. During the resolution, specialized pro-resolving mediators have been demonstrated to be crucial for the return to the homeostatic state. Moreover, the lipid analysis in the context of inflammation led to the discoveries of new uncharacterized compounds in the eicosanoid pathway but also in other lipid categories thanks to the mass spectrometry investigations.

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Part 3. Lysophosphatidylinositols: from simple metabolites to bioactive lipids

Part 3. Lysophosphatidylinositols: from simple metabolites to bioactive lipids

I. Introduction

For a long time, lysophospholipids were considered as simple membrane components. Indeed, lysophospholipids are present in the plasma membrane even if at lower levels compared to phospholipids. Actually it was considered that lysophospholipids were only able to alter the spontaneous curvature of the membrane, hence modulating the function of membrane proteins by altering the mechanical properties of these membranes.[1] Progressively, thanks to the developments in the fields of analytical chemistry, biology and chemical biology, these compounds are now also regarded as "bioactive lysophospholipids".[2] Among the lysophospholipids, some families, such as lysophosphatidic acid, have been studied in depth. However, much less studies were devoted to the other lysophospholipids such as LPI.

LPI were for the first time identified in the early 1960s by Keenan et al.[3] They are a subclass of lysophospholipids consisting of myo-inositol as its polar head group, one acyl chain and one glycerol backbone. LPI are ubiquitous lipids present in a large panel of animal cells and tissues. Indeed, LPI were found in several types of cells (mouse fibroblasts[4] and macrophages[5]), and tissues (rat brain[6], mouse spleen, liver, gastrointestinal tract, spinal cord…[7]).

The rat brain was shown to contain relatively large amounts of LPI (37,5 nmol/g tissue for all detected LPI), with 18:0 LPI (18,9 nmol/g tissue) being the most abundant followed by 20:4 LPI (8,3 nmol/g tissue).[6] We have found similar results in mice brain (see Results, Part 2).[7] As a comparison, LPCs the most abundant lysophospholipid in the mouse brain is present at ten times higher levels than LPI.[8] LPI levels were measured in humans as well, and found to be around 0.3-1µmol/L in plasma.[9,10] LPI were also detected in several human cells (platelets[11], neutrophils[12], endothelial cells[13]) and human cancer cell lines.[14-16]

In the tissues LPI can be found with two different structures depending on the position of acyl chain. Interestingly some acyl chains are more frequent at one position compared to the

other. As an example, arachidonic acid and stearic acid are more often detected as 2-acyl and 1-acyl LPI respectively (**Figure 1**).

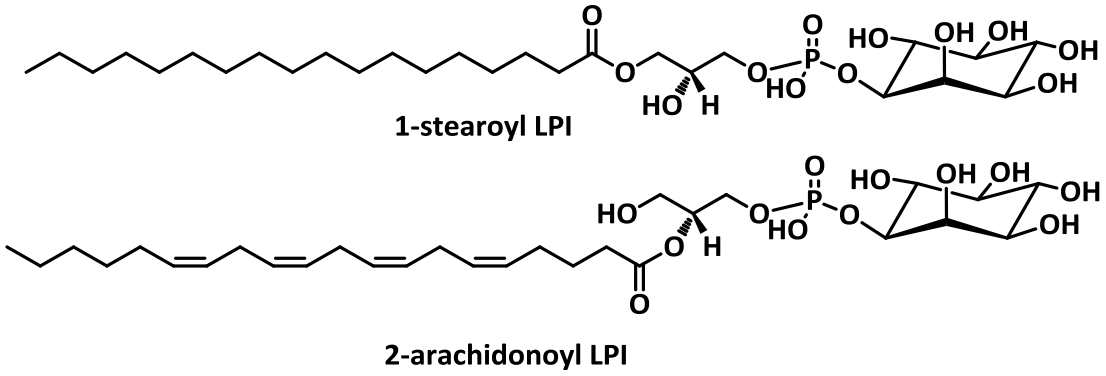

Figure 1. Structures of LPI. 1-stearoyl LPI and 2-arachidonoyl LPI as examples of the main forms of 1-acyl and 2-acyl LPI, respectively.

As we will discuss here, the recent works on LPI and on its molecular target, that is the GPCR GPR55, support the bioactivity of this lipid family. Therefore, we will first summarize the metabolism of LPI, before outlining the cellular pathways activated by this lipid family. We will then review the physiopathological actions described for LPI and GPR55.

II. Biosynthesis, degradation and efflux of LPI:

As mentioned, LPI can be found as *sn*-1 or as *sn*-2 lysophospholipids. These two isomers share the same phosphatidylinositol (PI) phospholipids as precursors but are produced by distinct phospholipases. PLA_1 will cleave at the *sn*-1 position of PI leading to the production of 2-acyl-lysophosphatidylinositols, while PLA_2 enzymes will produce 1-acyl-lysophosphatidylinositols upon hydrolysis of PI's *sn*-2 acyl chain (**Figure 2**).

1-acyl-lysophosphatidylinositols are essentially generated by the activity of the $cPLA_2$ (GIV PLA_2) enzymes, and more specifically the $cPLA_{2\alpha}$.[17] The enzyme activity is regulated by Ca^{2+} intracellular levels as well as by phosphorylation. Upon activation, $cPLA_{2\alpha}$ translocates from the cytosol to intracellular membranes were PI are located and will release arachidonic acid (its major product) and consequently a LPI.[18,19]

Similarly to the PLA_2, PLA_1 is a large family of enzymes. PLA_1 can be classified depending on their distribution (intracellular or extracellular). While, in mammal, extracellular PLA_1 has

been implicated in the production of some lysophospholipids (lysophosphatidic acid and lysophosphatidylserine)[20,21], intracellular PLA_1 is involved in the biosynthesis of LPI. Among intracellular PLA_1 members, DDHD domain containing 1 (DDHD1) is now clearly implicated in production of LPI. Even if this enzyme, first identified as PA-PLA_1[22], is not specific for LPI biosynthesis it clearly shows a PI-PLA_1 activity hydrolyzing PI and producing LPI.[23] Moreover, since the PI 1-stearoyl-2-arachidonoyl is very abundant in mammalian tissues[24,25], PI-PLA_1 seems to have an important role in the synthesis of 2-arachidonoyl-LPI.

While the production of LPI appears quite straightforward its catabolism can take several routes as outlined below.

In 1985, Murase et al. described for the first time a degradation pathway for LPI. In porcine platelets membranes, LPI were shown to be deacylated by lysophopholipase A (lyso-PLA) generating glycerophosphoinositol and a fatty acid.[26,27] Another catabolic pathway involves a lysophospholipase C activity (LPI-specific phospholipase C, lysoPI-PLC) which has been described as having high affinity for LPI, using porcine platelet membranes and synaptic plasma membranes from the rat brain.[26,28] Lyso-PLC is responsible for LPI hydrolysis into acylglycerol and inositolphosphate.[29] This lysoPI-PLC seems quite specific for LPI since very little hydrolysis of LPC, LPS and LPE was noticed.[30] Moreover this lysoPI-PLC is able to hydrolyze both 1-acyl and 2-acyl LPI.[30] LPI produced by activated platelets were also demonstrated to be converted into LPAs via a lysophospholipase D (lyso-PLD or Autotaxin, ATX) dependent manner.[31,32] Of note this lyso-PLD has also been shown to represent the main enzyme involved in LPA synthesis from other lysophospholipids.[33]

Furthermore, more recently, α/β hydrolase domain 6 (ABHD6) was suggested to hydrolyze lysoglycerophospholipids, including LPI, into fatty acids. In vivo, the silencing of ABHD6 in mice liver led to significantly increased levels of LPI.[34]

Besides their hydrolysis, LPI are also involved in the so called “remodeling” of PI. Indeed, LPI are also considered as “acyl acceptor compounds” leading to the reformation of the parent phospholipid.[35] This system is very important for the balance of fatty acids and has a crucial impact on phospholipid homeostasis. It is well established that fatty acids in cellular phospholipids have an asymmetrical distribution: generally, saturated fatty acids are esterified at the *sn*-1 position while polyunsaturated fatty acids (and mainly arachidonic acid

for PI) are esterified at the *sn*-2 position.[36] This point is clearly important for the ability of acyltransferases to remodel PI from fatty acids and LPI.

MBOAT7 (membrane-bound O-acyltransferase 7) is a LPI-specific acyltransferase (LPIAT) able to add a fatty acid to the free alcohol of the glycerol to generate the corresponding PI. MBOAT7 is thought to catalyze the formation of arachidonic acid-containing PI, mainly in the *sn*-2 position.[37,38] Lee et al. showed in 2008, using *caenorhabditis elegans*, that MBOAT7 is required for the incorporation of PUFAs (mainly arachidonic and eicosapentaenoic acids) into PI.[39] Moreover in 2012, MBOAT7 was shown to play a crucial role in brain development in mice. Indeed, MBOAT7$^{-/-}$ mice show practically no LPIAT activity and reduced arachidonic acid contents in PI and PI phosphates. These mice showed atrophy of the cortex and the hippocampus and lifespan limited to one month.[40] Since 38:4 PI is the major PI in the brain, MBOAT7 is now shown to play a key role in sustaining the synthesis of this crucial PI.[41]

The fatty acid moieties of LPI can also be esterified by another enzyme called "1-acylglycerol-3-phosphate acyltransferase 8" (AGPAT8) (**Figure 2**). Indeed, among other lysophospholipids, AGPAT8 is reported to possess acyltransferase activities for LPI.[42] After analysis of the yeast and *C. elegans* homologs of AGPAT8, Le Guédard et al. suggested that the physiological substrates of AGPAT8 were 2-acyl LPI.[43] Indeed, a disruption of a yeast homolog of AGPAT8 caused the disappearance of stearic acid at the *sn*-1 position of PI. Later on, Imae et al. also reported the involvement of AGPAT8 in the *sn*-1 fatty acid remodeling of PI.[44] Moreover, using AGPAT8-deficient mice, the fatty acid composition of PI in mammals was shown to be determined by AGPAT8.[45] These findings demonstrated that AGPAT8 is implicated, at least, in the *sn*-1 stearic acid remodeling of PI and that 2-acyl LPI are physiological substrates. Moreover, AGPAT8 was also demonstrated to be involved in the *sn*-2 acylation of lysophospholipids showing the wide possibilities of physiological substrates for the enzyme.[46]

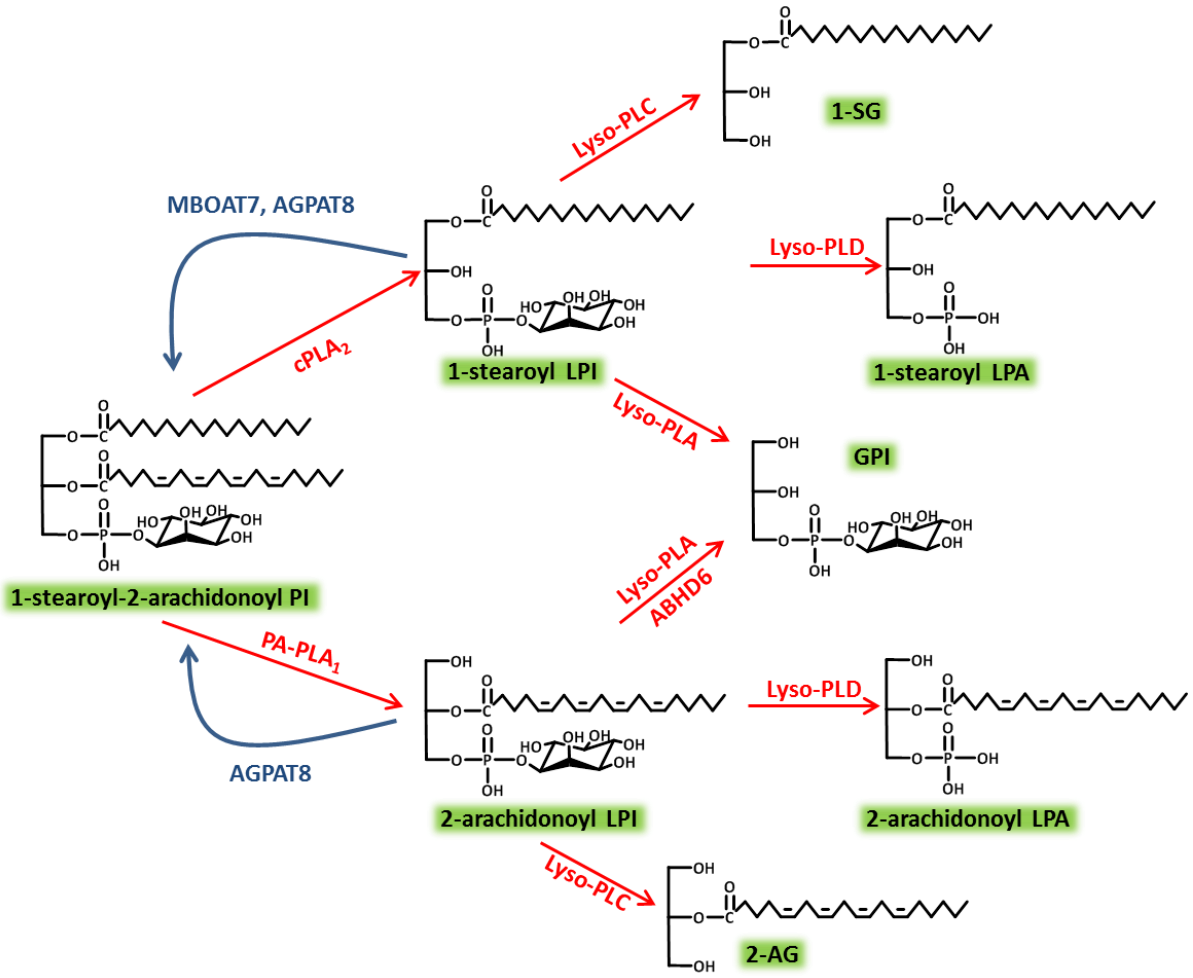

Figure 2. Schematic view of the LPI metabolism. Phosphatidylinositols (PI) are able to release lysophosphatidylinositols (LPI) from cell membrane via the action of phospholipase A_2 (cPLA$_2$) and phospholipase A_1 (PA-PLA$_1$) generating 1-acyl LPI and 2-acyl LPI respectively, Here as an example the hydrolysis of 1-stearoyl-2-arachidonoyl PI (one of the most abundant PI) will lead to 1-stearoyl LPI and 2-arachidonoyl LPI. These LPI can be metabolized by several enzymes: lysophospholipase A (Lyso-PLA), lysophospholipase C (Lyso-PLC) and lysophospholipase D (Lyso-PLD) giving glycerophosphoinositol (GPI), acylglycerols (here, 2-arachidonoylglycerol, 2-AG and 1-stearoylglycerol, 1-SG), and lysophosphatidic acid (LPA) respectively. LPI can be reacylated into phospholipids via acyltransferase activities: MBOAT7 and AGPAT8 reacylate 1-acyl LPI but only AGPAT8 is able to reacylate 2-acyl LPI into parent PI.

Moreover, even if little is known about the existing export system for LPI, the metabolism or actions of these lipids could also be dependent on their transport across membranes.[47] It was recently shown, in human prostate cancer PC-3 cell line, that ABCC1 (ATP-binding cassette transporter) is clearly implicated in the export of LPI into the extracellular medium. LPI levels were decreased (by more than 50%) after using siRNA to knock down ABCC1 mRNA.[48] Even if the exact mechanism is still unknown, the authors proposed an autocrine pathway that could regulate cell proliferation in cancer cells. 1-acyl LPI is synthesized by

$cPLA_2$, this newly generated LPI is released by ABCC1 and can lead to activation of membrane receptor to stimulate cell proliferation.

III. Biological activities of LPI:

Several years ago, Pineiro et al. classified LPI's biological activities in "non-receptor" and receptor-mediated effects.[49] We will here briefly summarize the biological activities of LPI, keeping the same formal separation used by Pineiro. Note that, here, non-receptors means that LPI effects are not mediated by a direct binding to a receptor, not that a receptor is not involved at all in the reported effect.

1. Non-receptor mediated effects:

Among the non-receptor effects, it is known that lysophospholipids are able to modify the properties of cell membranes by alteration of the membrane curvature. Indeed, while phospholipids are essentially of cylindrical shape (excepted for short alkyl chains, considered as lysophospholipids in terms of shape in the membrane), most lysophospholipids, including LPI, are considered to have an inverted cone shape (**Figure 3**). This is due to the large head group relatively to the smaller hydrophobic domain. This structure leads to a stabilization of convex surfaces and favors the formation of micelles rather than lipid bilayers with phospholipids. For these reasons, LPI are considered as non-bilayer forming lipids.

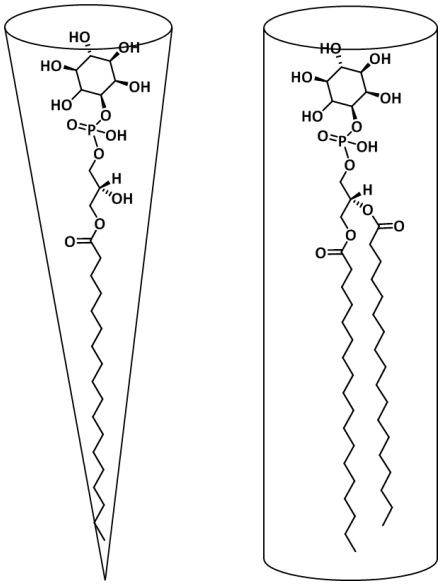

Figure 3. Structure of inverted cone-shaped 18:0 LPI and cylindrical 18:0/18:0 PI. LPI constituted by a large head group and a small hydrophobic domain with only one acyl chain are structured as invert cone-shaped while PI with two acyl chains are organized as cylindrical shape leading to the formation of micelles and lipid bilayers, respectively.

However, when lysophospholipids are mixed with phospholipids, it leads to a modification of the physical properties of the bilayer by introducing lateral stress or strain within the bilayer.[50] For instance, data obtained using fluorescent membrane dyes suggest that LPI has a direct effect on plasma membrane properties.[51] These changes in membrane conformation can result in changes in protein properties (e.g. ion channels) and therefore cellular effects that are not mediated by a direct binding of LPI to a receptor. As another example, 18:0 LPI, with its large polar head compared with others lysophospholipids, is able to modulate endocytic processes affecting clathrin-dependent endocytosis and numbers of invaginations at the plasma membrane.[52] Most likely similar properties should be found for other fatty acyls LPI.

Moreover, LPI were shown to play a role as regulators of membrane proteins. For instance, TREK-1 and TRAAK K^+ channels are activated by several lysophospholipids, including LPCs and LPI.[53,54]

2. <u>Receptor mediated effects:</u>

For the first time, in 2007, an orphan seven-transmembrane G protein-coupled receptor, GPR55, has been suggested as potential LPI receptor.[55] GPR55 is expressed in various mammalian tissues such as adipose tissues, testis, breast, spleen, gastrointestinal tract (e.g. esophagus, stomach, colon…), spinal cord and in the brain (e.g. hippocampus, brain stem, cerebellum, frontal cortex, hypothalamus, striatum…).[56-59]

In 2006, GPR55 was already shown to be able to respond to cannabinoids, suggesting that this receptor may represent a new cannabinoid receptor.[60] However, despite the fact that it was reported that GPR55 could be activated by several cannabinoids (e.g. anandamide, see **Table 1**), the small sequence homology with cannabinoid receptors (13% for CB_1 and 14% for CB_2) is not high enough to clearly affiliate this receptor with the established cannabinoid receptor family.

While the ability of the endogenous cannabinoids (such as anandamide and 2-arachidonoylglycerol) to bind and activate GPR55 is still debated, LPI's agonist activities at GPR55 (and especially for 20:4 LPI) were demonstrated in several studies thus supporting the relation between LPI and GPR55.[6,61,62] Therefore GPR55 was proposed to be considered as a LPI receptor - protein name LPI1 and gene name *LPIR1* (human) and *Lpir1* (non-human) -

in a nomenclature review for lysophospholipid receptors[63,64] (although it is still viewed as a cannabinoid receptor is numerous papers).

Several species of LPI were found to be able to induce the phosphorylation of ERK in a dose-dependent manner, in GPR55-expressing human embryonic kidney (HEK293) cells.[55] Moreover, in these same cells, LPI were shown to induce rapid transient increase of the intracellular Ca^{2+} concentration. The potencies of the different fatty acid species of LPI were also established showing differences between species. The highest level of activity was found for 2-arachidonoyl LPI with an EC_{50} of 30 nM. This activity was shown to be 15 times greater than 1- and 2-oleoyl LPI and 1-stearol LPI but also 8 time greater than 2-linoleoyl LPI, while 1-palmitoyl LPI was shown to be a weak partial agonist (see **Table 1**).[6] Note that according to IUPHAR (the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification), the endogenous ligand for GPR55 are LPI. The data regarding AEA binding to GPR55 (see table 1) were only reported once, in the cited reference.

Agonists	**EC_{50}**	**Output**	**Reference**
AEA (*N*-arachidonoylethanolamide)	18 nM	GTPγS	[57]
18:0 LPI	450 nM	ERK phosphorylation	[6]
18:1 LPI	450 nM	ERK phosphorylation	[6]
18:2 LPI	240 nM	ERK phosphorylation	[6]
20:4 LPI	30 nM	ERK phosphorylation	[6]
O-1602	13nM - 1.4 nM	GTPγS	[57,65]
Abnormal cannabidiol	2523 nM	GTPγS	[57]
Antagonists	**IC_{50}**	**Output**	**Reference**
cannabidiol	445 nM	GTPγS	[57]
CID16020046	210 nM	Ca^{2+} mobilization	[66]

Table 1. Activity of some GPR55 ligands. The table shows reported ligands activities at the GPR55 receptor.

In 2013, Kotsikorou et al. suggested that LPI can act by binding through its polar head group to a residue in a binding pocket within the transmembrane domain 2 of the GPR55.[67] Of note, to date no routine binding assay has been described for GPR55. Therefore most of the available data have been obtained through functional assays.

Regarding the intracellular signaling (**Figure 4**), it is established that GPR55 binding leads, via $G\alpha_{12/13}$ and $G\alpha_q$, to the activation of several downstream signaling pathways.[68]

Indeed, $G\alpha_q$ was shown to stimulate PLC activity, after LPI stimulation, inducing Ca^{2+} release from the endoplasmic reticulum and activating different protein kinase C (PKC) isoforms.[69] Upon LPI binding, $G\alpha_{12/13}$ leads to the activation of small GTPases, such as Ras homologue gene family member A (RhoA), resulting in the activation of Rho-associated protein kinase (ROCK) leading to PLC activation.[70,71] This ROCK activation[59], but also the activation of PKC, is able to catalyze the phosphorylation of intracellular proteins such as p38 mitogen-activated protein kinase (MAPK).[72] Moreover, the regulation of gene transcription was also investigated after GPR55 activation with LPI. It was shown that extracellular signal regulated kinase 1/2 (ERK1/2) can be activated[73,74] leading to the activation of transcription factors such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or the activating transcription factor 2 (ATF-2). [72,75] Of note CREB (3'-5'-cyclic adenosine monophosphpate response element-binding protein) activation was found in a HEK293 cell line[76] but not in human endothelial colony-forming cells.[77] As numerous GPCR, GPR55 was found to be able to form oligomers. A recent study in HEK293 cells has shown that the cannabinoid receptor 1(CB1) can interact with GPR55 forming a heteromer and playing on subsequent signaling: while GPR55 activation using LPI in vitro induce nuclear factor of activated T cells (NFAT) stimulation[76], in presence of CB1, this activation of NFAT by LPI was significantly reduced.[78]

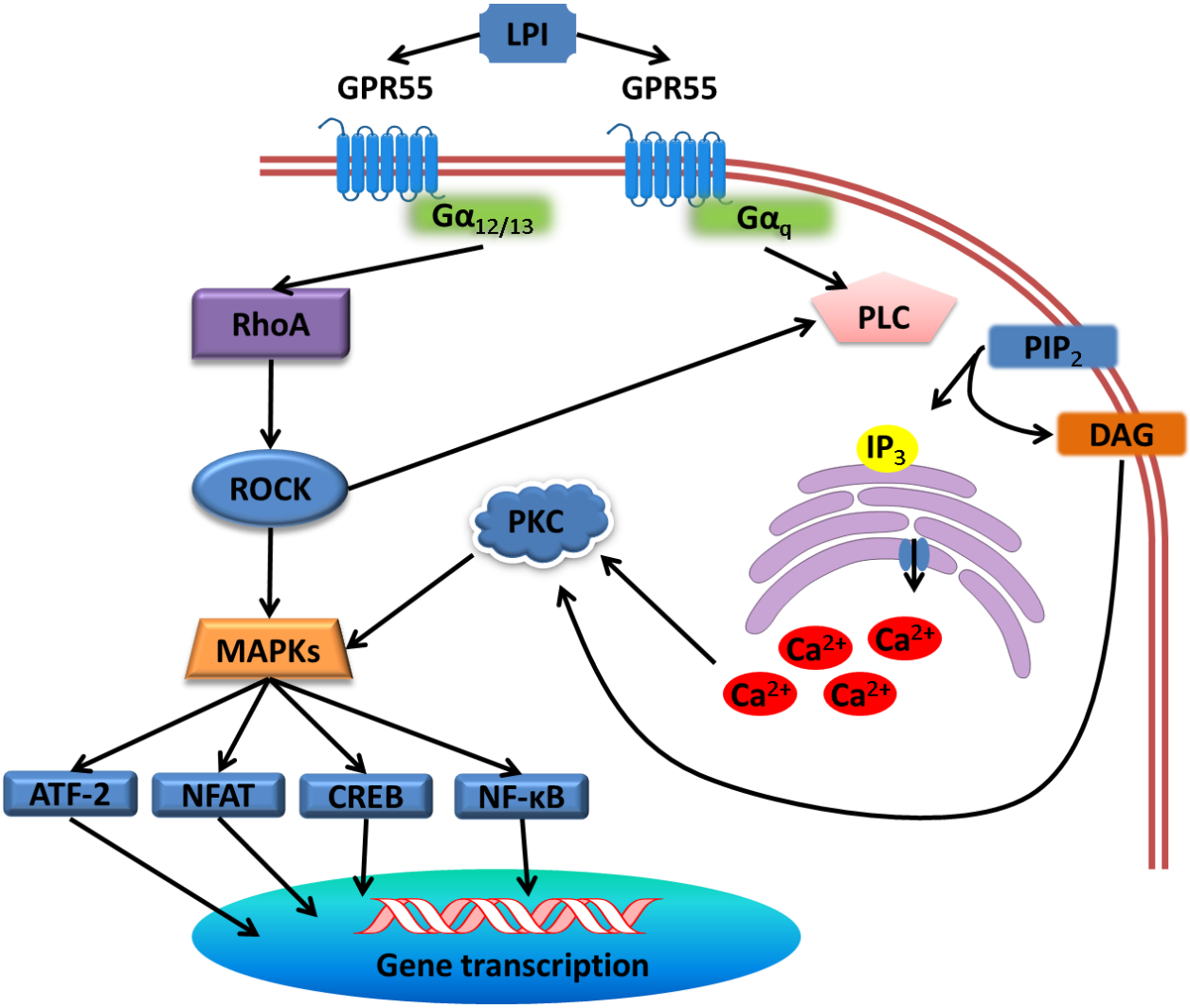

Figure 4. GPR55 cellular signaling. GPR55 can couple to $G\alpha_q$ and $G\alpha_{12/13}$ and upon GPR55 activation, the $G\alpha_{12/13}$ subunit stimulates the Ras homologue gene family member A/Rho-associated protein kinase (RhoA/ROCK) pathway that can regulate phospholipase C (PLC) but also mitogen-activated protein kinases (MAKPs) such as p38 or ERK 1/2 leading to modulation of a range of gene transcription factors including activating transcription factor 2 (ATF-2), nuclear factor of activated T-cells (NFAT), cAMP response element-binding protein (CREB) and nuclear factor kappa-light –chain-enhancer of activated B cells (NF-κB). Activation of ROCK could also lead to elevation of intracellular Ca^{2+} through PLC-induced phosphatidylinositol bisphosphate (PIP_2) hydrolysis and inositol 1,4,5-tris phosphate (IP_3)-mediated calcium from the endoplasmic reticulum. Upon activation, GPR55 could also be associated to the $G\alpha_q$, stimulating PLC activity which leads to release of Ca^{2+}. Released Ca^{2+} but also diacylglycerol (DAG) can activate protein kinase C (PKC), which can then also regulate DNA transcription and gene expression through MAPKs.

In endothelial cells isolated from the rat mesenteric artery, it has been suggested that LPI utilizes especially ROCK-RhoA pathways to elevate intracellular Ca^{2+}.[71,79]

Because the RhoA/Rock and p38 MAPK signaling pathways are involved in the control and the regulation of numerous cellular functions (cell division and proliferation, apoptosis…) and were shown to be altered in several cancers, the LPI/GPR55 axis seems to be very interesting in terms of physiological comprehensions but also in potential pharmacological therapeutic indications. We will briefly outline these in the following pages.

3. Effects induced by the LPI/GPR55 system

a) Ca^{2+} metabolism

Ca^{2+} is widely considered as a key intracellular mediator in several signaling pathways and is involved in a number of intracellular physiological processes. As illustrated earlier, GRP55 activation by LPI affects Ca^{2+} signaling. The first reports on LPI showed that LPI were able to promote insulin release, at least in part via mobilization of Ca^{2+}.[80,81] Consistently, Rustenbeck et al. showed that LPI increased the free Ca^{2+} concentration in mouse pancreatic islets.[82]

An effect of lysophospholipids, and LPI in particular, on Ca^{2+} transport has also been shown in rat liver mitochondria where the interaction of LPI with the mitochondrial membrane bilayer was suggested to induce release of Ca^{2+}.[83] LPI have also been demonstrated to be involved, via a GPR55, in the Ca^{2+} regulation in endothelial cells.[84] However, more recently GPR55-independent effects of LPI on Ca^{2+} levels were also described. For instance, in an *in vivo* study, LPI were shown as expected to elevate the concentration of Ca^{2+} in islets of mice. Surprisingly however, these effects were still present in islets from $GPR55^{-/-}$ mice suggesting stimulatory effects of LPI on islet function by a GPR55-independent pathway(s).[85]

b) Cell migration and proliferation

Cell migration is a fundamental process involved in both physiological and pathological situations (embryo development, inflammation, angiogenesis, cancer…). Thus it is important to understand how cells move or adhere and how cells can interfere with other cells and react to modification of their microenvironment. Lysophospholipids are known to be involved in the regulation of cell migration and among them LPI are now reported as playing a role in cell migration and proliferation, in normal cells but also in cancer cells. For instance,

GPR55 activation by LPI was shown to induce migration of human peripheral blood neutrophils.[86]

In cancer cells, LPI are also able to modulate migration and proliferation. In a breast cancer cell line (MDA-MD-231), LPI have been shown to increase cell chemotaxis towards serum. In GPR55 overexpressing cells, LPI were also able to enhance a robust migratory and invasive response.[16] Moreover, in this study, cannabidiol, a known GPR55 antagonist (see **Table 1**), and use of siRNA for GPR55 were used to successfully block the migratory response, thus supporting GPR55 as the receptor mediating this effect. Similar effects of LPI were reported in other cell types. In human prostate cancer cell line (PC-3), LPI were able to increase cell migration.[15] More recently, LPI were also shown to induce migration in GPR55 overexpressing colon cancer cells.[87]

LPI were also reported as a mitogenic factor, enhancing cell number.[88,89] Elevated levels of LPI were found in ras-transformed cells and these higher levels might play a role in the modulation of k-ras transformed cell proliferation.[88] More recently, also on breast cancer cells, both in vitro and in vivo, activation of GPR55 by LPI has been reported to confer pro-invasive features, suggesting that GPR55 could promote breast cancer metastasis.[90]

In human studies, clinical investigations showed elevated LPI levels in the blood and ascites of patients with ovarian cancer or peritoneal cancer compared to healthy patients.[91] Total LPI in the plasma sample collected preoperatively from patients with stages 1 to 4 ovarian cancer were 2,98 µmol/L; 4,58 µmol/L; 4,25 µmol/L and 2,96 µmol/L respectively, in comparison with 1,51 µmol/L for the healthy controls.[92] Note that LPI blood levels have been suggested by some authors as potential markers of ovarian cancer.[10,91-93] However, until now, specific roles of LPI are not so clear, and it should be considered only as *potential* marker for ovarian cancer. In 2015, Gaul et al. showed altered levels of 18:1 LPI and 15 other analytes (certain are still unknown) in ovarian cancer, and suggested that this mixture of analytes should be better than only LPI.[93]

Thus the data on hand suggest that both LPI and GPR55 are important players in cancer development.[16,47,77,94-97] Interestingly a positive correlation was found between the aggressiveness of the cancer and the expression of GPR55, in different human tumors types.[98] Furthermore, GPR55 knockdown was shown to reduce tumor growth in vivo since

using selective siRNA for GPR55 allowed to significantly reduce the growth of tumors generated in nude mice with glioma cells compared with the corresponding controls. All these evidences suggest that GPR55 and LPI could play an important role in several cancer types.

c) Bones turnover

Osteoblast and osteoclasts were also demonstrated to express GPR55[73] and a role for LPI/GPR55 axis has been described in bone morphogenesis. Indeed, one study reported that GPR55 activation (using LPI and O-1602 (**Table 1**)) inhibits osteoclast formation and stimulates osteoclast polarization and resorption activity. Supporting these effects, the GPR55 antagonist cannabidiol was reported to increase osteoclast formation. Furthermore, a culture of macrophages obtained from the bone marrow of $GPR55^{+/+}$ mice, in presence of LPI, presented an inhibition in osteoclasts formation while this effect was not observed using macrophages from $GPR55^{-/-}$.[73] This study suggests an implication of the LPI/GPR55 axis in bone turnover. Further studies will have to determine whether this system could be an interesting target in the context of osteoporosis.

d) Cardiovascular diseases

A potential role for GPR55 has also been suggested in the cardiovascular system. It has been shown that $GPR55^{-/-}$ mice exhibit maladaptive adrenergic signaling and systolic dysfunctions in comparison with control mice suggesting a role for this receptor in the control of adrenergic signaling in the heart but also in the pathogenesis of heart failure.[99] Moreover, cardiac arrest induces whole-body ischemia, leading to damage to multiple organs and Kim et al. found a substantial increase in 16:0 and 18:0 LPI in a rat model of asphyxia-induced cardiac arrest.[100] However, in the same model, LPI levels were decreased if a cardiopulmonary resuscitation followed the cardiac arrest suggesting that PI metabolism is sensitive to cellular conditions altered by ischemia and resuscitation.[100] Interestingly from a translational point of view, total LPI levels (including 16:0, 18:0, 18:1, 18:2, 20:4, 22:6 LPI) were found to be elevated in plasma of acute coronary syndrome patients.[101] All these studies only show altered level of LPI and obviously further studies are needed to complete these first results to support the hypothesis of the potential implication of GPR55 and LPI in cardiovascular disorders.

e) Energy homeostasis

Energy homeostasis is a metabolic process providing a balance between the energy requirements of cells (growth, metabolism…) and energy input from food intake. Disturbances in this system, provisions exceeding requirements, could lead to metabolic disorders as type 2 diabetes mellitus or obesity.

The potential role of LPI/GPR55 system has also already been investigated in human adiposity demonstrating the presence of GPR55 in human visceral and subcutaneous adipose tissue.[9] Moreover, authors showed that GPR55 expression was enhanced in the adipose tissue of obese subjects and that circulating LPI levels are also increased in obese patients.[9] More recently, several LPI (16:1, 18:1, 18:2, 20:3, 20:4 and 22:6 LPI) were shown to be significantly elevated in individuals with type-2 diabetes, in comparison with controls.[102] A possible role for LPI in type-1 diabetes has also been suggested as significantly higher circulating LPI levels were found in a mouse model of type-1 diabetes compared to control animals.[103]

Already in 2011, GPR55 was thought to play a role in the regulation of food intake because a missense polymorphism in the GPR55 gene was associated with anorexia nervosa, with low body weight.[104] More recently, McKillop et al. showed that administration of the GPR55 agonist abnormal cannabidiol reduces by 20% the food intake of NIH Swiss mice.[105] However, the actual role of the GPR55 pathway is not clear as several other studies have demonstrated the absence of difference (in both of male and female mice), between $GPR55^{-/-}$ and control mice or the increase of the food intake after treatments with an GPR55 agonist (O-1602).[106,107]

Another study found that $GPR55^{-/-}$ mice have increased adiposity and insulin resistance that are not due to changes in feeding behavior but potentially to decreased physical activity.[108]

f) Inflammation

Since the availability of arachidonic acid for eicosanoid production is partly regulated by the cycles of deacylation-reacylation of phospholipids, it is plausible that LPI levels would be affected by inflammation. This was indeed found in a model of inflammatory pain where 18:0 LPI and 20:4 LPI levels were increased in rat brain after facial carrageenan injection.[109]

Our data (see Results, part 3) also show a link between activation of inflammatory cells and changes in LPI levels.

Interestingly, GPR55 was also shown to be involved in inflammation processes. Several anti-inflammatory cytokines, interleukin-4, interleukin-10 and interferon-γ were increased following the intraplantar administration of complete Freund's adjuvant (CFA) in paws from CFA-injected $GPR55^{-/-}$ mice when compared with the CFA-injected $GPR55^{+/+}$ mice, suggesting that GPR55 signaling can influence the regulation of cytokines.[110]

Some indications that GPR55 may be involved in the response of the gut to intestinal inflammation were suggested by the lower GPR55 expression in control rats than in animals treated with lipopolysaccharides.[58] Supporting this assumption, blocking the GPR55 receptor, using the antagonist CID16020046 (**Table 1**), decreased the hallmarks of experimental intestinal inflammation.[111] This possible suggested role of LPI-GPR55 axis, in the gastrointestinal tract, was also put forth using $GPR55^{-/-}$ mice. Indeed the severity of the experimental colitis was lower in the knockout mice compared to the wildtypes mice.[112]

Of note, it has been shown that GPR55 could represent a future target for the treatment of colonic motility disorders since GPR55 activation results in the inhibition of contraction in the colon and the ileum with respectively 60% and 25% of reduction.[113,114]

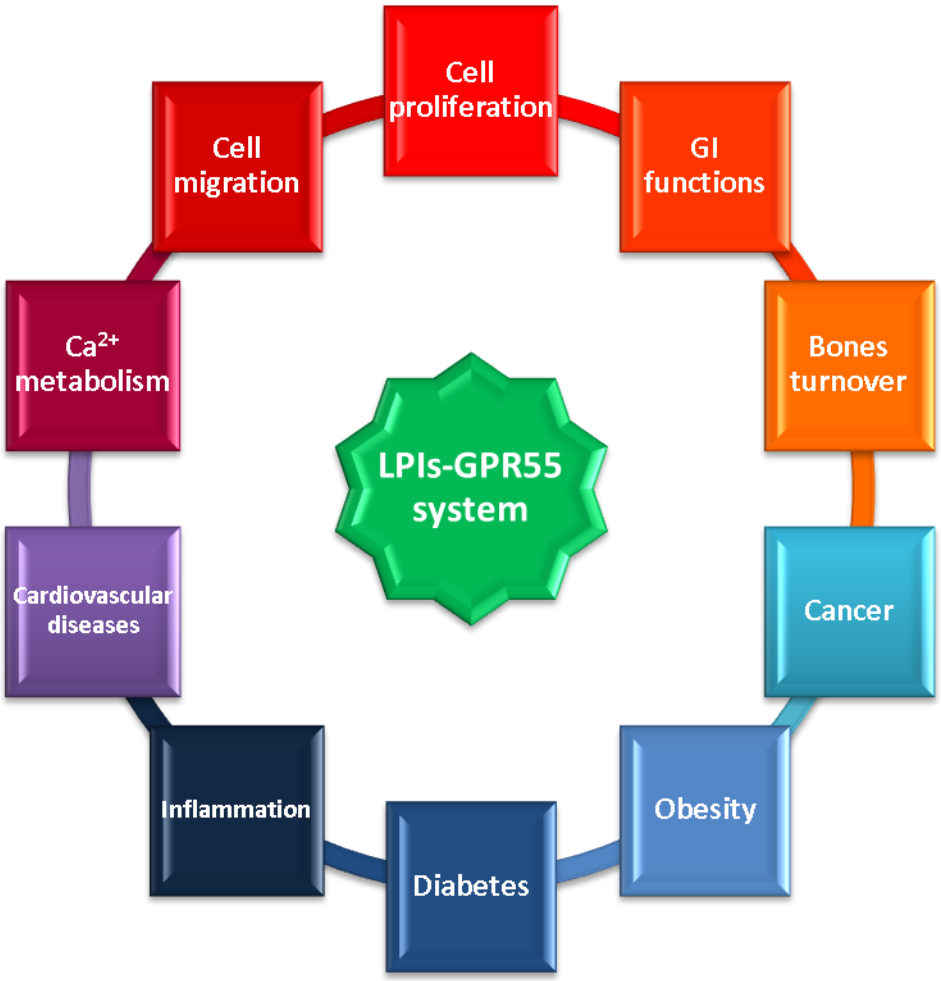

Figure 5. LPI/GPR55 main implications diagramme. The main multiple involvements of LPI and its receptor are represented including "cell signaling" such as cell migration and proliferation, calcium metabolism, bones turnover and gastrointestinal (GI) functions but also "diseases and disorders" such as cardiovascular diseases, inflammation, diabetes, obesity and cancer.

While the studies briefly outlined above strongly suggest a role for the LPI/GPR55 axis in physiopathology (**Figure 5**), it clearly appears that additional studies are needed to better pinpoint the actual role of these ligands and receptor. It is quite evident that the development of potent and selective agonists and antagonist will help in this process. Indeed, while cannabidiol and related compounds were useful in the early stages; more potent and selective tools would help move the field forward.

IV. Conclusion

As discussed here, LPI besides being important partners in the biosynthesis and remodeling of membrane glycerophospholipids are also key lipid mediators activating at least one receptor, namely GPR55. Numerous functions have been described for LPI/GPR55 since the deorphanization of the receptor. The effects of LPI and GPR55 activation on cancer are among those more studied in the literature. However in number of cases the molecular mechanisms underlying these different processes remain to be clearly identified. The GPR55$^{-/-}$ mice and the development of selective antagonists will allow to progressively delineate what are the effects mediated by LPI and GPR55 and those mediated by LPI via GPR55-independent pathways. Indeed, as discussed earlier, LPI can alter membrane properties which in turn can induce physiological changes independent of GPR55. Alternatively, it is possible that LPI activate additional receptors besides GPR55.

As evidence supporting LPI and/or GPR55 importance is continuously accumulating and because LPI levels were demonstrated to be altered in several pathologies the next years should bring new exciting discoveries on this bioactive lipid family.

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Part 4. The toolbox of lipid analysis.

Part 4. The toolbox of lipid analysis.

I. Introduction

As described in the previous sections, lipids play multiple roles in an organism. They are the main constituents of cell membranes, behave as hydrophobic barriers to isolate cellular compartments, contribute to energy storage, and participate in intracellular and extracellular signaling thus acting as bioactive molecules.[1,2] Differently from other biomolecule classes, lipids do not share a common structure. Actually, the LIPID MAPS (LIPID Metabolites And Pathways Strategy) consortium uses eight categories to classify the over 180 000 lipid species (See Introduction, Part 1).[3,4]

Changes in lipid levels and in lipid metabolic pathways are involved in numerous pathologies including diabetes, obesity, cardiovascular disease, cancer, inflammatory disease and neurodegeneration.[5-8] Thus, dysregulation of lipid metabolism is a capital contributor to many pathologies and lipid analysis is nowadays considered as essential to a full understanding of most physiological and pathological processes.

As a part of metabolomics, lipidomics is intended as a global analysis of lipid species eventually leading to the understanding of their biological roles in diseases and pathologies. Indeed a global lipid analysis, as opposed to a targeted one, will allow identifying the mechanistic details by which alterations in lipid metabolism are linked to metabolic homeostasis.[9] Two major ways of analyzing lipids can be envisioned. One is a targeted analysis of selected, but limited, lipid species while the other one consists in a global and untargeted analysis of all the main lipid species in the studied sample. The latter approach is becoming widespread, especially in translational research for the study of new biomarkers and discovery of disease mechanisms. However, because of the diversity of lipid species, lipidomics still constitute a challenge to the analytical chemists.[10,11] Steps that could appear as trivial are actually crucial to avoid biased quantifications. These start with the sample collection, its workup, to the choice of the analytical method – from the separation to the detection – and so forth. Although lipid analysis could use simple tools such as thin layer chromatography, nowadays ion mobility coupled to liquid chromatography (LC) and mass spectrometry (MS) gives unprecedented opportunities to unequivocally detect and quantify the lipids of interest. Regardless of the method used for lipid analysis, the key steps of

sample treatment are similar, including sample collection, preparation, extraction, purification, separation (except for shotgun lipidomics), or the rationale choice of the standards to use. Here we will outline the main principles of sample treatment - from collection to injection – and discuss the methods used for lipidomics (**Figure 1**).

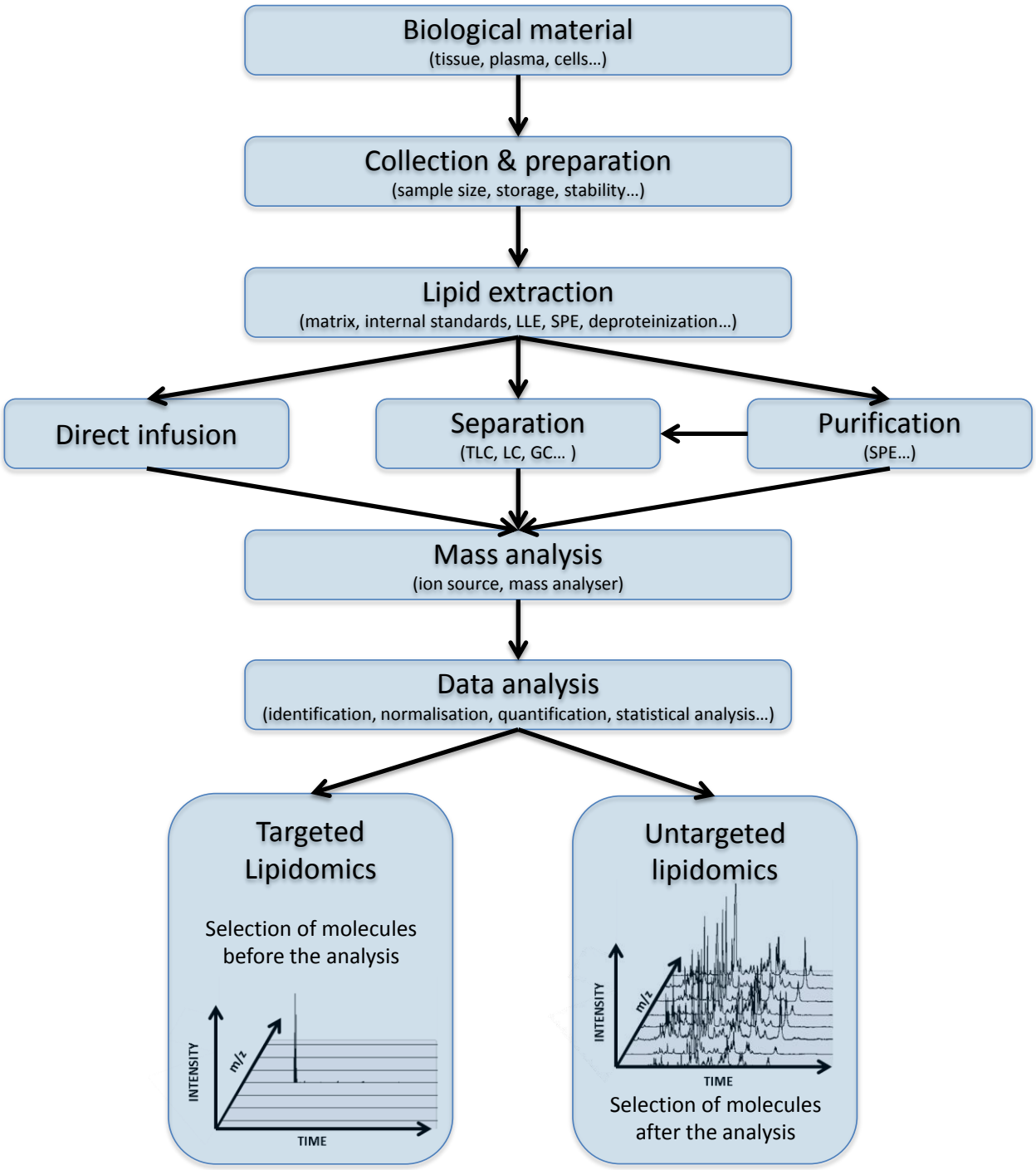

Figure 1. Illustration of the workflow of global lipid analysis of lipidomes from crude extracts of biological material. First, biological samples as animal or plant tissues, cells or fluids are collected and prepared prior the lipid extraction. The enriched lipid fraction obtained can be directly infused,

purified after extraction and/or separated by chromatography depending on the aim of the analysis. Then, mass analysis results in raw files that can be used for identification and quantification of lipids. Targeted and untargeted lipidomics are characterized by a selection of molecules of interest "prior" or "after" the lipid analysis, respectively. The explanations on the different steps are found in the main text.

II. From sample collection to its purification

1. Sample collection and preparation

Sample collection is the first step of any analysis. Biological samples can be solid (cells, animal or plant tissues…) or more or less complex fluids (serum, plasma, urine…). Two key parameters guiding the amount of sample to be used are the expected amount of analyte(s) and the sensitivity of the analytical method. Nevertheless one has to keep in mind that increasing the sample size (from few mg to 100 mg) comes with the cost of increasing the risk of matrix effects. Although the increased sensitivity of several techniques allowed for a reduction in sample size (a few milligrams can be sufficient), the sample used should be representative of the heterogeneity of the tissue/organism studied. This is especially important with large tissues, such as liver or fat depots, where a few milligrams will have to represent the whole tissue. Once the sample collected, storage is a crucial parameter as over time – due to temperature, oxygen, enzymatic activity, etc – the lipid composition can be altered. Thus snap freezing the sample upon collection and storage below - 20°C, ideally - 80°C, is critical. If this is not possible, quenching the tissue in organic solvents might represent an alternative for some tissue and analytes (e.g. analysis of eicosanoids in plasma samples).

Direct analysis by LC or MS is possible however most frequently the sample has to be prepared and the lipids extracted before analysis. This could help minimize interferences and concentrate the analytes of interest if needed. Numerous lipid families tend to adsorb on plastic tubes making glassware and Teflon caps the preferred option when working with lipids. Of note this will also minimize the contamination due to phthalates and plasticizer-extraction when organic solvents are used. A cautionary note also on pipette tips where lipids can adsorb and from which phthalates and plasticizer can be extracted by the solvent used. The contamination by these elements is particularly critical when working in an untargeted mode as contaminating peaks will appear on the mass spectrum.[12]

Besides, another element is the stability of the analytes during the workup. This is critical for several lipid families that are particularly sensitive to oxidation and for which air and temperature will result in decreased or increased levels depending on the fact that the lipid is degraded by oxidation (e.g. polyunsaturated fatty acids) or produced by oxidation (e.g. 5-cholesten-3β-ol-7-one and 7β-hydroxycholesterol can be produced from cholesterol-containing samples). Using stabilizers in the solvents (e.g. butylated hydroxytoluene, EDTA, …) as well as nitrogen when evaporating solvents or to flush the vials will help reducing oxidation.[13,14]

2. Lipid extraction

Extraction of lipids from complex matrices is often necessary before analysis as this will help removing proteins, saccharides and small polar molecules from the analyzed sample. Several strategies can be envisioned, including protein precipitation, and liquid-liquid (LLE) or solid (SPE) phase extractions. Several parameters should be considered as the ease of the protocol, protein removal efficiency, lipid recovery, repeatability.[15-17] Protein precipitation using an organic solvent (e.g. acetone, acetonitrile, and methanol) and cold temperatures is easy to apply but does not remove the smaller polar analytes present. The resulting extract can then be analyzed as such, further concentrated or purified.

LLE remains one of the most common techniques for lipid extraction. For untargeted lipidomics the ideal system should extract the largest number of lipid families with the highest efficiency. Given the diverse lipid structures this remains a challenge. However four systems are generally used and allow for a relatively good coverage of the diversity of lipids. Two systems, originally developed by Folch and by Bligh and Dyer, based on the use of chloroform and methanol in the presence of an aqueous phase (added or coming from the sample) are still commonly used. Although differences in the protocols exist, those two methods give quite similar results in terms of lipid extraction.[18] However, beside the toxicity of the solvents used, lipid partition in the lower phase makes it difficult to apply the methods in automated high-throughput analysis of lipids. An alternative method, developed by Matyash et al., uses methyl-*tert*-butyl ether (MTBE) instead of chloroform[19] resulting in the presence of the lipids in the upper layer thus highly simplifying sample handling and automated processing of biological samples. Indeed, here the sample is homogenized in

methanol before adding MTBE and incubating the sample for 1h at room temperature. The phase separation is induced by adding 1.25 mL of water. After centrifugation the upper layer is recovered. More recently, Löfgren et al.[20,23] developed a new, simple, chloroform-free and rapid method for tissue lipid extraction. A mixture of butanol and methanol (3:1) (BUME) is used to homogenize the tissue before adding a mixture of heptane, ethyl acetate and acetic acid resulting in the lipids extracted in the upper phase. When comparing the "BUME method" to the Folch and MTBE methods a similar pattern of lipid extraction was found using liver and heart.[20]

With these two phase extraction system, some lipids may more or less evenly partition between the organic and the aqueous layer resulting in low extraction. To avoid poor and inconsistent yields, especially in the extraction of strongly hydrophilic glycerolipids, the aqueous phase can be acidified or its content in electrolytes increased (i.e. salting out). It is however important to take into account the stability of the species. For instance, lipids such as plasmalogens can rapidly be degraded at low pH due to the cleavage of their *sn*-1-enol ether chain resulting in the corresponding *sn*-1 hydroxyl group (e.g. *Sn*-2-acyl-lysophosphatidylcholines and *sn*-2-acyl-lysophosphatidylethanolamines).[21] The resulting s*n*-2-acyl-lysophosphatidylcholine and *sn*-2-acyl-lysophosphatidylethanolamine, because they could be isobaric species of others lysophospholipids, could yield the same major fragment ions increasing the risk of misidentification.[21,22]

3. Sample purification

Solid–phase extraction (SPE) is a key step for sample purification. This sample preparation technology uses solid particles and chromatographic packing material (often contained in cartridges) to chemically separate different components from biological complex matrices. In most cases, samples are liquids and the exact appellation is "liquid-solid phase extraction", with a solid (chromatographic particles) and a liquid (sample) state. Through the differential interaction of the analytes between the two phases, selective retention of the analytes on the solid phase can be achieved.[24] SPE is often used as a selective remover of interferences including in lipid analysis (e.g. SPE for selectively trapping proteins & phospholipids). The benefits of SPE in the context of sample purification are multiple:

(i) simplification of complex sample matrix along with compound purification (simplifying the sample matrix leads to an improved quantitation accuracy),

(ii) reduction of the ion suppression or of the ion enhancement,

(iii) possibility to fractionate the lipid extract in the context of targeted analytes (complex samples can be easily separated into fractions using step gradient of increasing strength solvents)

(iv) trace concentration of very low level compounds could be detected because SPE allows analyte enrichment (In some cases, lipid levels could be lower in the raw sample than the sensitivity capacity of analytical instruments).

Optimal selection of the SPE strategy requires a good knowledge of the physicochemical properties of the analytes and of the matrix composition. Several SPE options are available, the normal-phase SPE (with a polar stationary phase and a much less polar (or non-polar) mobile phase and the reversed-phase SPE (with a polar mobile phase and a non-polar (hydrophobic) stationary phase are amongst the most frequently used. For lipid analysis reversed-phase SPE represents more than 60% of the literature. Ion exchange (with stationary phases characterized by the charge on their surfaces and types of ions that they attract/retain) could also be used. For SPE method development, the relationship with the pH can also be very powerful.

Of note SPE can be combined with LLE, as an additional clean-up step, to select specific classes of targeted lipids from biological complex matrices.

III. Sample separation

After the preparation, extraction and purification of the biological complex matrices, the following key step is the detection and the analysis of the lipid species present in the samples. Some lipidomic applications (e.g. “shotgun lipidomics” consisting in the direct infusion of the sample into the MS) do not use HPLC or GC separation of the analytes prior to their analysis. However, the absence of separation come with several drawbacks including ion suppression due to the amount of matrix injected in the same time and inefficiency to separate structural isomers. Thus, although time-consuming a separation step is often used.

Several separation techniques, such as thin layer chromatography (TLC), liquid chromatography (LC) and gas chromatography (GC) are usable for lipid analysis.

1. Thin layer chromatography (TLC)

TLC is a very simple and rapid analytical separation method that can be used without expensive equipment, to obtain acceptable resolution of different classes of lipids.[25] Even if the results obtained by TLC are less accurate than those that can be achieved using GC or HPLC, this method, allowing the separation of lipids with different polarities in a single run, could generate a great deal of useful information, with a very little effort and in a limited time. TLC could be used to separate several lipid families such as steroids, free fatty acids, phospholipids, or diacylglycerols.[26] The use of (semi)automated sampling tools to apply the samples on the plate allows to increase reproducibility and accuracy of the results compared to manually applied samples.

Separating samples in two orthogonal directions, 2-D TLC, is also possible and is often used to increase resolution and peak capacity. A refined version of TLC, high-performance thin-layer chromatography (HPTLC), with a reduced particle size (5 µm for particles in comparison with 20-50 µm for "classical" TLC) and thickness of the layer, also increases the resolution in comparison with TLC.[27] The solid phase (stationary phase) of the TLC has a great influence on the performance. Classical stationary phases for lipid samples separations are silica gel or alumina, with the first one being the most popular. TLC could be combined with MS with an "indirect" coupling -offline mode- to the MS (the spot corresponding of the target analyte is eluted from the silica gel with appropriate solvents and the resulting fractions are later characterized independently by MS) or with a "direct" coupling –online mode- to the MS (extraction interface combined with HPLC pump used with ESI.[28] Of course, other ionization sources such as APCI of MALDI can also be used in combination of TLC. Of note, TLC systems are poorly isolated from the environment with the risk of increasing the contact between samples and air thus leading to the self-oxidation and degradation of lipids.

2. Gas chromatography (GC)

GC is also used for lipid analysis but has some limitations. Indeed, due to the fact that most lipids are non-volatiles and some of them are easily degraded at high temperature, GC is

more easily adapted to targeted quantifications (e.g. for fatty acids, sterols,…) than for untargeted lipidomic analysis. Fatty acids are among the most studied lipid by GC and GC-MS has played a pivotal role in the identification and quantification of eicosanoids in biological samples.[29] Indeed for fatty acids analysis, the resolution ability of GC is much higher than liquid chromatography.

However, an important difficulty for the use of GC is the need to derivatize analytes from the sample, to reduce their polarity and increase their separation. Moreover, chemical derivatization could lead to potential degradation during the derivatization process and obviously cause an increase of the number of peaks resulting in highly complex chromatograms very difficult to interpret. Derivatization may also significantly eliminate much structural information about lipid molecular species.[30]

For complex mixtures two dimensional GC (GC x GC), based on the coupling of two capillary columns, each contributing in a different way to the resolving power of this technique, can also be an adequate method for lipid analysis.

3. Liquid chromatography (LC)

Among all the separation methods, HPLC is certainly the most widely used in lipidomics. Compared to the other separation techniques, or to direct infusion, HPLC coupled with MS allows good reproducibility and high resolution. Is also allows detection of isobars species and of isomers, reduction of ion suppression, enriching low-abundance lipid species and eliminating the inferences from the high abundance species before ionization etc. Even though HPLC has been a common analytical tool for several years in lipid analysis, the power of LC-MS has been enhanced by the development of ultra-HPLC (UHPLC) leading to new high-throughput applications. In comparison with HPLC, UHPLC makes use of columns with smaller particle size and operates at higher pressures, at normal flow rates.[31] The main gains are less solvent employed, reduced analysis time due to the high resolution chromatograms with peak widths limited to a few seconds. In the recent years, the core-shell technology was developed in the chromatographic field. This technology uses a solid silica core and a porous outer silica shell to provide the same high efficiency separation than the sub 2µm UHPLC particles, but with potentially lower backpressure.[32] Moreover, the porous shell fused to a solid core decreases the diffusional mass transfer path in comparison with totally porous

particles resulting in an enhanced chromatographic resolution. In the lipidomic field, reverse-phase LC (RPLC), normal-phase LC (NPLC) but also hydrophilic interaction chromatography (HILIC) are the most frequently used LC-based separation methods.[33] While RPLC method is regularly used to obtain a separation between different molecular species in one particular class, based on the difference of the fatty acyl chains (mechanism of action based on lipophilicity), NPLC is more often used for the separation of different classes based on the polar head groups (mechanism of action based on the lipids hydrophilic functionalities). Although much less frequently used, HILIC method can be used for more "polar" lipids, representing an alternative to NPLC.[34]

Nevertheless RPLC certainly remains the most used method for global lipidomics studies (> 70% of lipidomics studies).[35] Typical methods consist in gradient of acetonitrile-water or methanol-water solvent systems in combination with C8 of C18 columns with flow rate of 0.1-0.4 mL/min. To ameliorate the separation efficiency (and the detection), numerous modifiers (e.g. formic acid, acetic acid, ammonium acetate…) can be used and added to the mobile phase. Changing the modifier or its concentration can have profound effects on the separation.[36] In RPLC the carbon-chain length and the number of double bonds are crucial elements determining the retention. Lipid species with longer acyl chains are more retained on the LC column than shorter chain lipids and saturated acyl structures are also eluted later than (poly)unsaturated species.

NPLC methods are used in less than 20 % of the lipidomics papers[35] and this is explained by the fact that the mobile phases used have a low ionization capacity when using an electrospray and are highly volatiles. The weak mobile phase consists of heptane, chloroform of solvent mixtures (with methanol, isopropanol…) while stronger eluting mobile phase consists of solvents with increased polarity (chloroform/methanol, acetonitrile/methanol…). Classically, chromatographic runs are longer in NPLC compared to RPLC because the columns are often longer. The stationary phase is polar in nature and consists in either bare silica/alumina or polar ligand bonded to the particles.

The HILIC method, sometimes used in lipidomics analysis, can be defined as a separation mode that combines stationary phases usually used in the NPLC mode and mobile phases used in RPLC separations. This separation method often uses a gradient starting with a high

percentage of acetonitrile followed by increasing proportions of water. Acetonitrile can be used alone or mixed with another organic solvent (methanol, isopropanol) and often contains at least 5% of water, as a minimum of 2% is essential for the formation of the aqueous layer involved in the separation.[37] Columns used for HILIC analysis are the most frequently made of silica with a broad range of different functional groups chemically bonded to silica surface to achieve a modified stationary phase for HILIC method including aminopropyl, amide, cyano groups and polyols depending on the applications.

Regardless of the LC system used, the choice of the solvent used for the resuspension of the extract prior to the LC-MS analysis is crucial. Samples ready to be injected into the LC-MS system should be in a solvent (or solvent mixture) which is compatible with the initial composition of the mobile phase to avoid peak distortion or solubility issues and which is able to avoid degradation of lipids in solution. Frequent mixtures contain water, methanol, acetonitrile, chloroform, isopropanol…in different proportions.

4. Ion mobility

Ion mobility (IM) represents a new development in the separation techniques that is applied to lipid analysis in combination with MS. With IM, lipid ions cross an ion-mobility separation cell just before their MS detection. The separation occurs on a timescale of milliseconds, making it appropriate for coupling with MS. In contrast to the other separation methods, IM is a post-ionization technique. It differentiates ions, in the gas phase, by their mass, charge but also size and shape based on the different mobility in electrical fields within a chamber pressurized with a buffer gas (e.g. nitrogen).

IM can be used with several lipidomics strategies such as after liquid chromatography[38-40], direct infusion[41], desorption ionization (MALDI, see Sample analysis, ionization techniques).[42,43] In each case IM brings an additional separation step thus increasing the selectivity of the analysis. The use of IM in lipidomics opens new possibilities. Typically identification of novel lipid species is allowed since IM allows the separation of isobars, isomers and conformers (**Figure 2**).[44-46]

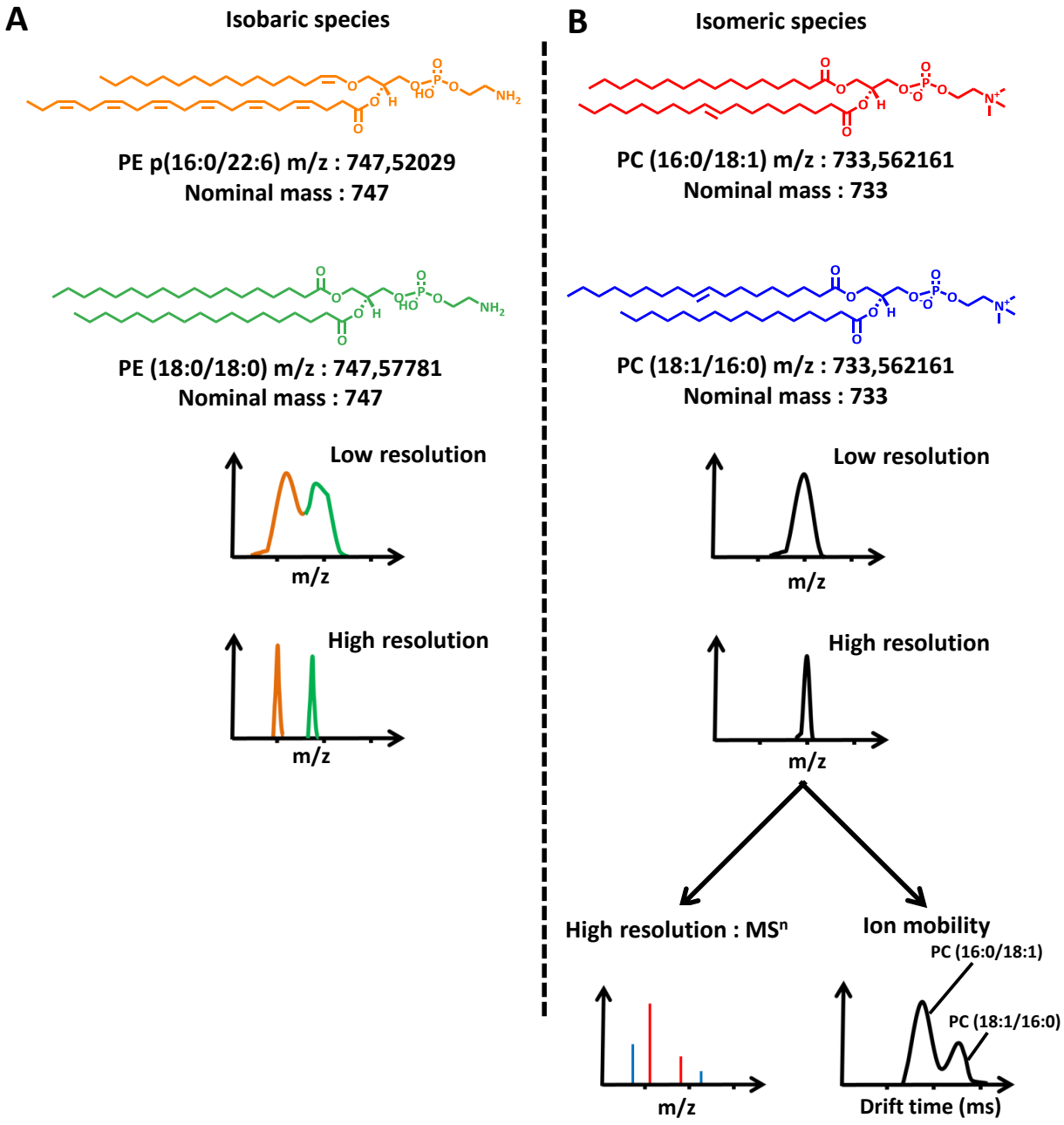

Figure 2. Isobaric and isomeric species study in lipid analysis. Since isobaric species are defined as two (or more) ions with the same nominal mass but different exact masses, the discrimination is not easy in routinely used mass spectrometers. (**A**) However, depending of the resolution of the mass spectrometer, these compounds can be distinguished based on their exact mass, with high resolution mass spectrometers. (**B**) For isomeric species which are compounds with exactly the same exact mass but with different positions in the molecule, it is very hard to separate them with classical HPLC conditions. However, these compounds can be easily separated with ion mobility or they can be discriminated studying product ions after fragmentation.

IV. Sample analysis

Besides nuclear magnetic resonance spectroscopy (NMR), sometimes used in lipid analysis (allowing structure elucidation, quantitative and qualitative analysis of targeted analytes in biological samples) but of limited application due to its low sensitivity, MS is a key technique in lipidomics. The principle of MS is the generation of molecular ions and the related fragments, differentiation of these generated ions depending on their *m/z* and measurement of the intensities of individual ions. A range of ion sources for ionization of lipids from biological matrices and of mass spectrometers with different benefits and disadvantages are available and will be briefly outlined in the next pages.

1. Ionization techniques

Electron impact (EI) is often considered to be a "hard" ionization method because it can cause considerable fragmentation and its usefulness is very limited for large lipids causing too much fragmentation and often barely detectable molecular ions. In comparison with this technique, "soft" ionization techniques have been developed and employed for lipid analysis and are more useful for this kind of compounds because they produce molecular ions and result in limited in-source fragmentation.

Electrospray ionization (ESI) MS-based lipidomics analysis is nowadays a crucial and essential tool for measuring lipidomes (plants, cells, fluids, tissues…).[47-49] The ESI source was developed by Fenn and al. in the 1980's.[50] The liquid mobile phase containing the analytes of interest flows through a metallic capillary contained inside the probe. An electric field is applied between the tip of this capillary and a counter electrode. Droplets are formed by nebulization when the liquid phase leaves the capillary, in the ionization chamber, forming the Taylor cone. Electrosprayed droplets will possess an excess of negative or positive charges according to the capillary bias polarity. When used in the negative mode, the droplets carry net negative charges because a negative electrical potential is applied to the end of the capillary and a positive electric potential is applied to the entry of the analyzer. Due to the presence of a heated gas, the droplet size decreases by evaporation of the mobile phase and increases the repulsive forces between the excess charges in the droplet leading to disintegration into many smaller droplets. At the end, the analyte molecule stays with residual charges attached. In the positive mode, ESI tends to produce only molecular adduct

ions, $(M+X)^+$ (e.g., H^+, K^+, Na^+, Li^+, NH_4^+) while it produces deprotonated molecular ions, $(M-H)^-$ or adduct ions, (e.g. Cl^-, formate, acetate) in the negative mode. The ESI ionization technique is among the least destructive methods and analytes become ionized with minimal in-source fragmentation. This negligible in-source fragmentation contributes to the higher detection sensitivity of ESI-MS, compared to other techniques with higher in-source decay. This is a big advantage of the use of ESI for quantitative analysis of lipids. ESI is also very often used because individual species of certain lipid classes having identical acido-basic properties possess similar ionization efficiencies.[51,52] However, the main limitation of using ESI is the ion enhancement and suppression effects resulting from co-eluting analytes.

Atmospheric pressure chemical ionization (APCI) is another well-established ionization technique for liquid samples. Actually ESI and APCI can be seen as complementary in lipid analysis. Indeed, whereas polar lipids such as phospholipids or many sphingolipids are preferably ionized by ESI, APCI is more suited for the analysis of relatively non-polar molecules and can also be used in the lipid analysis.[14,53,54]

In the APCI, the liquid mobile phase flows through a silica capillary inside the probe and droplets are produced by nebulization of the mobile phase into a spray. Contrary to ESI, a heater in the probe is used to evaporate the solvent and to produce gas-phase molecules. A corona discharge needle is present in the ion source, generating electrons and ionizing gaseous solvent molecules that form species (H_2O^+, N_2^+, O_2^+) that rapidly results in the formation of H_3O^+. Proton transfer from or to the ionized solvent can result in the chemical ionization of the analytes. Since APCI is a gas-phase ionization method, it seems to be less affected by ion suppression and ion enhancement than the ESI. However, considerable care is needed when using the APCI due to the possible huge in-source fragmentation.

For nonpolar lipids, which cannot form charged droplets in solution, APCI but also atmospheric pressure photoionization (APPI) can be very suitable. In the latter ionization technique, ion production is made possible by ionization under the effect of UV radiation produced by a krypton discharge lamp. Even if, APPI, involving absorption of a photon by molecules having the appropriate chromophore, is more used for targeted analysis, polar and non polar lipids can be easily ionized and it could be used as ESI for more large scale analysis.

A fourth, soft, ionization MS technique very often used in lipid analysis but also widely applied for proteomics is the Matrix-Assisted Laser Desorption Ionization (MALDI).[55-58] Here, the first step involves the absorption by the matrix material of the ultraviolet light (laser energy) and the conversion to heat energy. This lead to the very rapid (nanoseconds) heating of a very small part of the matrix (less than 1 µm from the top outer surface) leading to the ablation of a part of the matrix and production of a "plume" containing matrix molecules (ionized or not). In the second step, the molecules are ionized. This ionization method is rapid and easily used since it is now very simple (and automatable) to spot lipid sample on plates but has also several drawbacks. The presence of a lot of adducts complicates the analysis, the presence of lipid aggregation in the sample spot, and the fact that it is very difficult to have precise quantitative analysis in biological complex matrices, even if use of matrix such as 9-aminoacridine could be used for lipid quantification with a limited adduct formation. To improve the lipid analysis, recent advances as coupling (HP)TLC with MALDI try to resolve these difficulties, decreasing the total amount of matrix in the analyzed spot.[59,60]

2. Mass analyzers

Mass analyzers will separate the ions formed by the source according to their respective *m/z*. Even if the triple quadrupole (tandem quadrupole) is considered as the "gold standard" for lipids quantitation and remain the most widely used for that purpose[61,62], some other mass analyzers can be used for quantification but also for identification, providing a full range of spectral information with a high mass resolving power.[63]

As mentioned, triple quadrupole (TQ) mass analyzer represents the best choice for most routine targeted quantification assays. It is organized as three consecutive quadrupole stages. The first quadrupole (Q1) works as a mass filter transmitting only selected ions to the second quadrupole (Q2) that is used as collision cell. Inside this cell, the transmitted ions are fragmented by collision with an inert gas (helium, argon, nitrogen…). The last quadrupole (Q3) works, as the Q1, as a mass filter transmitting selected fragments to the detector. The general principle for this analyzer is the selection of a precursor "parent" ion, its fragmentation, and the measurement of the mass-to-charge ratio of the "product" ion formed. TQ offer a lot of benefits for lipid quantification: excellent sensibility and selectivity[64], fast positive and negative ionization, ability to run multiple analytes

simultaneously, very moderate cost. However, the main drawbacks of using TQ in lipidomics are the limited resolution (about 1500 FWHM) and low mass accuracy (typically 100 ppm at *m/z* 1000).

Even if TQ is the most appropriate instrument for quantification it is not quite the same for identification. Actually high resolution mass analyzers are more appropriate for large untargeted analysis, based on the *a posteriori* processing of the data using libraries or databases for identification of analytes, as opposed to the pre-programmed masses in the acquisition method usually used for TQ quantification. The Time of Flight (TOF) analyzer and the Orbitrap are both high resolution analyzers. TOF mass analyzers are able to separate different analytes based on the different "flight time" that they have. An electrical field is used to accelerate ions traveling with a same potential and the time taken for each analyte to reach the detector is used to determine the m/z ratio. The heavier ions reach the detector after the lighter ions. TOF is widely used for lipid analysis with high efficiency and high mass accuracy/resolution.[65-67]

The Orbitrap mass analyzer consisting in two electrodes (an outer barrel like electrode and a central spindle like electrode along the axial axis) shows a very high resolution (up to 150 000 FWHM) providing very good selectivity using exact mass measurement.[68] Before entering the Orbitrap, ions are delivered into a curved rf-only quadrupole (C-trap) filled with nitrogen. After focusing in this trap, ions are accelerated and converge into a tight beam to enter in the Orbitrap. Ions are captured in the Orbitrap by increasing the electrical field and have different frequencies of oscillation depending on their m/z ratio. Signals from electrodes are transformed into a frequency spectrum by fast Fourier transformation and the resulting frequency spectrum is converted into a mass spectrum. Orbitrap can, as TOF or TQ, also be used for lipid analysis, from quantification to identification of new lipid species.[69-71]

Other mass analyzers such as FT-ICR (Fourier Transform Ion Cyclotron Resonance) principally use cyclotron frequency in a fixed magnetic field for the determination of the ions mass to charge ratio (m/z) can also be used for lipid analysis. Indeed, these analyzers possess very high resolution and sensitivity. However, they are very expensive and highly cumbersome explaining that its use in routine is limited.

V. Possible causes affecting exact lipid quantification

1. Matrix effects and ion suppression:

The matrix refers to all the components present in the sample, other than the target analyte(s). Tissue samples, blood, plasma, bile, urine are common matrices frequently encountered by bioanalytical scientists. The "matrix effect" is a generic name used to describe the problems met during (lipid) analysis of complex biological matrices. This effect is usually caused by endogenous biological components (endogenous metabolites, proteins, other lipids) or by exogenous substances introduced during sample processing and analysis (e.g. mobile phase additive or contamination during sample preparation, such as polymers extracted from plastic containers). Depending on their properties, it may be necessary or not to remove these interfering species from the sample.

Several common components are present in most matrices but their levels may vary among individuals (genetics, disease state…). A matrix effect is often encountered when a compound from the matrix (for example, a phospholipid) co-elutes with the targeted analyte and leads to an increase (enhancement) or a decrease (suppression) in ionization efficiency, in comparison with the analyte elution without matrix. This could lead to an erroneous estimate of the concentration.[72-74] Several strategies are conceivable to reduce or suppress matrix effects. It is possible to optimize the chromatographic separation to separate the target analytes from the more interfering compounds. Another possibility is to optimize the sample preparation, to obtain "as clean as possible" samples. However, sometimes these approaches are not effective enough and can lead for instance to analyte losses or result in long chromatographic run times. Sample dilution is also possible and constitutes a very simple method to reduce the amount of coexisting matrix components in a sample.[75] However this is possible only if the analyte is present at levels compatible with the sensitivity of the method used. Another strategy to decrease the impact of the matrix effect is to quantify the analytes by standard addition. However, this is practically feasible for a limited number of samples and of compounds.

The ionization methods used for the LC-MS method could also impact the matrix effect. The concentration and the chemical structure of the analytes and co-eluting matrix components could determine whether they influence each other during the ionization process. Several

parameters of the source can be modulated to decrease the matrix effects. For instance, it is possible to change the ionization mechanism (positive or negative polarity), but also the ionization mode (e.g. ESI vs APCI) or the source design (depending of the manufacturers and including several spray orientations).[76]

2. In-source fragmentation

This phenomenon corresponds to the fragmentation of an analyte in the source, during the ionization process, and not in the mass analyzer. Some analytes (e.g. oxysterols) are more sensitive to this phenomenon than others. This phenomenon can result in qualitative and quantitative artifacts. For instance, if a phospholipid loses its head group during the ionization, the resulting phosphatidic acid (PA) might not be differentiated from the endogenous PA. Here the chromatographic separation is a crucial element to avoid this type of artifact. An accurate determination of the endogenous PA levels is possible by using liquid chromatography to separate PA and PA moiety generated by more complex phospholipids, with different retention times.[77] Clearly using hard ionization conditions (e.g. high ionization voltage or temperature) could favor in-source fragmentation. In addition to add complexity in the qualitative analysis (see above), in-source fragments can also add variability for quantification (fragmentation depending on the different molecular species). Thus during process development the sensitivity of the lipid analytes to this phenomenon should be accounted for.

3. Choice and availability of IS

Internal standards are crucial in lipid analysis from complex biological matrices. The choice and the availability of the internal standard used is critical but also the number of internal standards used for quantification, depending on the type of analysis.

As for others quantification technics (such as UV), no direct link exists between the real concentration of an analyte and the absolute intensity of its molecular ion obtained by mass spectrometry.[78] Several factors as sample extraction, ionization, analysis conditions could affect the ion intensity and negligible changes of one of these factors could modify the ion intensity. Because it seems impossible to exactly repeat an experiment from time to time, exact quantification should be made by comparison to internal or external standard.

External standard being analyzed separately, internal standard mimics better all the steps of an analysis.

Internal standards are necessary for quantitative analysis of lipids contained in biological matrices by mass spectrometry to be able to control the variability in recovery from the biological material and the factors that could affect ion yield.[79] The amount of internal standard should be optimized to have acceptable relation intensity between the internal standard peak and the ion peak corresponding to the most abundant species in the class. Indeed, if the internal standard amount is to low, the experimental error is amplified while if this amount is too high, an ion suppression effect can appear. With this approach, the internal standard and the analyte are analyzed at the same time.[80] The quantification is made by ratiometric comparison with the internal standard and the analyte of interest.

Ideal quantification of the analyte can be achieved accurately only if the internal standard, almost chemically identical to the analyte, has a very similar response factor[a] compared to the analyte. Because it should not be present in any of the samples to be analyzed and should be as similar in physiochemical properties to the analytes of interest as possible, the ideal internal standard is a stable isotope-labeled version of the analyte of interest.[81] However, having such standard for each compound of interest is not possible regarding the large number of analytes examined in lipidomics. It is also impractical to use thousands of internal standards for quantitative analysis of the complex lipidome.[82] Consequently, an alternative approach is to identify internal standards that are related in structure, ionization and fragmentation characteristics for each of the different categories of studied compounds (**Figure 3**). Again, because stable isotope-labeled standards are not available for all lipid species (e.g. lysophospholipids or phospholipids) odd number of carbons on the acyl chain is also often use in lipidomics (e.g. LIPID MAPS Mass Spectrometry Internal Standards).[21] Addition of one standard for each lipid class was shown to be sufficient for the lipid quantification since the ionization of lipids is mainly dependent on the class specific head group, more than the fatty acyl chains.[83]

[a] that is a same amount of analyte and internal standard will result in a same signal intensity for both compounds

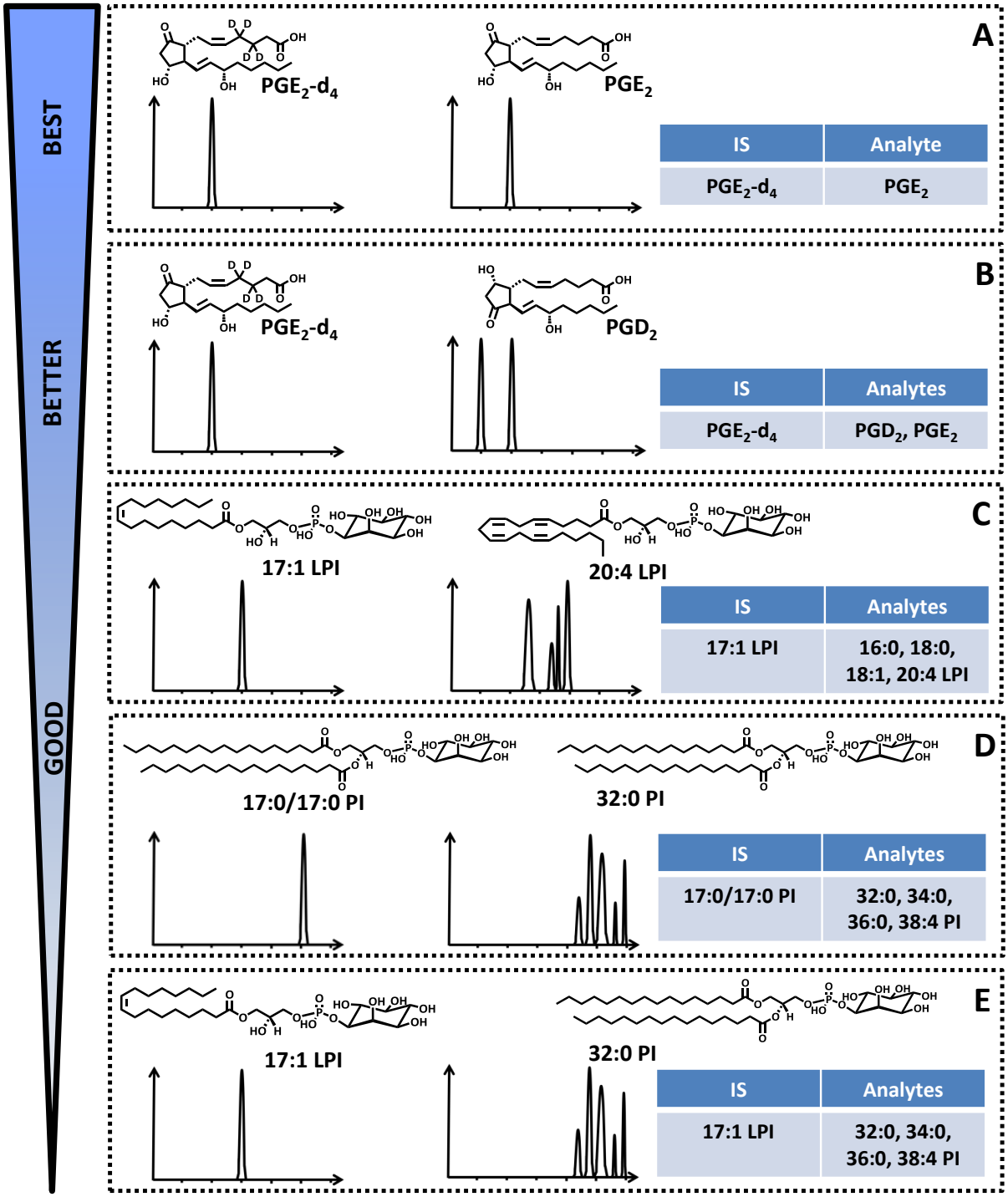

Figure 3. Use of internal standards for lipidomics studies. From the perfect internal standard to what is possible to use for a daily wide lipidomics analysis. (**A**) In limited target analysis, the best option is the use of the corresponding SIL to avoid ion suppression or matrix effect since both analytes are eluted in the same time: PGE_2-D_4 for PGE_2. (**B**) Often a SIL is used for all the family of compounds: PGE_2-D_4 for PGE_2 and PGD_2. When SIL are not available, structural analogues are used (very often in glycerophospholipids analysis) : (**C**) 17:1 LPI for LPI and (**D**) 17:0/17:0 PI for PI but also sometimes (**E**) the lysophospholipid corresponding of the phospholipids with 17:1 LPI for PI.

For sure, it is necessary to add all the internal standards to each sample as early on in the analytical procedure as possible. Typically this could be done at the beginning of the experiment, prior to the lipid extraction to correct for partial recovery and to correct for any losses during sample preparation. It can also be used to account for a number of method variables as recovery during clean-up and transfer stages, injection volume variability, matrix suppression…

Ideally, lipid standards (analytical and internal standards) should be stored in glass containers at relatively high concentration (mM to M) to avoid variation of concentration in absence of light and at temperatures below 20° C. Lipid standards removed from cold storage should be allowed to reach room temperature before use.

The choice of internal standard itself can have a significant effect on the results obtained by LC-MS. For some compounds, using two deuterium (D2), five deuterium (D5) or three carbon 13 enrichment (C13) give different results. This is why the effects of the combination of chromatography and internal standard choice should be investigated during method development and the same internal standard should be kept for all the experiment.[84]

VI. Untargeted Lipidomics vs targeted lipidomics

As discussed in the previous points, nowadays, practically all lipidomics methods are MS based. TQ, TOF and Orbitrap can be used for lipid analysis with different advantages and drawbacks for each analyzer. All these analyzers can be combined with several types of sample introduction systems and ionization sources. Lipidomics strategies can be divided into two main approaches - untargeted and targeted lipidomics – each one with its own advantages and limitations.

Global lipidomics, also called untargeted lipidomics (**Figure 4**), use an unbiased way for detecting analyte. The mass spectrometer analysis is performed with a broad mass range for a global detection of lipids. This kind of analysis is conducted without *a priori* on lipid-classes and all ionized lipids will be detected (without preliminary information on the molecular ions and their fragmentation). To be able to identify all the lipids in the studied sample, these analyses are very often performed using high resolution analyzers (TOF, Orbitrap…) reaching ppm mass accuracy, allowing detecting lipids based on their exact mass.[85,86] However, in this

scanning mode it is very difficult to easily distinct molecular lipids with the same mass and it is why MS scans could be combined with fragmentation to increase the capability of identification of molecular lipids. Moreover, accurate quantification could be hard due to the complex sample matrix (and possible ion suppression) and due to the possible absence/inadequacy of internal standards. Again, because matrices are complex, runs can be very long in untargeted lipidomics to achieve acceptable separation.

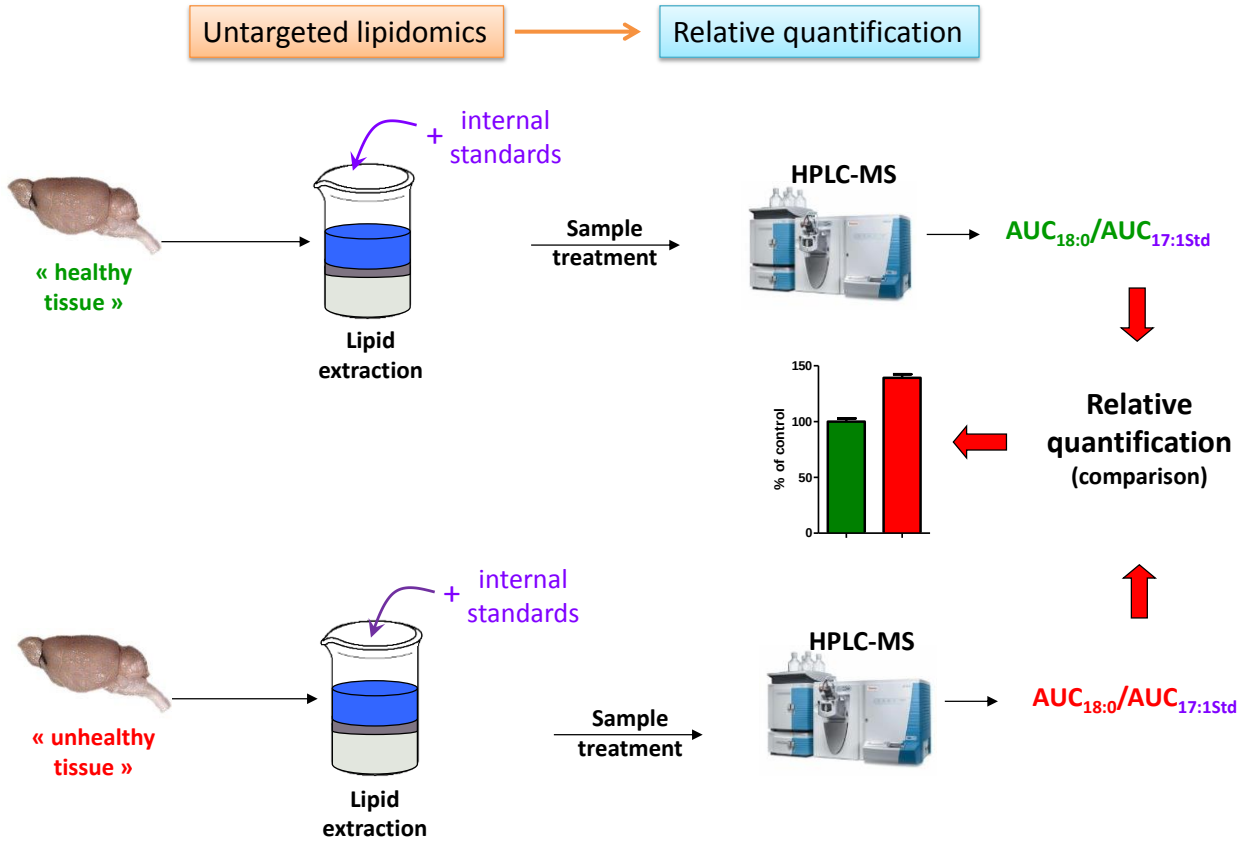

Figure 4. General view of untargeted lipidomics.

Targeted lipidomics is focusing on a selected lipid class or on selected lipids using specific fragments (both parent ions and their specific product fragments ions) (**Figure 5**). Selective MS methods as multiple reactions monitoring (MRM) or neutral loss scan (NLS) on TQ are typically used, drastically increasing the sensitivity of the method. In these analyses, both Q1 and Q3 are locked on specific selected masses allowing very sensitive and selective detection.[87] With this approach quantitative data for one single analyte to hundreds of lipids can be realized keeping in mind that the number of monitored lipids should be compatible with the instrument. Indeed if a too large number of lipids are monitored in the same time using MRM, it is possible that not enough data points per chromatographic peak are

generated leading to inaccurate quantification. Recent advance in instruments as development of faster instruments or the use of scheduled MRM depending of the retention time after LC (MRM transition is limited to a certain time window) allow to increase the number of detected lipids in the same run.

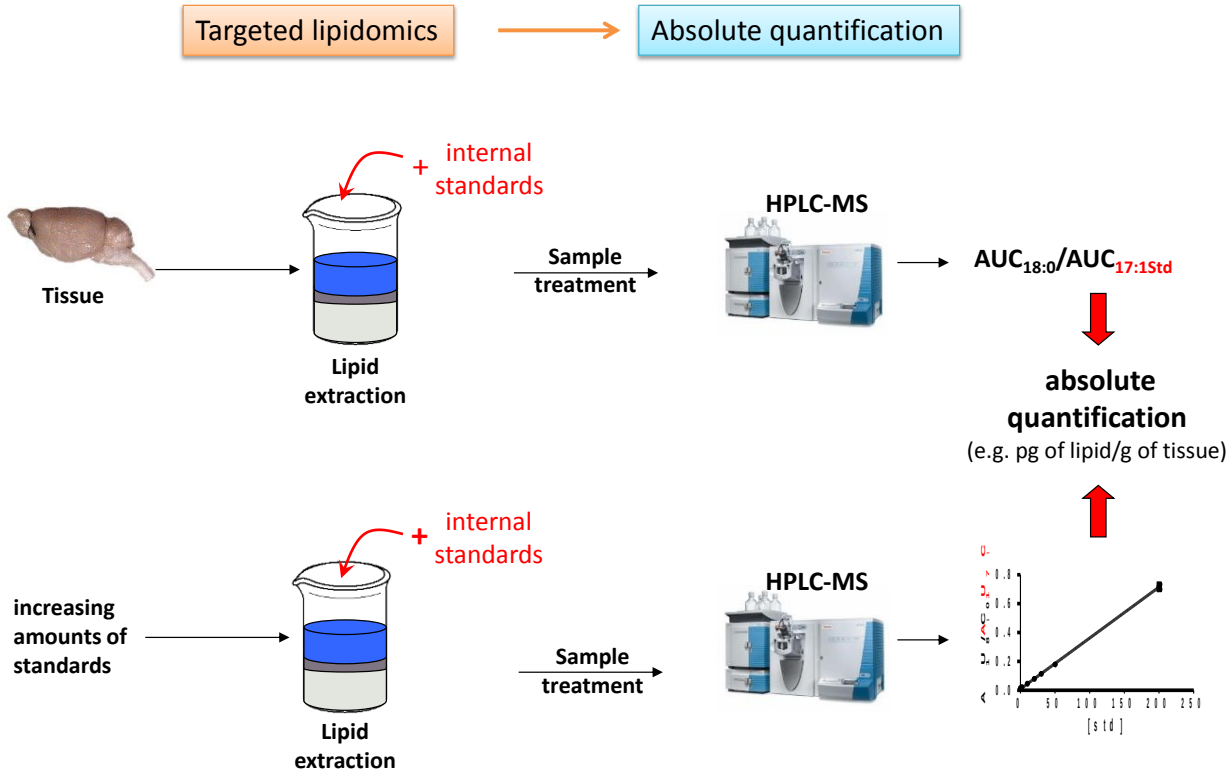

Figure 5. General view of targeted lipidomics.

The untargeted lipidomics method gives the better number of individual analytes that can be analyzed in a unique experiment having the capacity for discovering unexpected molecules but due to the complex spectrum obtained, it is less sensible than targeted lipidomics. In the contrary, targeted lipidomics approach shows a very high sensitivity during analysis of a limited number of individual molecular species with a very small capacity to find unexpected analytes (**Figure 6**).

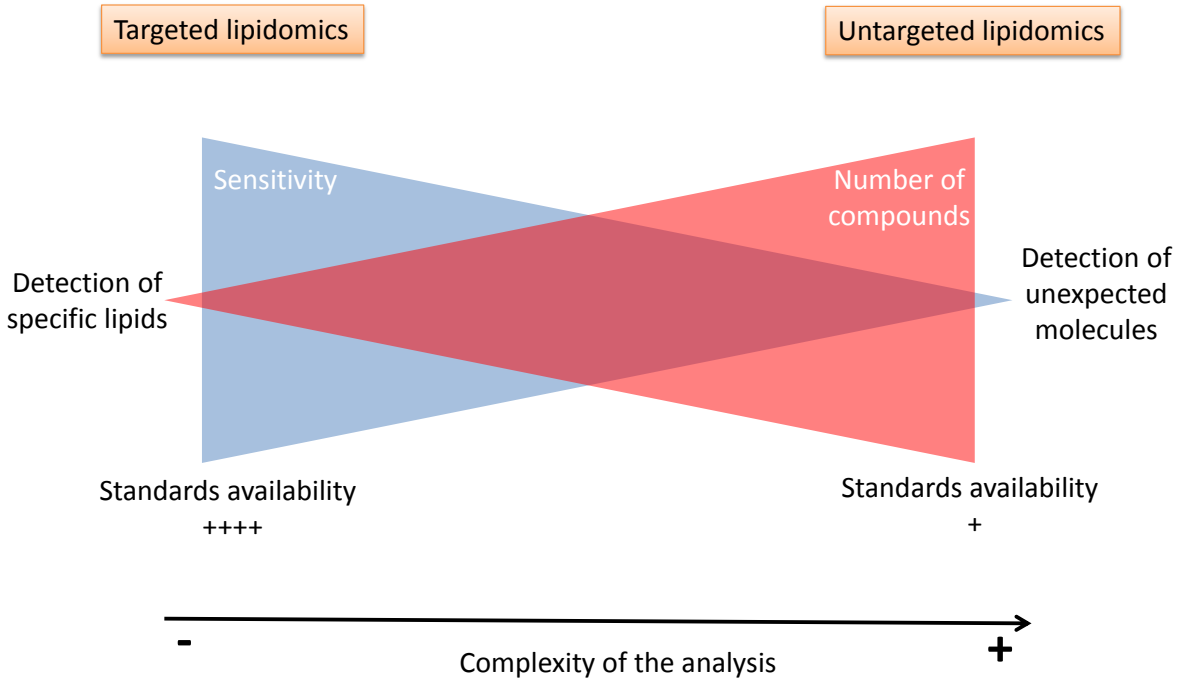

Figure 6. Advantages and drawbacks for both analysis.

VII. Conclusion

Lipidomics is still a young and emerging "omics" field with new appearing strategies and lipid analysis clearly became essential in the study of physiopathology (from discoveries of mechanisms to study of treatments and of diseases evolution). As discussed here, the potential analytical methods to be used in lipidomics are numerous and can be very different. Nevertheless, beside the MS step, special attention should be given to the entire process from sample collection to sample separation to reach an accurate quantification and a precise identification of lipids species. New efficient approaches in extraction (BUME method, MTBE method…), in separation (ion mobility…) or in detection (scheduled MRM to increase the number of detected lipids simultaneously) help to develop lipid analysis (simultaneous analysis of different lipid classes) and facilitate identification and interpretation of lipidomics data. Further developments targeting an increase of the throughput of this technology through global automation, along with standardization, will certainly place MS based lipid analysis in the heart of many discoveries from target elucidation to clinical programs.

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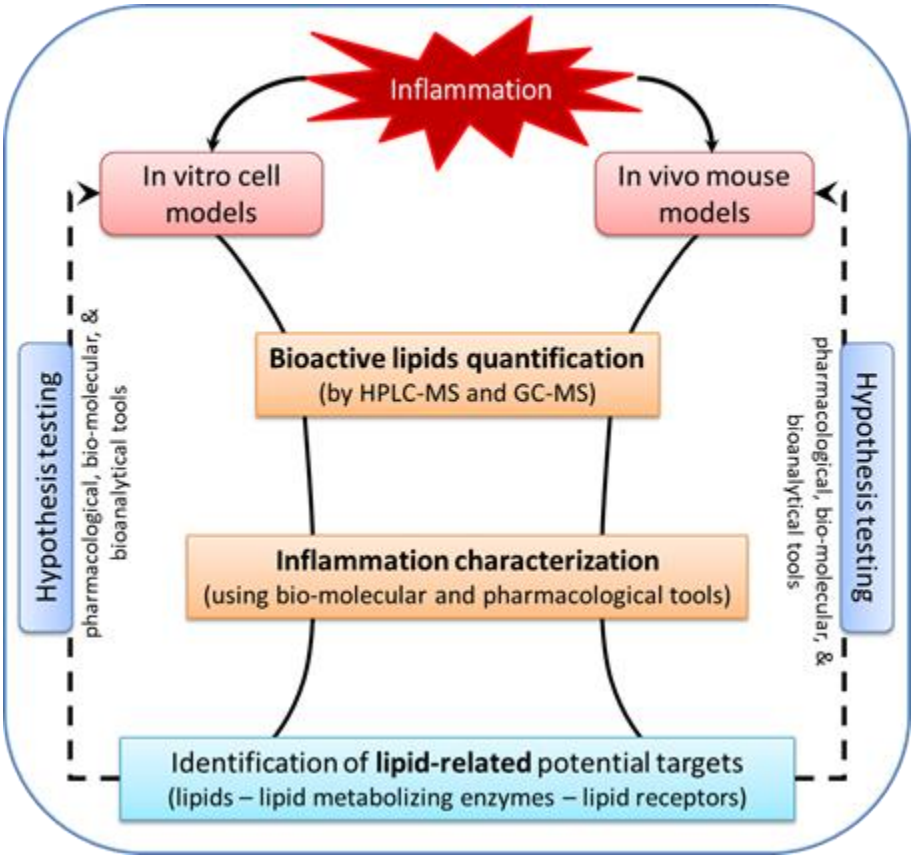
AIMS & OBJECTIVES

The general aim of the "Bioanalysis and Pharmacology of Bioactive Lipids" (BPBL) research group, is to explore the role of bioactive lipids both in physiological and pathological situations mainly related to inflammation as a first step, to be able, in a second time, to suggest possible novel therapeutic targets for inflammatory diseases.

To do so we combine expertise in bioanalytical methods and in inflammatory models as outlined below.



As it clearly appears from this scheme, being able to quantify the bioactive lipids is a central part of the strategy developed in our group. **Thus the main focus of this thesis is to develop several types of approaches allowing for the quantification of the bioactive lipids of interest from biological samples** (cells and tissues). As discussed in the introduction, two approaches can be used, either an "untargeted" approach or a "targeted" approach. Thus, the first part of the work was devoted to the set up and application of an untargeted approach for the identification and characterization of lipids by HPLC-ESI-MS. Next, because we were interested in the properties of lysophosphatidylinositols, we decided to set up and

validate an HPLC-MS method to quantify their levels in cells and tissues. Both methods were largely used to study the effect of inflammation on bioactive lipid levels.

In the last part of the work, based on preliminary data from the laboratory and on the fact that lysophosphatidylinositols are produced by cPLA2 activity, we thought it would be interesting to explore the properties of lysophosphatidylinositols on macrophage activation.

Thus, the specific aims of this thesis can be outlined as follows

(i) Development of an untargeted approach for the identification and characterization of lipids by HPLC-ESI-MS and application to macrophage activation.

- Using an HPLC-LTQ-Orbitrap XL, the aim is to set-up an HPLC-ESI-MS method to allow the analysis of several phospholipids, lysophospholipids, (dihydro-) ceramides, galactosylceramides, (hydroxylated-) sulfatides, and fatty acids from the same biological sample.
- Apply the developed method to the characterization of the effects on lipid levels of LPS-induced J774 macrophage activation

(ii) Development and validation of an HPLC-ESI-MS method for the quantification of lysophosphatidylinositols

- The objective is to develop and validate a specific and sensitive HPLC-ESI-MS targeted analysis method for quantification of lysophosphatidylinositols and applied it to the determination of their levels in mice tissues.

(iii) Study of the effect of macrophage activation on lysophosphatidylinositol levels and the effect of lysophosphatidylinositols on macrophage activation.

- the first aim is to characterize how macrophage activation will affect lysophosphatidylinositol levels
- the second aim is to determine if and how lysophosphatidylinositol incubation will affect LPS-induced macrophage activation.

Taken together these studies should contribute to a better understanding on how inflammation affects bioactive lipids.

RESULTS

This results section is divided into 3 parts :

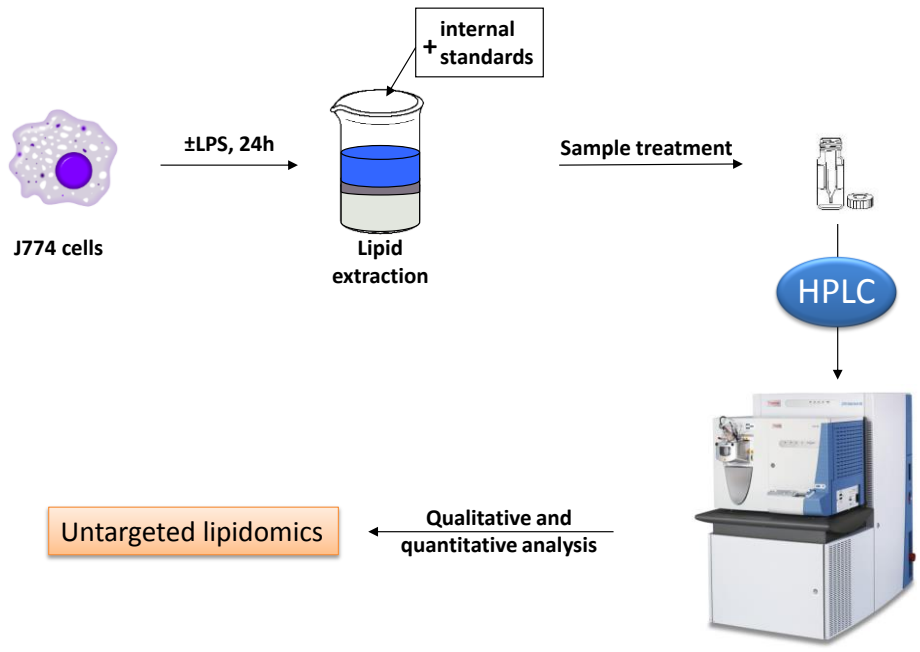
Part 1. Development of an untargeted approach for the identification and characterization of lipids by HPLC-ESI-MS and application to macrophage activation.

Part 1. Development of an untargeted approach for the identification and characterization of lipids by HPLC-ESI-MS and application to macrophage activation.

I. Set up of the methodology: identification and characterization of lipids by HPLC-ESI-MS

The first part of our work consisted in finding conditions allowing for a detection as broad as possible of the major lipid species present in mammalian tissues (representing most of the samples that our laboratory works with). As mentioned, we had access to a LTQ-orbitrap XL coupled to an HPLC system, equipped with both ESI and APCI sources. Therefore our work was driven by these elements. During the development of this method, we used several C18 HPLC columns explaining the variability in the retention times between experiments.

Global method for lipids analysis



The identification of the lipids present in the total lipid extracts was accomplished by high pressure liquid chromatography-electrospray-mass spectrometry (HPLC-ESI-MS) in ESI negative mode, ESI positive mode and APCI positive mode, depending of the goal of the analysis. In ESI negative ionization mode, the mode most frequently used here, the LTQ-Orbitrap XL tends to yield upon MS fragmentation fatty acid (FA) residue fragments and lyso-

lipid fragments depending on the parent analyte. Further fragmentations of these species yielded a broad range of information giving fragments as “head group” fragmentation, lyso-lipid formation, or FA fragments helping in the lipid identification. Some species, such as phosphatidylinositol (PI) or sulfatides, generate a large variety of product ions while some others result in less information rich spectra.

In negative ionization mode, some lipids are ionized as $[M-H]^-$ (e.g. Cer, sulfatides, PI, PE, PG, PS and respective lysophospholipids etc.) while some others (e.g. PC, SM, LPC…) are, as described in literature, detectable only after the addition of ammonium acetate $[M+CH_3COO^-]^-$ allowing the ionization and the detection.

Of note, for some lipids (PC are a typical example) ionization as $[M+H]^+$ ion in ESI positive mode is possible due to the quaternary ammonium polar moiety. Nevertheless, the ion pairing with formic acid or acetic acid allows their detection as $[M+RCOO^-]^-$ and therefore their detection along with the other lipid classes in ESI negative mode.

Because we worked with a LTQ-Orbitrap XL, unfortunately it was not possible to switch the polarity during the run. Indeed, to reach stability of the signal with this mass spectrometer, 90 minutes are needed after polarity switch. This is why it is important to select a polarity for our untargeted method.

In this section, we chose to show relative abundance and histogram of AUC to allow the visualization of all the pics. Indeed due to the huge difference in terms of ion numbers, we could not see all the pics in a same chromatogram without using 100% for each example.

HPLC-MS analysis of phospholipids and lysophospholipids often show 2 peaks due to the presence of *sn*-1 and *sn*-2 fatty acyl position for lysophospholipids and to the presence of 2 different acyl chains concerning phospholipids.

From a practical point of view, when looking at the detection of lysophospholipids (**Figure 1** & **2**) and phospholipids (**Figure 3** & **4**) it is clear that the ESI negative mode allows for a larger number of lipid species to be ionized. Indeed, LPG, LPI, and LPS were not detectable in ESI positive mode while they were detected in ESI negative. For the negative mode, a chromatographic gradient was used and mobile phases A and B were composed of MeOH-H_2O 85:15 (v/v) and MeOH, respectively, with 5 mM of CH_3COONH_4 while for the positive

mode, a chromatographic gradient was used and mobile phases A and B were composed of MeOH-H_2O 75:25 (v/v) and MeOH, respectively, with 0,1% of CH_3COOH.

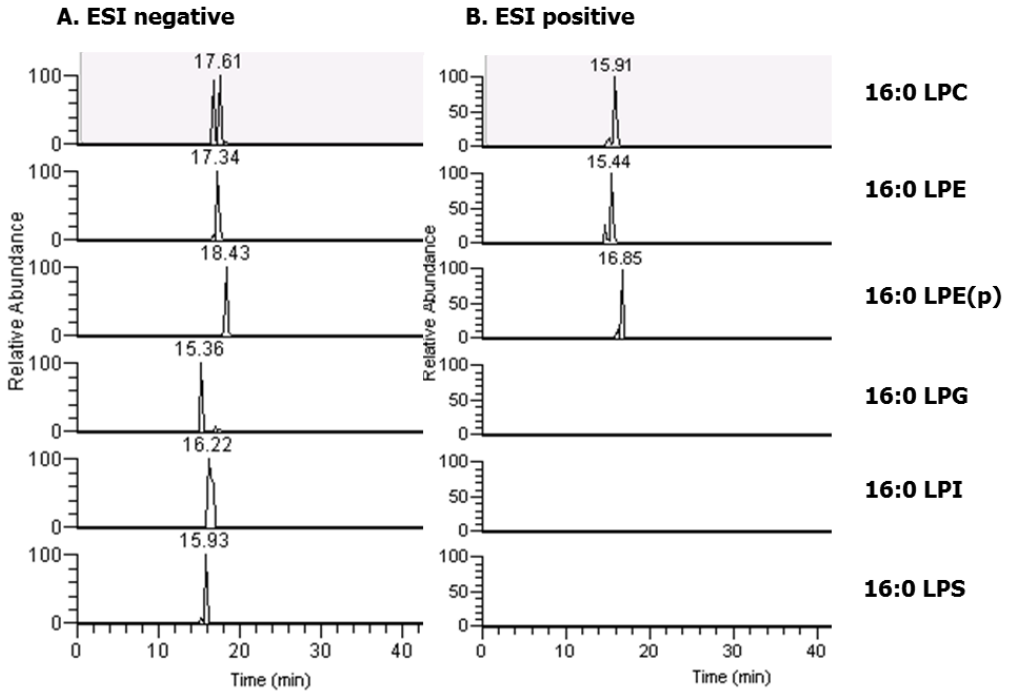


Figure 1. Comparison of lysophospholipids detection based on the ionization mode. ESI negative mode (**A**) allowed us to detect all the studied lysophospholipids while, in our conditions, in ESI positive mode (**B**) only LPC, LPE (and LPE(p) but more than 20 fold less than the others) were detected with MeOH, H_2O and 5 mM CH_3COONH_4 as mobile phase.

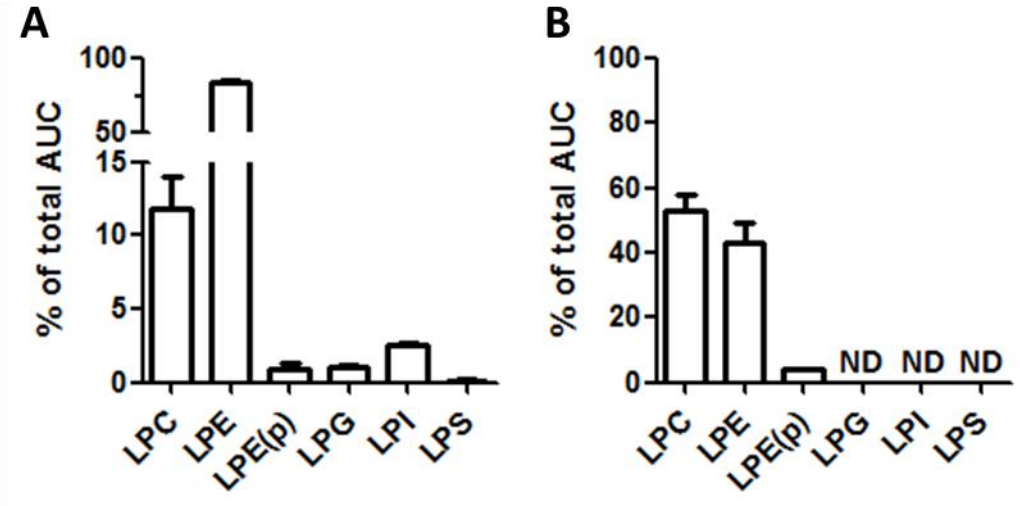


Figure 2. Total area under the curve (AUC) for 16:0 lysophospholipids in negative (A) and positive (B) mode. ESI negative mode allowed us to detect all the studied lysophospholipids while in our conditions, in ESI positive mode LPG, LPI and LPS were not detected: % of the total of AUC for 16:0 lysophospholipids.

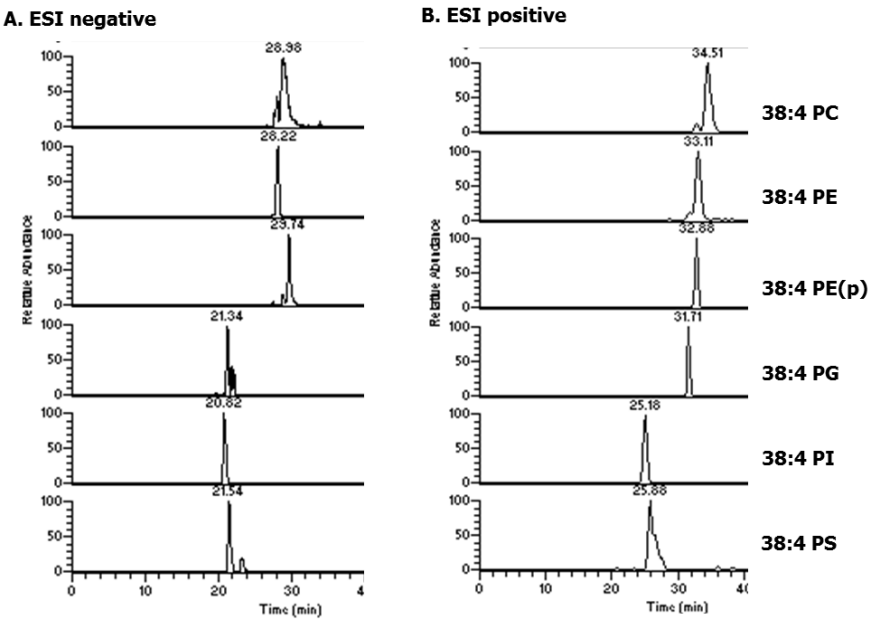


Figure 3. Comparison of phospholipids ionization mode. ESI negative mode (**A**) allowed us to detect all the studied phospholipids with similar intensities while, in our conditions, in ESI positive (**B**) mode only PC and PE were detected with high intensities (PG and PS are detected with 100 times lower intensities and PE(p) and PI with more than 20 times lower intensities in comparison with PC and PE).

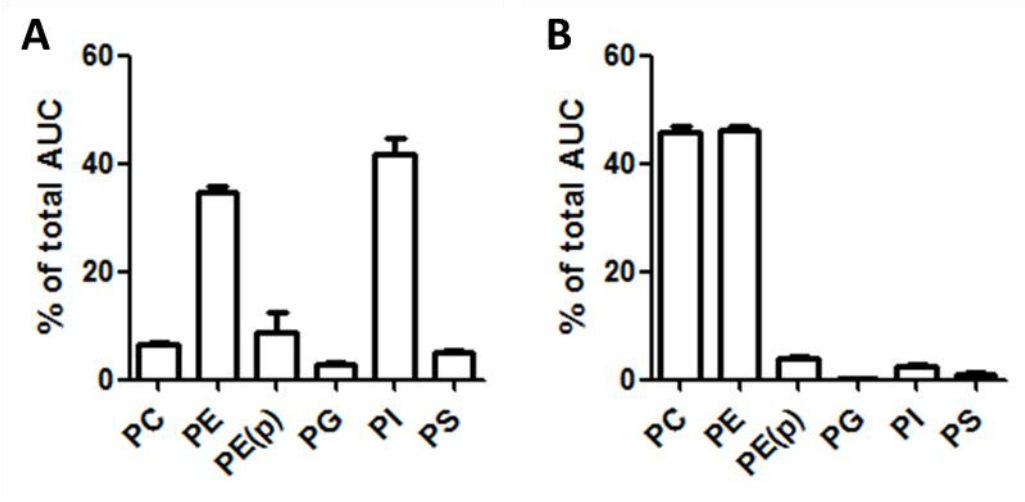


Figure 4. Total area under the curve (AUC) for 38:4 phospholipids in negative (A) and positive (B) mode. ESI negative and positive mode allowed us to detect all the studied phospholipids but negative mode was more constant in the detection represented by % of the total of AUC for 38:4 phospholipids.

Of course, during the development of the method and choice of the ionization mode, the mobile phase is also a crucial parameter. Therefore, we tested different mobile phases based on what is most often described in the literature. In ESI negative mode, among the numerous systems tested two were quite interesting. The first one consisted in a gradient

with water and methanol containing NH_4OH to assist in the ionization of (lyso)phospholipids (Panel B of **Figures 5** & **6**). The other one consisted in a gradient of water and methanol containing CH_3COONH_4 to assist in the ionization, as acetate adducts, of the choline moiety-bearing lipids (Panel A of **Figures 5** & **6**).

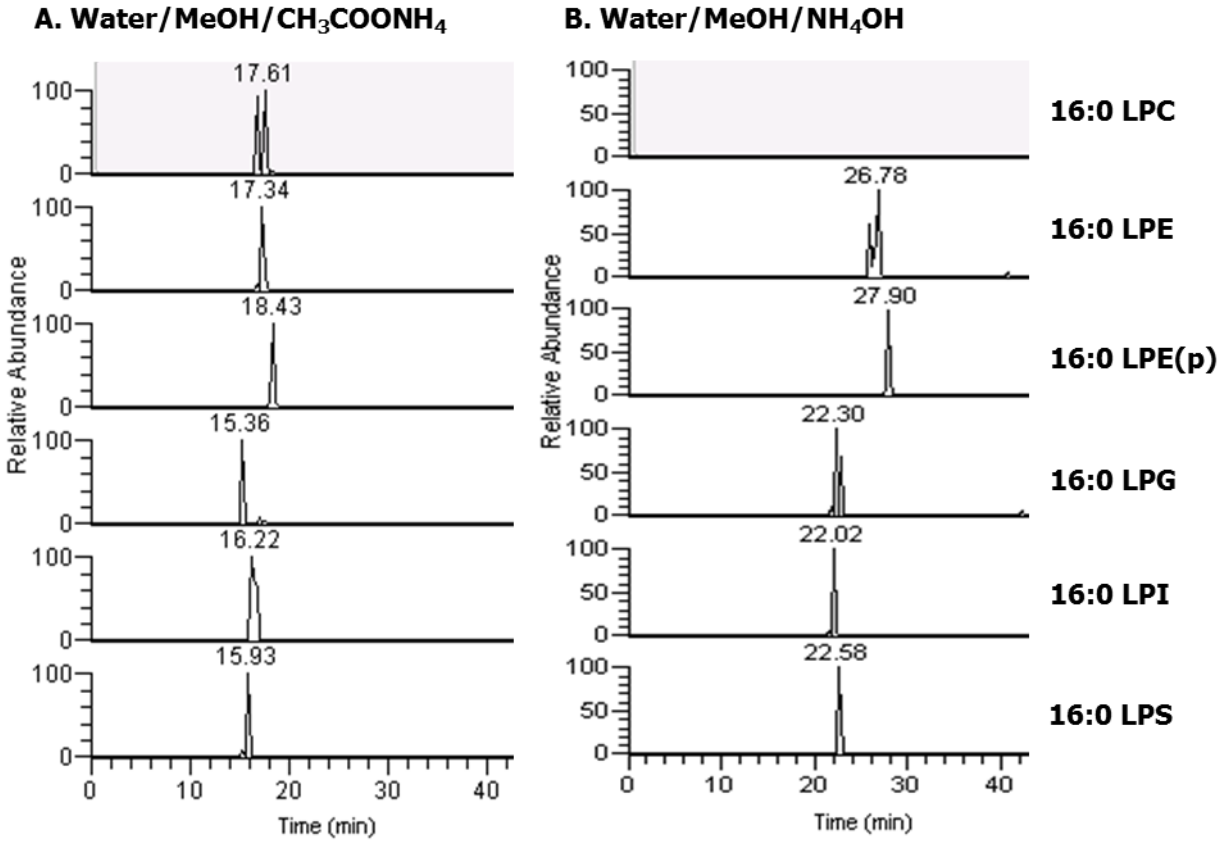


Figure 5. Comparison of lysophospholipids detection depending on the mobile phase. Two different mobile phases were tested in ESI negative mode: a mix of water and methanol with 5 mM of ammonium acetate (**A**) or with 0,1% of ammonium hydroxide (**B**) as modifier, with a better detection with ammonium acetate.

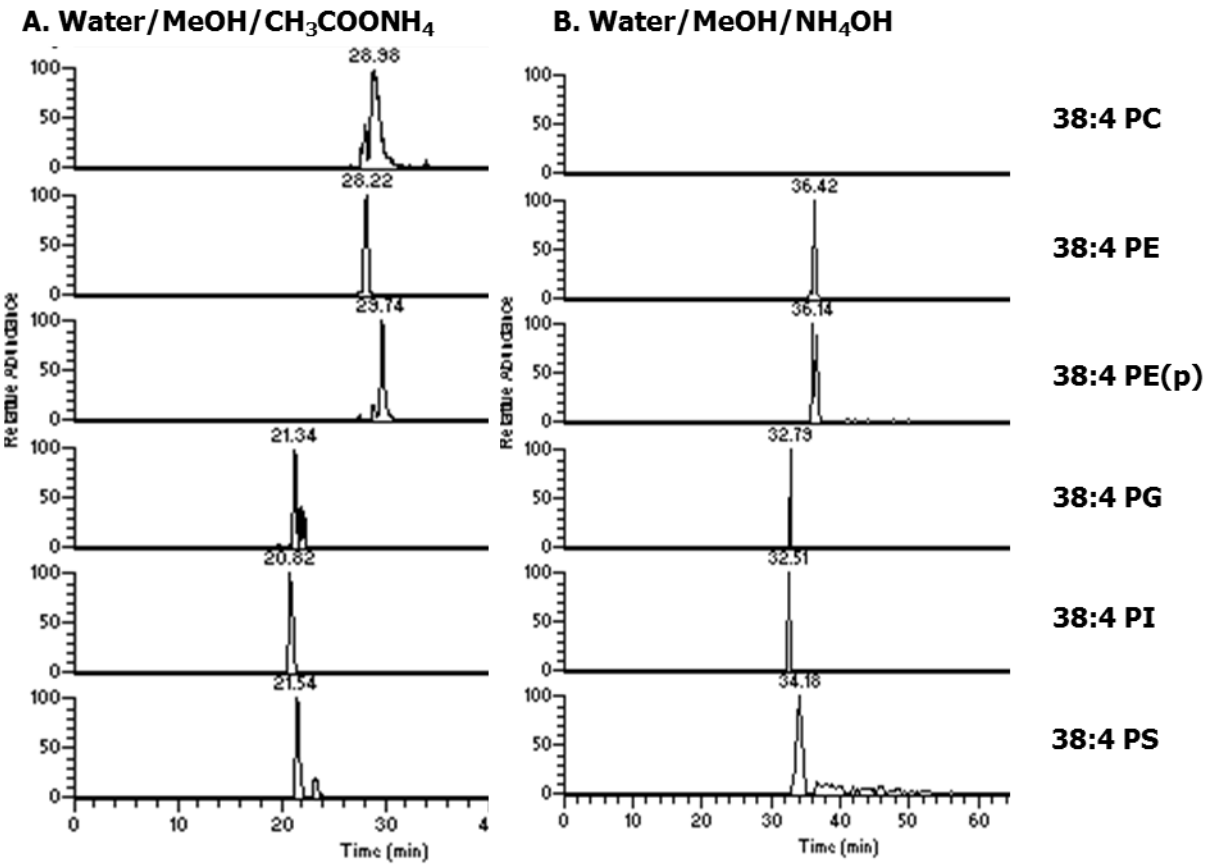


Figure 6. Comparison of phospholipids detection depending of the mobile phase. ESI negative mode was used to test two different mobile phases: a mix of water and methanol with 5mM of ammonium acetate (**A**) or with 0,1% of ammonium hydroxide (**B**) as modifier, with a better detection with ammonium acetate. With the NH_4OH mobile phase, only PE, PE(p) and PI were properly detected, PG and PS were only present as traces and PC was not detected.

The water/methanol/CH_3COONH_4 mobile phase, allowed us to detect PC, LPC but also SM which was not possible using NH_4OH as modifier of the mobile phase. We also noticed that in these conditions, sphingolipids were detected as acetate adducts as well.

Based on these results, as our aim was to detect as much lipids as possible in a single run, we selected the system containing ammonium acetate and ESI negative as ionization mode. Note that only PE, Cer and GalCer are lipids that can be analyzed with similar sensitivity regardless of the polarity ionization.

Before finalizing our choice, we also wanted to test the APCI source. Indeed this source allows a better ionization for several lipid species. However, APCI tends in some case to induce in-source fragmentation (see **figure 7** as an example) thus complicating the interpretation of the data, especially for untargeted methods.

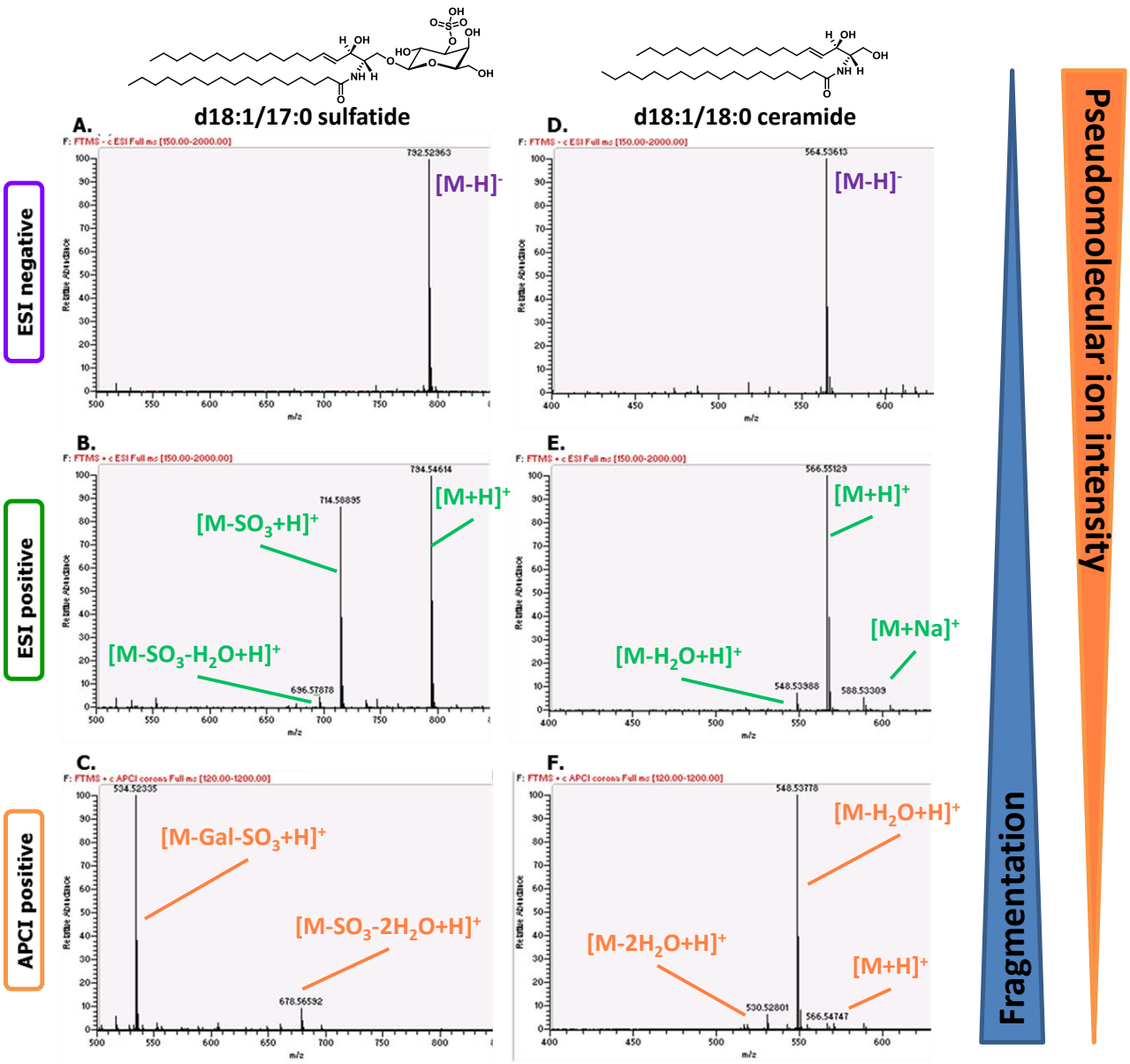


Figure 7. Examples of differences in ionization with d18:1/17:0 sulfatide and d18:1/18:0 Cer, using ESI in negative and positive mode and APCI in positive mode. For d18:1/17:0 sulfatide, while in ESI negative mode, the obtained ion correspond to $[M-H]^-$ only (**A**), in ESI positive mode ions correspond to $[M+H]^+$, $[M+H-SO_3]^+$, $[M+H-SO_3-H_2O]^+$ (**B**), and in APCI positive mode to $[M+H-SO_3-2H_2O]^+$ and [M+H-galactosulfate]$^+$ without presence of $[M+H]^+$ (**C**). For d18:1/18:0 Cer, while in ESI negative mode, the obtained ion correspond to $[M-H]^-$ only (**D**), in ESI positive mode ions correspond to $[M+Na]^+$, $[M+H]^+$ and $[M+H-H_2O]^+$ (**E**) and in APCI positive mode to $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ (**F**).

In untargeted methods, it is not common (and feasible) to confirm the nature of an ion (molecule) based on its co-elution with an internal standard. Thus, extra care should be taken in the interpretation of the HPLC-MS data and strategies should be used to support identification.

During this work, we used several elements in order to support our identifications.

- Thanks to the use of an LTQ-Orbitrap XL, we only used exact masses (m/z) with a high precision (less than 5 ppm)
- To confirm the nature of the peaks based on the retention time (e.g. **Figure 8**) and mass spectra (e.g. **Figure 9**), we always analyze standards of the compounds directly following our samples of interest, thus in the same chromatographic conditions.
- For some lipids, the behavior (e.g. retention time, fragmentation) of the internal standard is also informative.
- The elution order of the lipids is also very much informative. Indeed, the elution order is determined by several parameters such as the water solubility of the analyte, the carbon content, the presence of insaturations, the form and the interactions with stationary phase. Thus, for analogous species, the retention time is increased when the number of carbon atoms increases, while insaturations reduce the retention and "branched-chain" analytes are eluted more rapidly than linear isomers.

The following data obtained for LPI will illustrate these elements. As evident from **Figure 8**, the retention times of the LPI found in a mouse brain lipid extract have the same value than the retention time obtained for a standard LPI preparation (soy bean extract) (**Figure 8**). Moreover, we calculated the mass accuracy (ppm) for standard soybean and mouse brain LPI. We found 4,9 ; 4,3 ; 4,5 ; 4,6 ; and 4,7 for 16:0 LPI, 18:0 LPI, 18:1 LPI, 18:2 LPI and 20:4 LPI respectively for standard soybean LPI and 3,3 ; 3,2 ; 3,1 ; 3,6 and 3,3 for 16:0 LPI, 18:0 LPI, 18:1 LPI, 18:2 LPI and 20:4 LPI respectively mouse brain LPI.

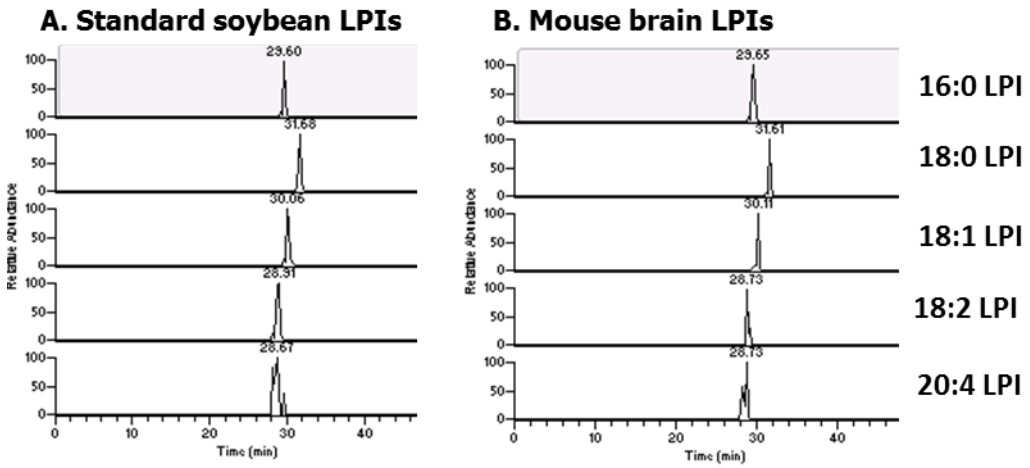


Figure 8. HPLC-ESI-MS extracted ion chromatograms (EIC) of standard soybean LPI and LPI in mouse brain. LC-MS separation of LPI from a standard LPI extract from soybean (**A**) and from mouse brain (**B**): detection as $[M-H]^-$ ions at m/z 571.28889 (16:0), 599.32019 (18:0), 597.30454 (18:1), 595.28889 (18:2), 619.28889 (20:4).

When looking at the MS fragmentation of a representative LPI, namely 18:0 LPI, we can see that the product ions obtained following parent ion fragmentation are strictly the same for the standard and the brain lipid extract (**Figure 9**).

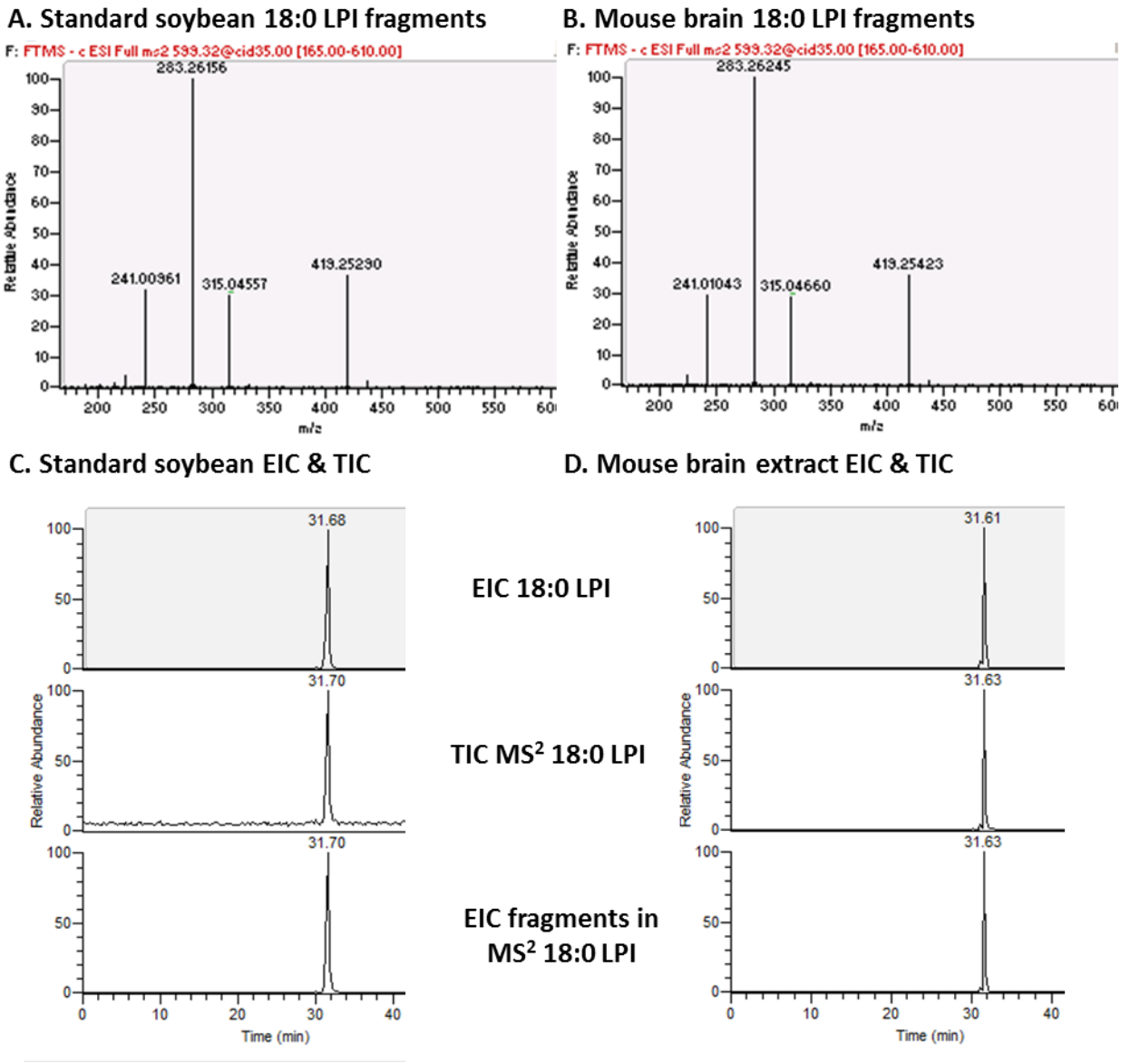


Figure 9. Spectra of fragments after MS^2 and HPLC-ESI-MS extracted ion chromatograms (EIC), total ion current (TIC) of the MS^2 and EIC of fragments from MS^2 for standard soybean 18:0 LPI (A and C) and mouse brain 18: LPI (B and D). In **A** and **B**, product ions after fragmentation of 18:0 LPI (599.32019) obtained for standard and mouse brain and in **C** and **D**, LC-MS detection of 18:0 LPI: detection as $[M-H]^-$ ions at 599.32019 for 18:0 LPI and 241.01243, 283.26425, 315.04921 and 419.26462 for fragments.

When looking at those fragments and at the structure of 18:0 LPI, they can be easily explained by quite straightforward fragmentation patterns (at least for 18:0 LPI) (**Figure 10**).

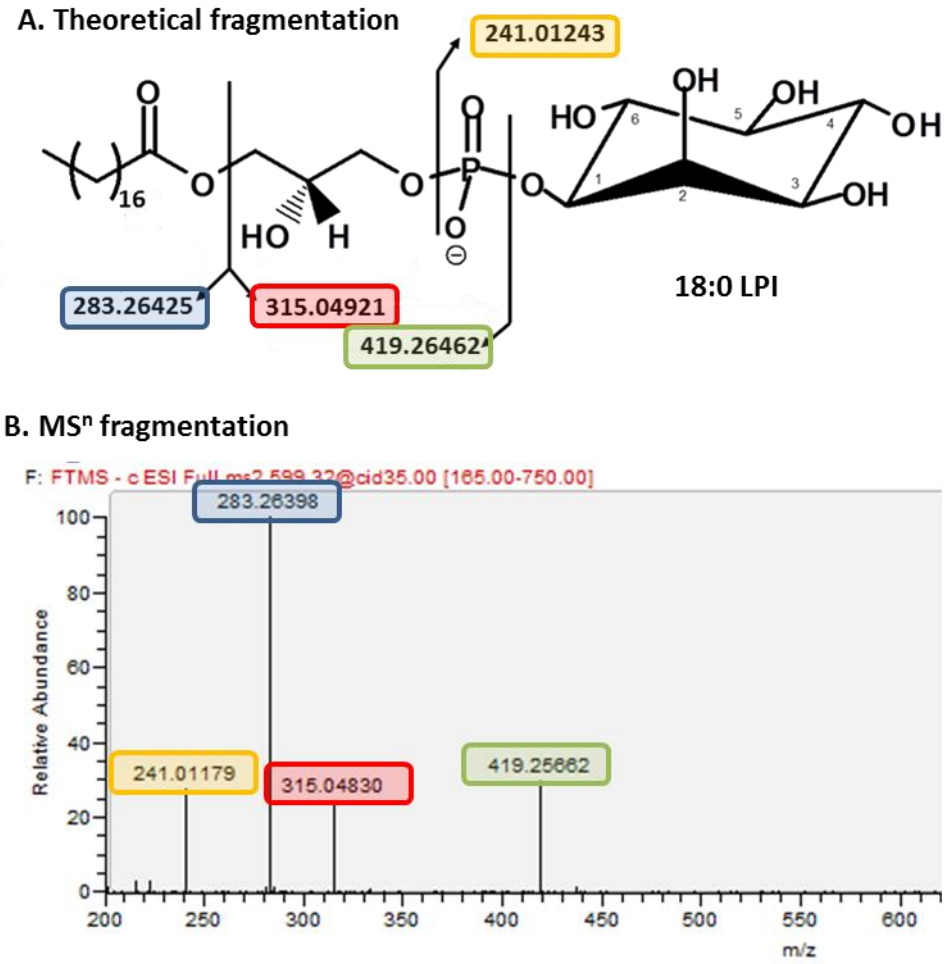


Figure 10. MS Fragmentation pattern and identification of fragments. Structure of 18:0 LPI (**A**) with an explanation of the different MS/MS fragments obtained (**B**).

As previously mentioned, the aim here was to develop a methodology allowing for the detection and identification of a large number of lipid families. In the following pages, we summarize for each lipid subclass the chromatographic and spectrometric behavior. Of note, in the literature, very often, peaks are smoothed and appear more “continuous” and “fluid”. Here we decided to show the chromatogram as “raw data”, that is without processing (**Figure 11**).

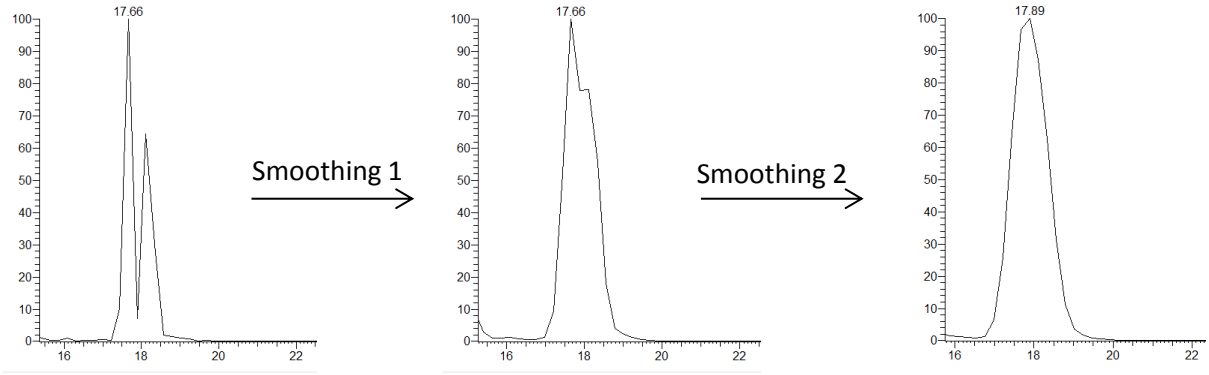


Figure 11. Example of post-data acquisition smoothing. Example of smoothing for *sn*-1 & *sn*-2 16:0 lysophosphatidylcholine. LTQ-Orbitrap XL is working with 1 scan/second at 60000 resolution for 400 m/z.

Below, we present an example of the organization of the identification patterns for all studied subclass of lipids.

For each species, the general structure, the name, and the exact m/z value of the analyzed ion are shown. Chromatograms for each chain length and an example of fragmentation and identification of product ions are also presented. The chromatograms were obtained by analyzing a lipid extract from 20 mg of mouse brain. Therefore some lipids are not visible on the chromatogram. Nevertheless, for consistency, we mention their m/z value in the table. For the fragmentation examples, for sure, generated ions are strongly dependent of the collision energy. Here, since the goal of this part is to have an unequivocal identification, we did not study several collision energies but only one for each compound.

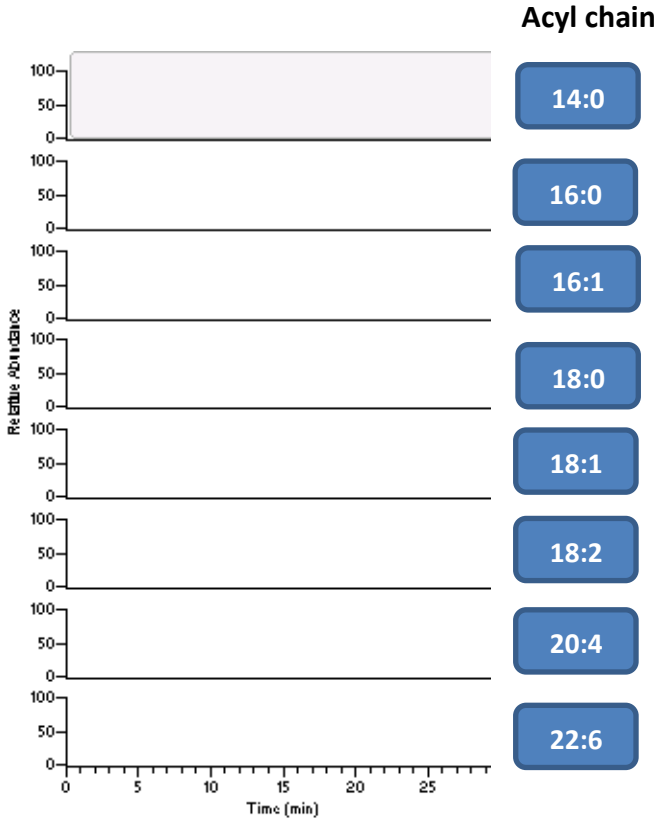
II. Lipid characterization using an HPLC coupled to a LTQ-Orbitrap XL mass spectrometer

General name of the lipid subclass

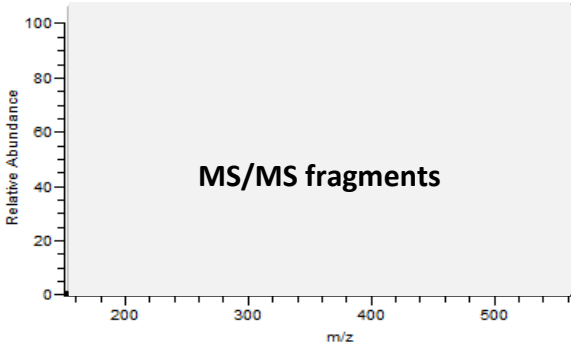
Example of structure for the lipid subclass

Name of the structure

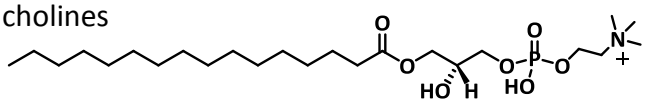
Acyl chain	Species (m/z)
14:0	
16:0	
16:1	
18:0	
18:1	
18:2	
20:4	
22:6	



	Parent mass	Fragment 1	Fragment 2	Fragment 3
Name	m/z	m/z	m/z	m/z

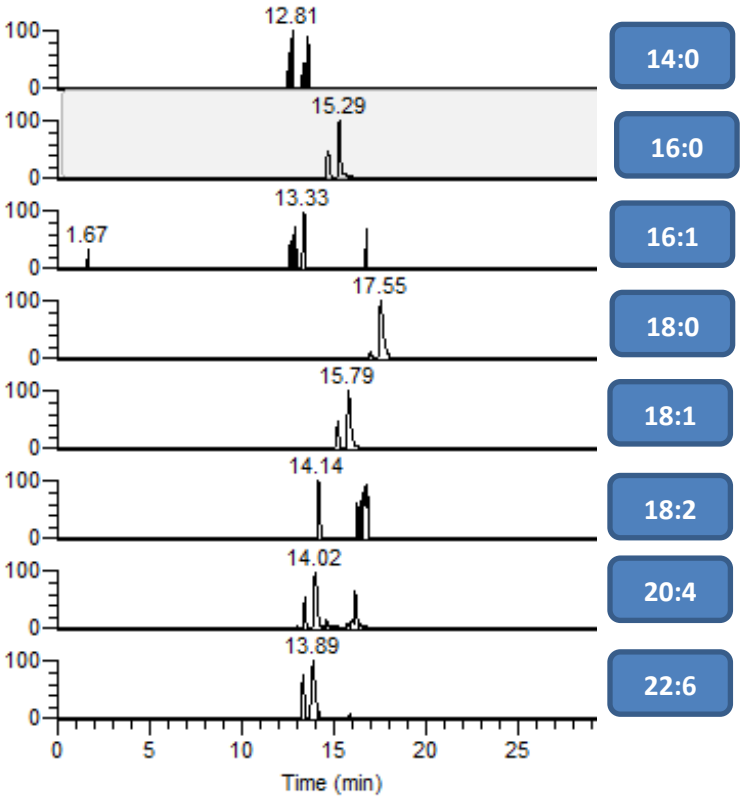


Lysophosphatidylcholines

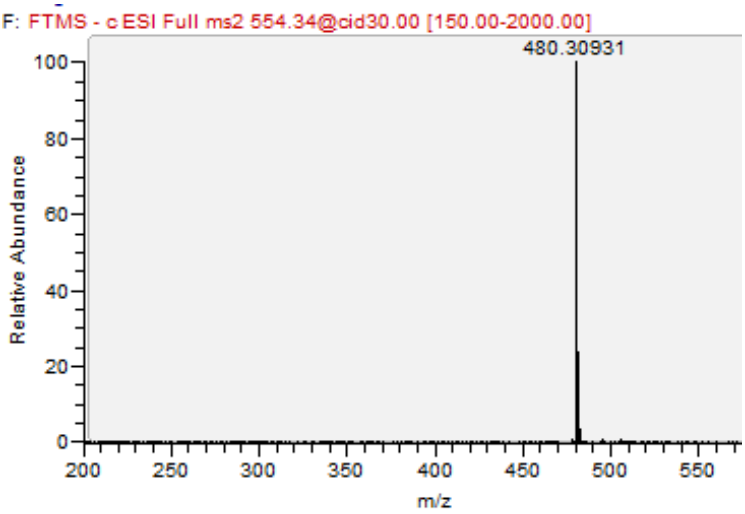


16:0 LPC

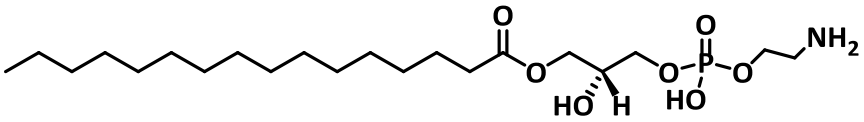
	LPC
14:0	526.31504
16:0	554.34634
16:1	552.33069
18:0	582.37764
18:1	580.36199
18:2	578.34634
20:4	602.34634
22:6	626.34634



	$[M+CH_3COO^-]^-$	$[M-CH_3]^-$
LPC 16:0	554.34634	480.30901

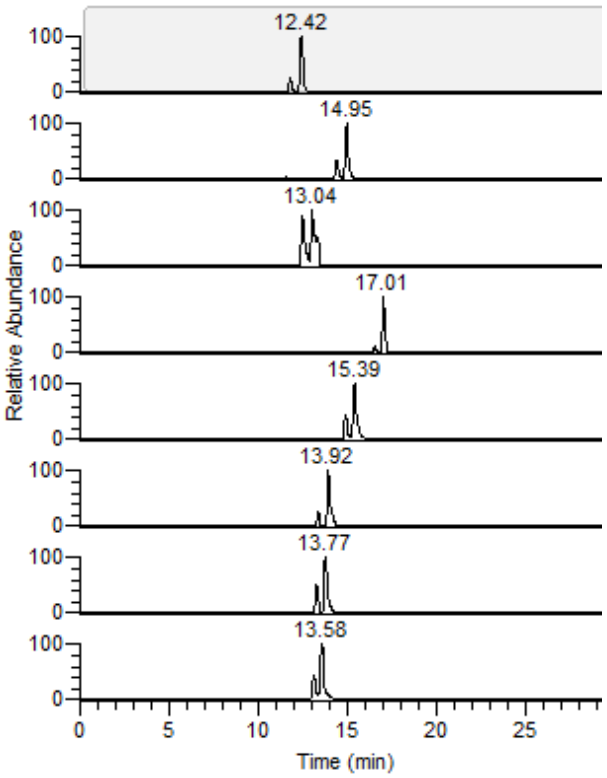


Lysophosphatidylethanolamines

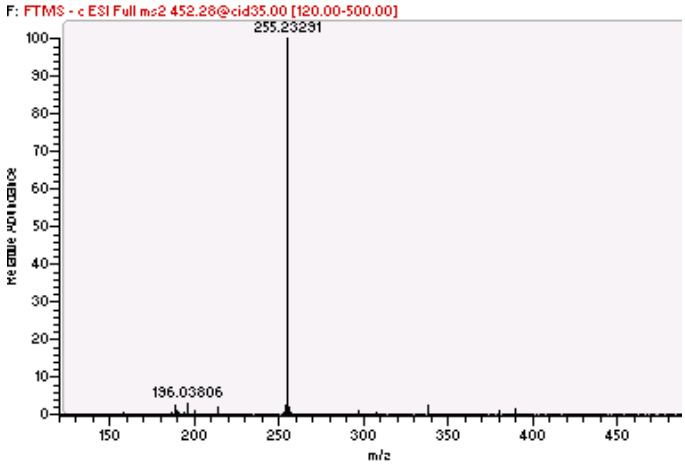


16:0 LPE

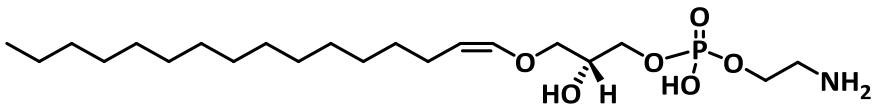
	LPE
14:0	424.24696
16:0	452.27826
16:1	450.26261
18:0	480.30956
18:1	478.29391
18:2	476.27826
20:4	500.27826
22:6	524.27826



	$[M-H]^-$	$[LPE-FA-H]^-$	$[FA-H]^-$
LPE 16:0	452.27826	196.04586	255.23241

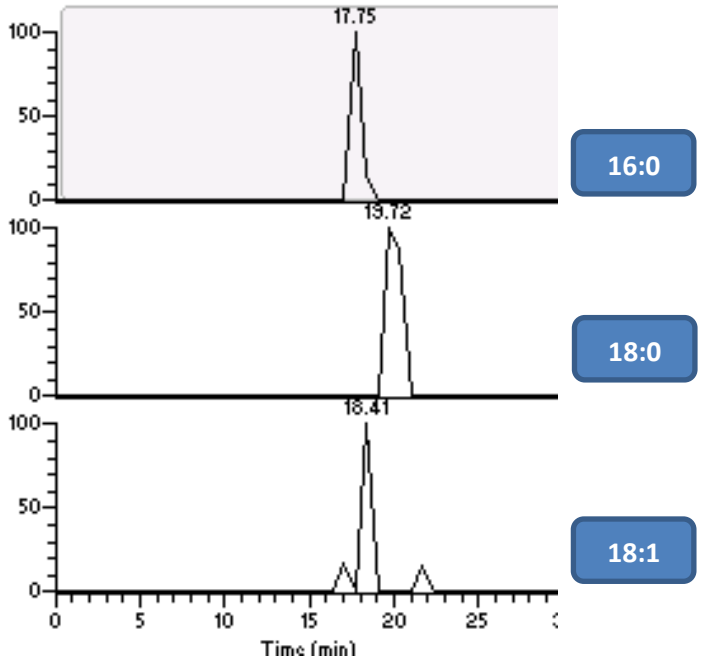


Lysophosphatidylethanolamines (plasmalogen)

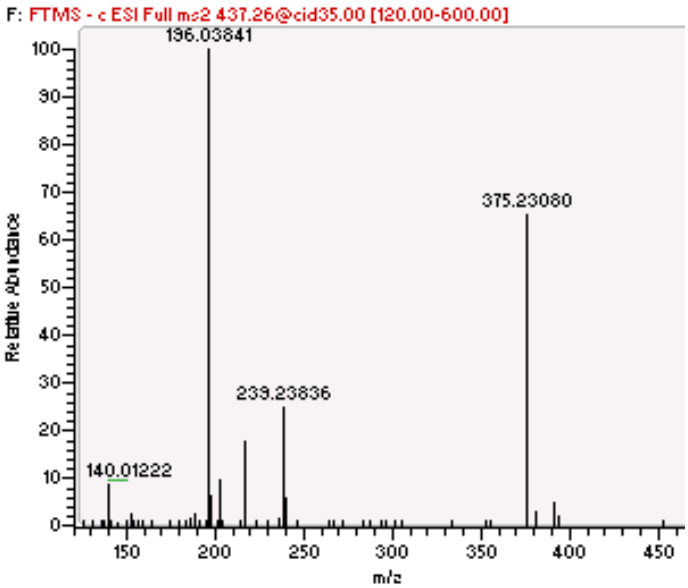


16:0 LPE(p)

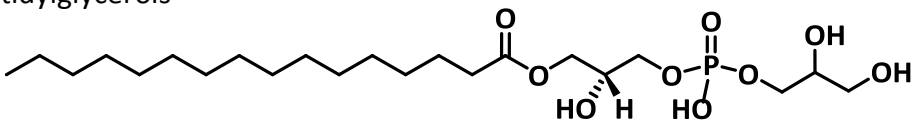
	LPE(p)
14:0	408.25205
16:0	436.28335
16:1	434.26770
18:0	464.31465
18:1	462.29900
18:2	460.28335
20:4	484.28335
22:6	508.28335



	$[M-H]^-$	$[PE-H]^-$	$[LPE(p)\text{-alkenyl acyl-H}]^-$	$[\text{alkenyl acyl-H}]^-$	$[LPE(p)\text{-ETA-H}]^-$
LPE(p) 16:0	436.28335	140.01127	196.04586	239.23749	375.23113

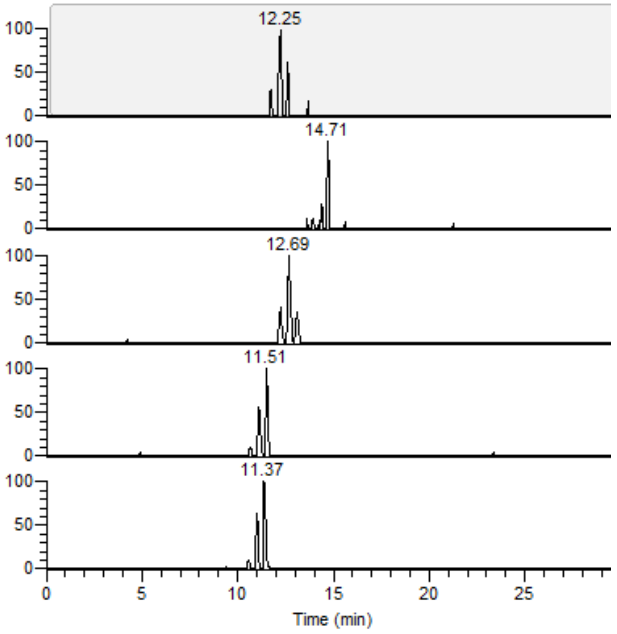


Lysophosphatidylglycerols

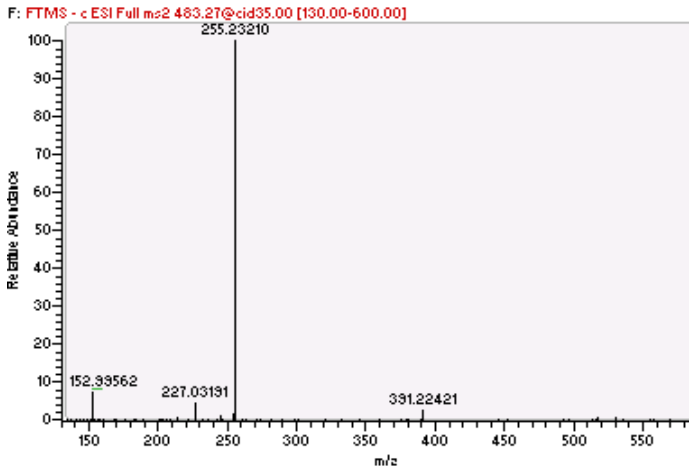


16:0 LPG

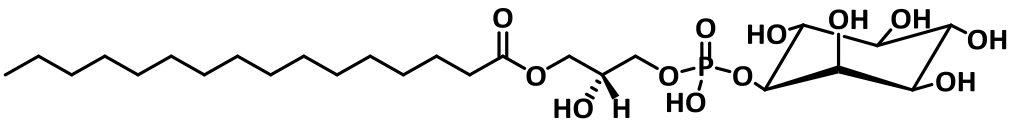
	LPG
14:0	455.24154
16:0	483.27284
16:1	481.25719
18:0	511.30414
18:1	509.28849
18:2	507.27284
20:4	531.27284
22:6	555.27284



	$[M-H]^-$	$[glycerol-H_2O-H]^-$	$[LPG-FA-H]^-$	$[FA-H]^-$	$[LPA-H_2O-H]^-$
LPG 16:0	483.27284	153.00366	227.04044	255.23241	391.22605

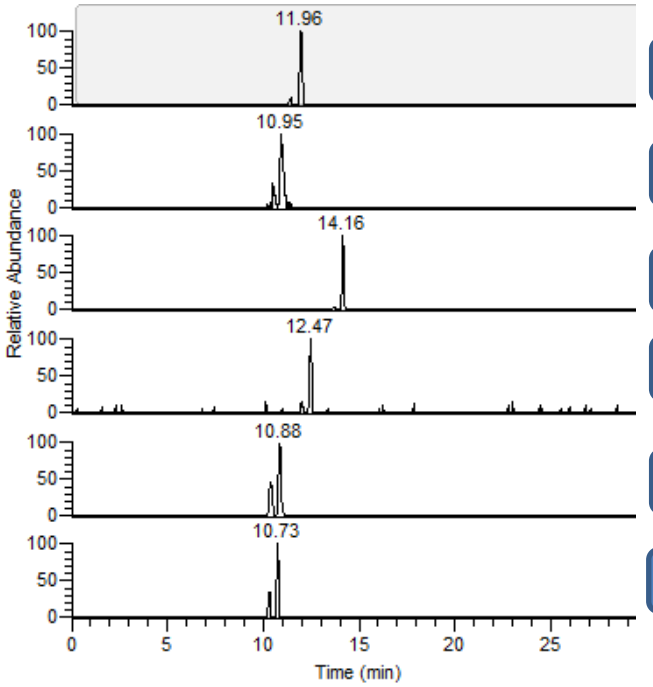


Lysophosphatidylinositols

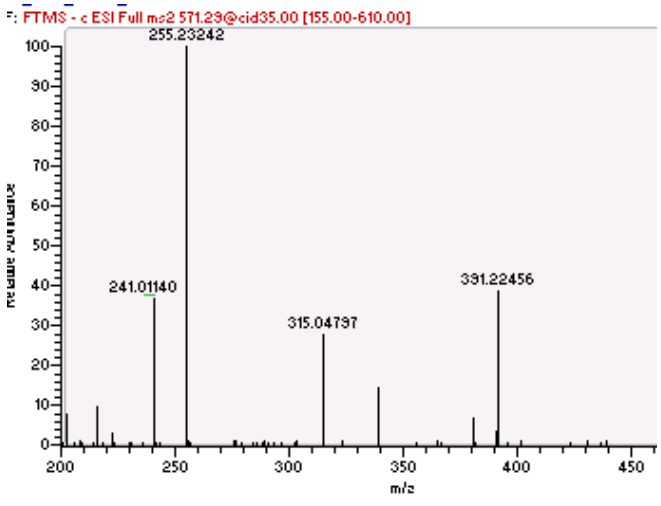


16:0 LPI

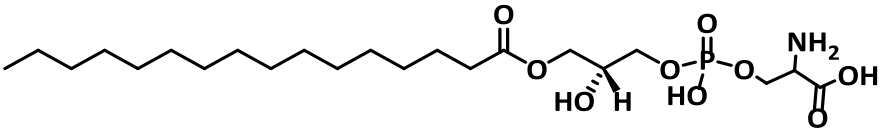
	LPI
14:0	544.26486
16:0	571.28889
16:1	569.27324
18:0	599.32019
18:1	597.30454
18:2	595.28889
20:4	619.28889
22:6	643.28889



	[M-H]$^-$	[inositolphosphate -H_2O-H]$^-$	[FA-H]$^-$	[GPI-H_2O-H]$^-$	[LPA-H_2O-H]$^-$
LPI 16:0	571.28889	241.01243	255.23241	315.04921	391.22605

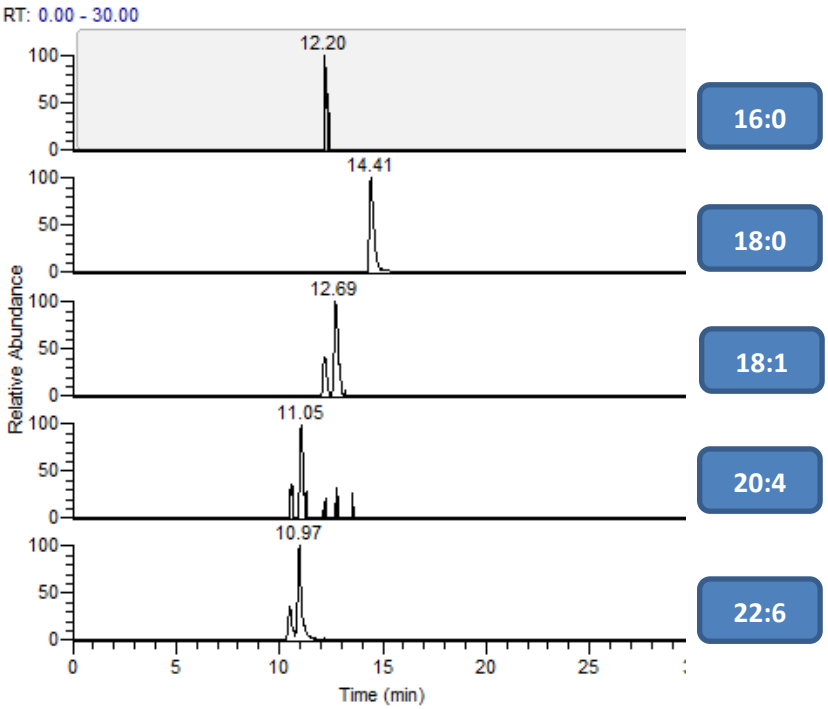


Lysophosphatidylserines

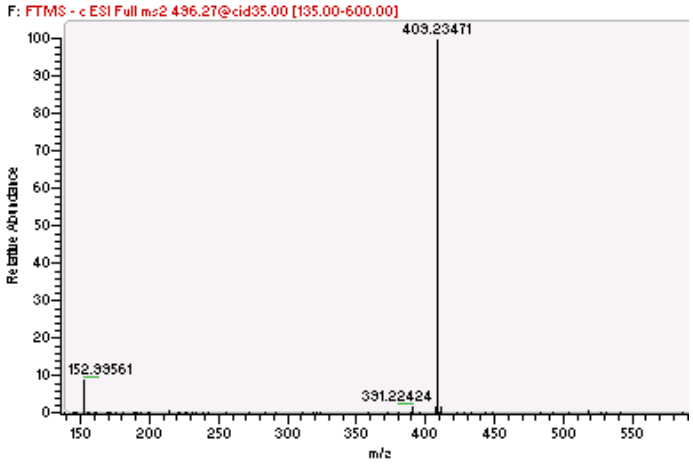


16:0 LPS

	LPS
14:0	468.23679
16:0	496.26809
16:1	494.25244
18:0	524.29939
18:1	522.28374
18:2	520.26809
20:4	544.26809
22:6	568.26809



	$[M-H]^-$	$[glycerol-H_2O-H]^-$	$[LPA-H_2O-H]^-$	$[LPA-H]^-$
LPS 16:0	496.26809	153.00366	391.22605	409.23606

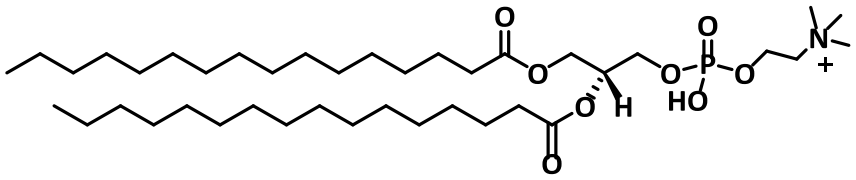


Phospholipids

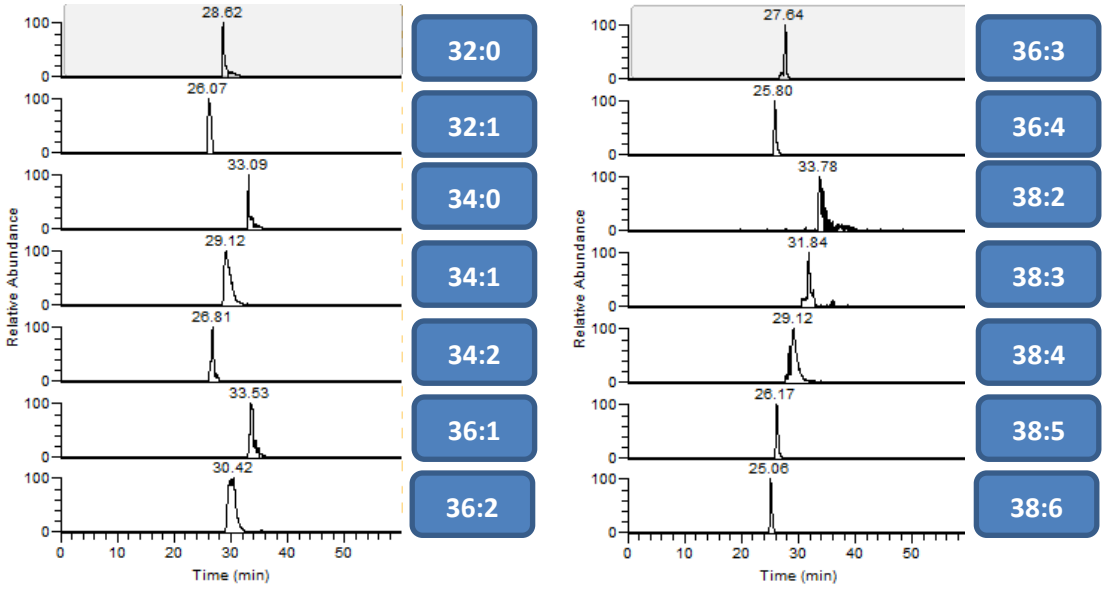
	PC	PE	PE(p)	PG	PI	PS
32:0	792.57656	690.50793	674.51301	721.50251	809.51855	734.49776
32:1	790.56091	688.49228	672.49736	719.48686	807.50290	732.48211
34:0	820.60786	718.53923	702.54431	749.53381	837.54985	762.52906
34:1	818.59221	716.52358	700.52866	747.51816	835.53420	760.51341
34:2	816.57656	714.50793	698.51301	745.50251	833.51855	758.49776
36:0	848.63916	746.57053	730.57561	777.56511	865.58115	790.56036
36:1	846.62351	744.55488	728.55996	775.54946	863.56550	788.54471
36:2	844.60786	742.53923	726.54431	773.53381	861.54985	786.52906
36:3	842.59221	740.52358	724.52866	771.51816	859.53420	784.51341
36:4	840.57656	738.50793	722.51301	769.50251	857.51855	782.49776
38:2	872.63916	770.57053	754.57561	801.56511	889.58115	814.56036
38:3	870.62351	768.55488	752.55996	799.54946	887.56550	812.54471
38:4	868.60786	766.53923	750.54431	797.53381	885.54985	810.52906
38:5	866.59221	764.52358	748.52866	795.51816	883.53420	808.51341
38:6	864.57656	762.50793	746.51301	793.50251	881.51855	806.49776

m/z values for the different phospholipids analyzed (see below)

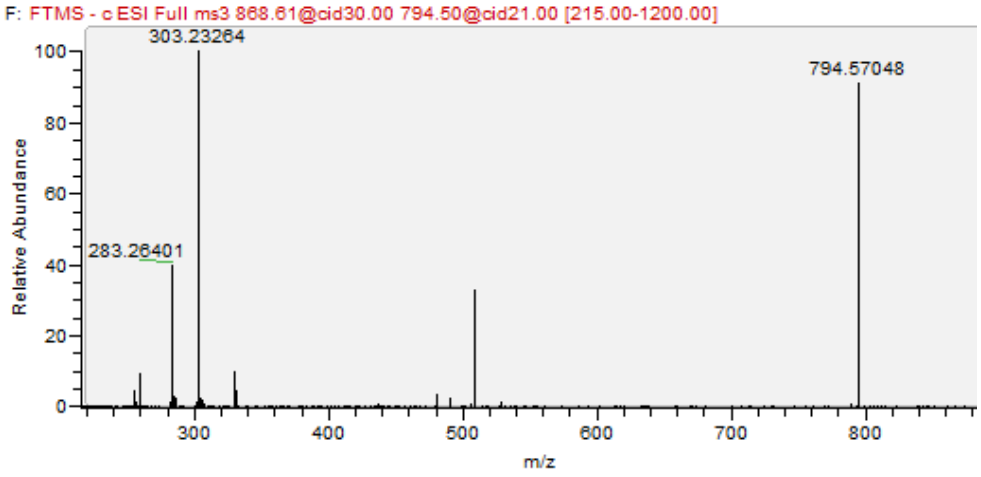
Phosphatidylcholines



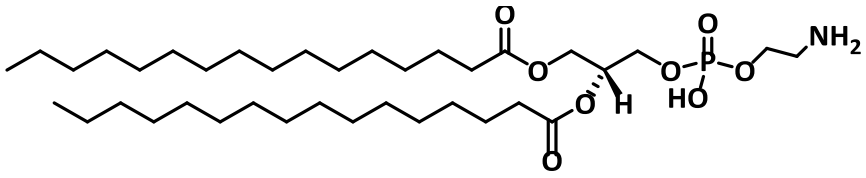
32:0 PC



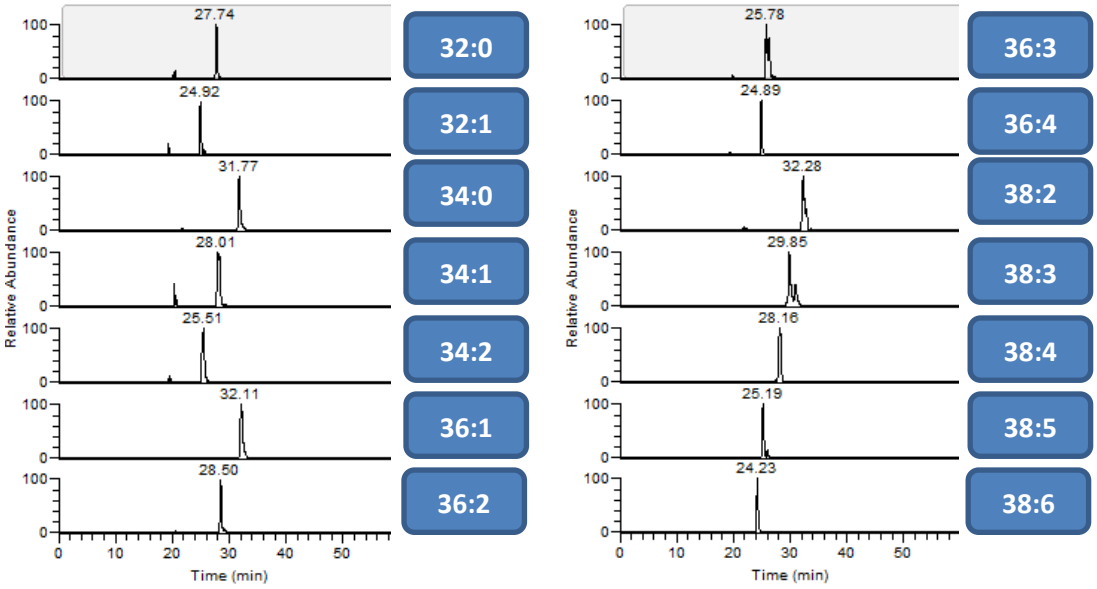
	$[M+CH_3COO^-]^-$	$[FA-H]^-$	$[PC-CH_3]^-$
PC 38:4	868.60786	283.26371 303.23241	794.56998



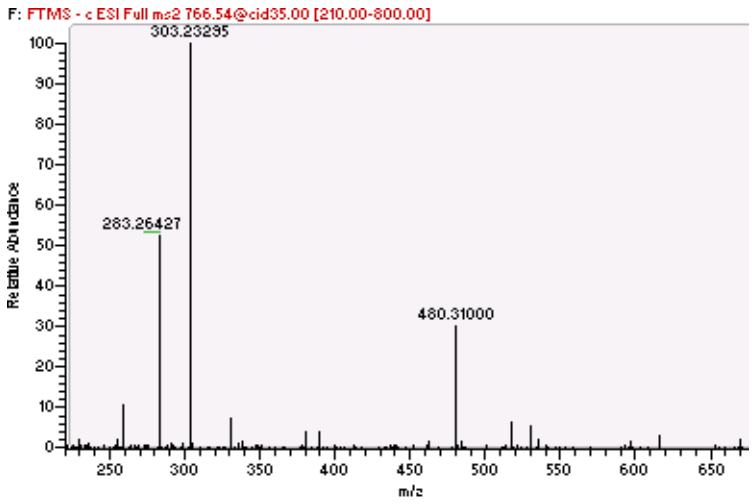
Phosphatidylethanolamines



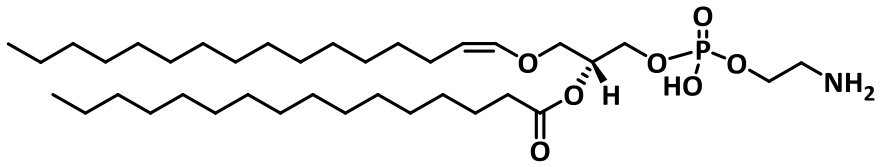
32:0 PE



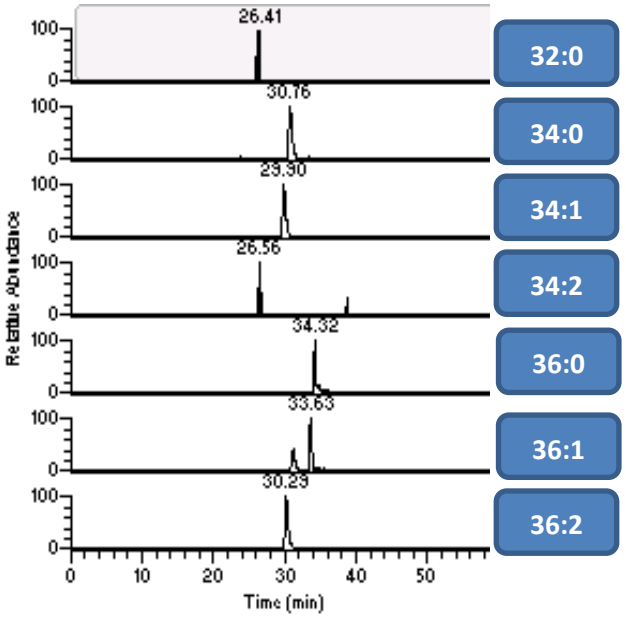
	[M-H]$^-$	[FA-H]$^-$	[PE-FA-H]$^-$
PE 38:4	766.53923	283.26371 303.23241	480.31187

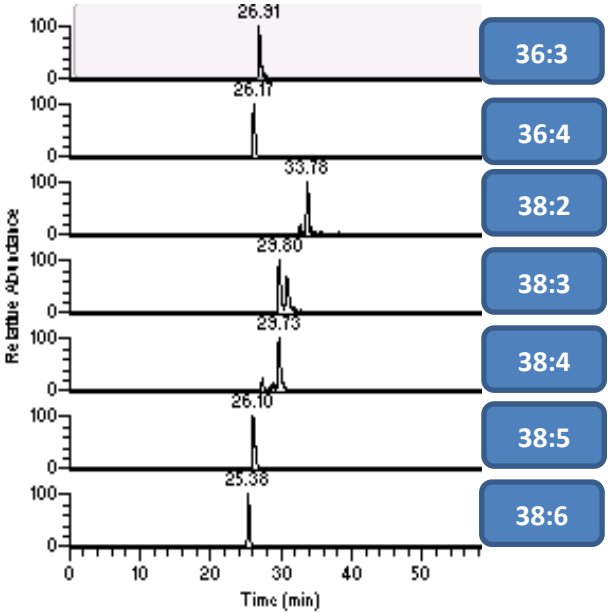


Phosphatidylethanolamines (plasmalogen)

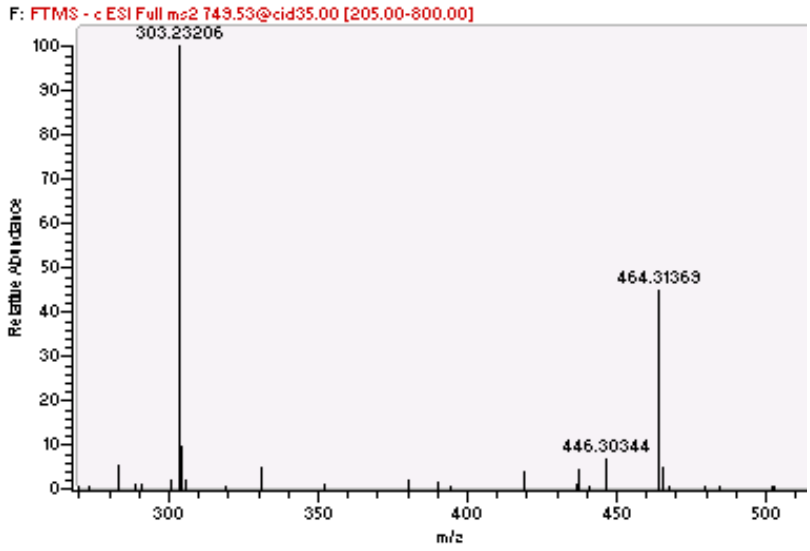


32:0 PE(p)

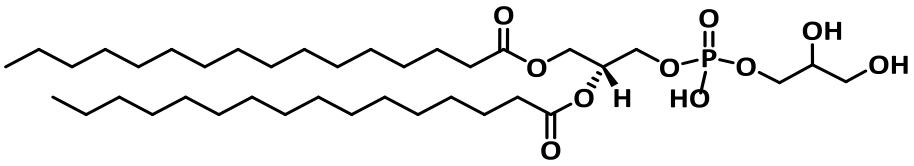




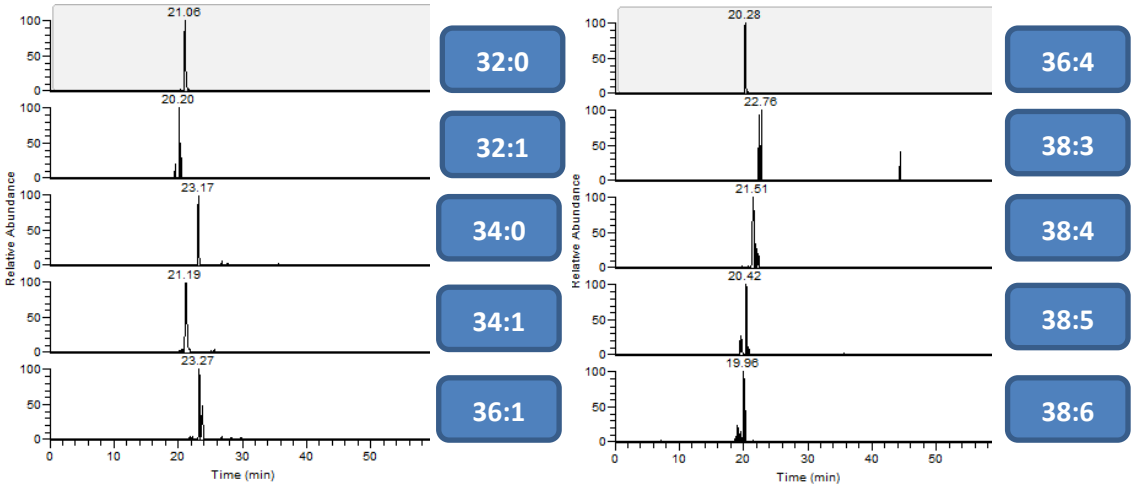
	[M-H]$^-$	[FA-H]$^-$	[PE(p)-FA-H]$^-$	[PE(p)-FA (CH=C=O)]$^-$
PE(p) 38:4	750.54431	303.23241	446.30408	464.31410



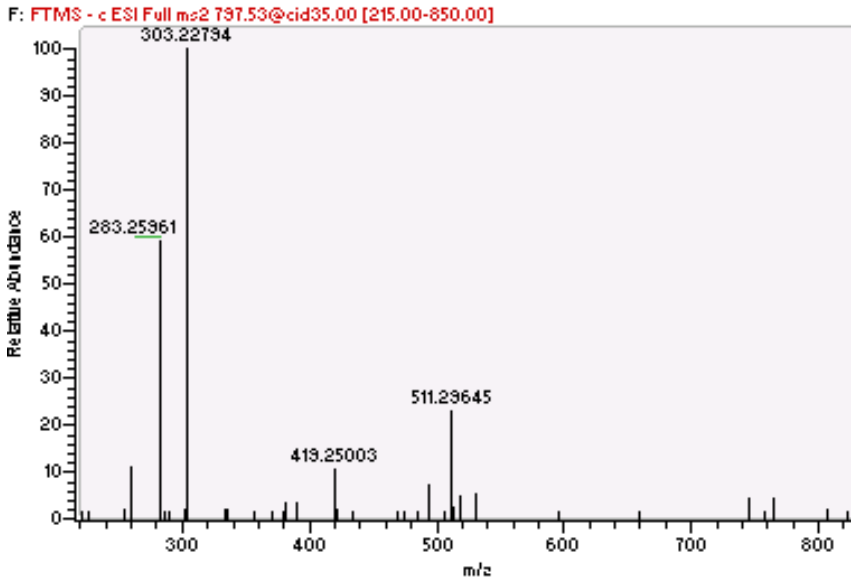
Phosphatidylglycerols



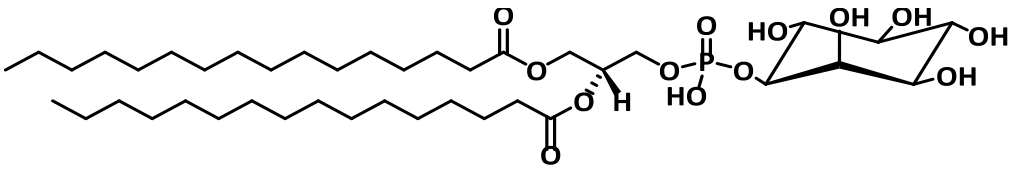
32:0 PG



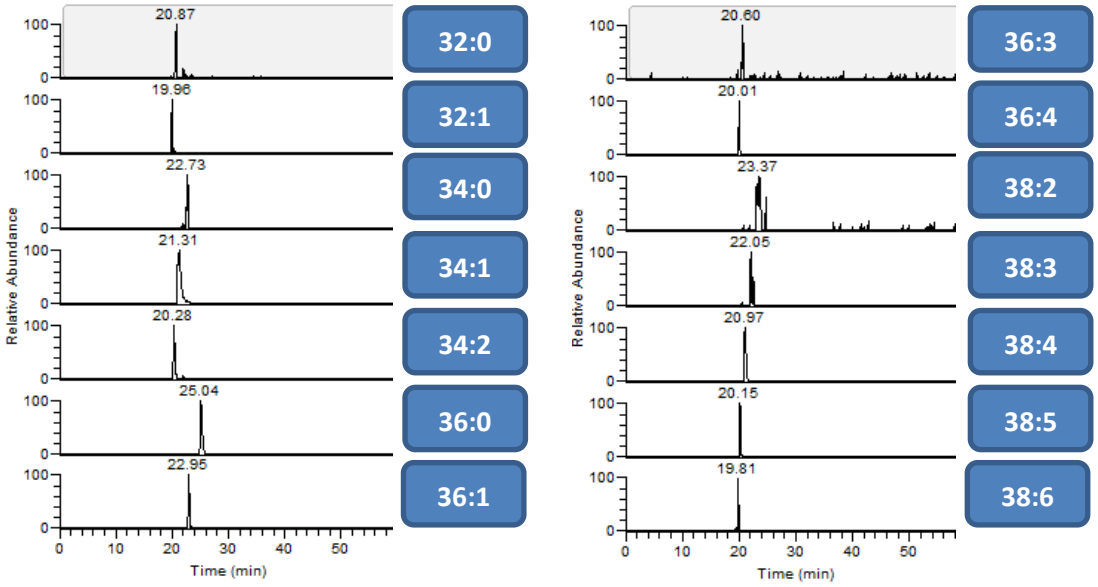
	[M-H]⁻	[FA-H]⁻	[LPA-H₂O-H]⁻	[PG-FA-H]⁻
PG 38:4	797.53381	283.26371 303.23241	419.25739	511.30676



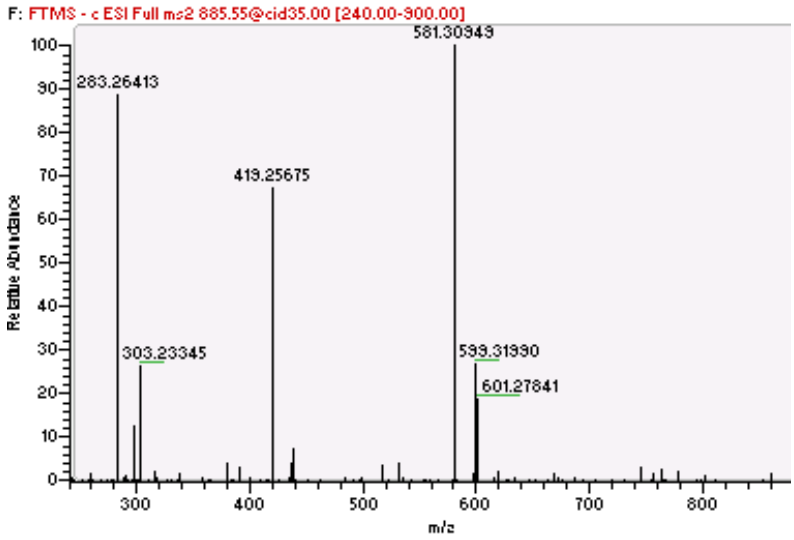
Phosphatidylinositols



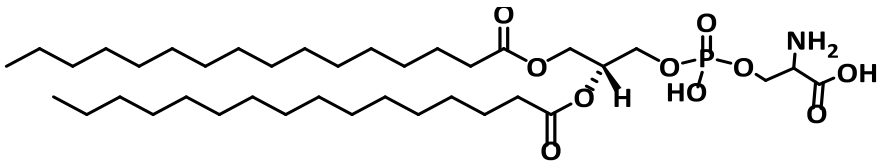
32:0 PI



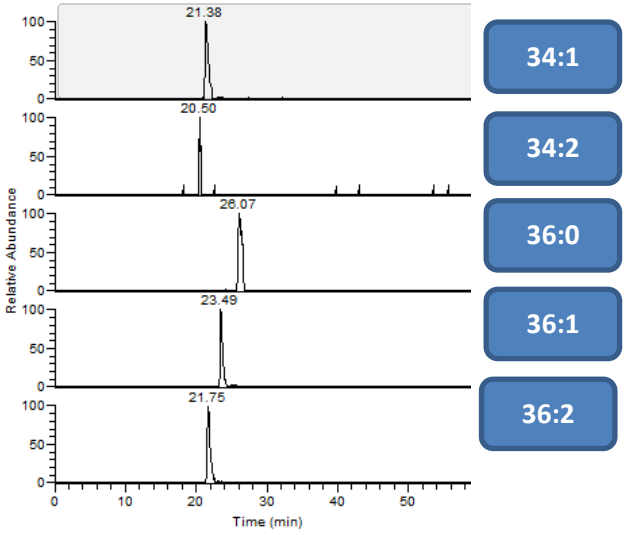
	[M-H]$^-$	[FA-H]$^-$	[LPA-H_2O-H]$^-$	[PI-FA-H_2O-H]$^-$	[PI-FA-H]$^-$
PI 38:4	885.54985	283.26371 303.23241	419.25739	581.30992 601.27791	599.32019

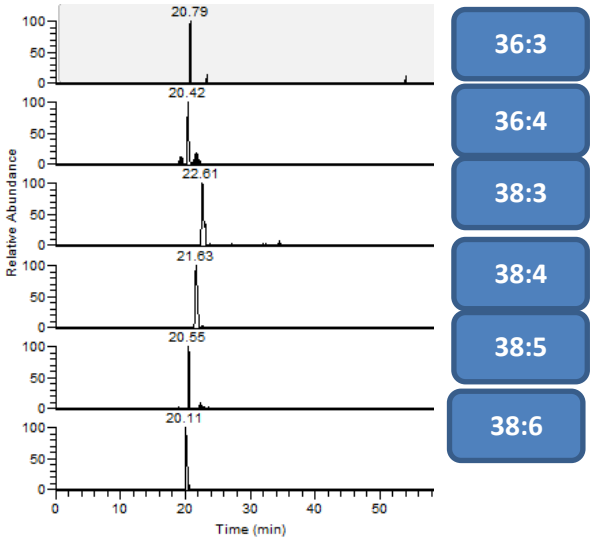


Phosphatidylserines

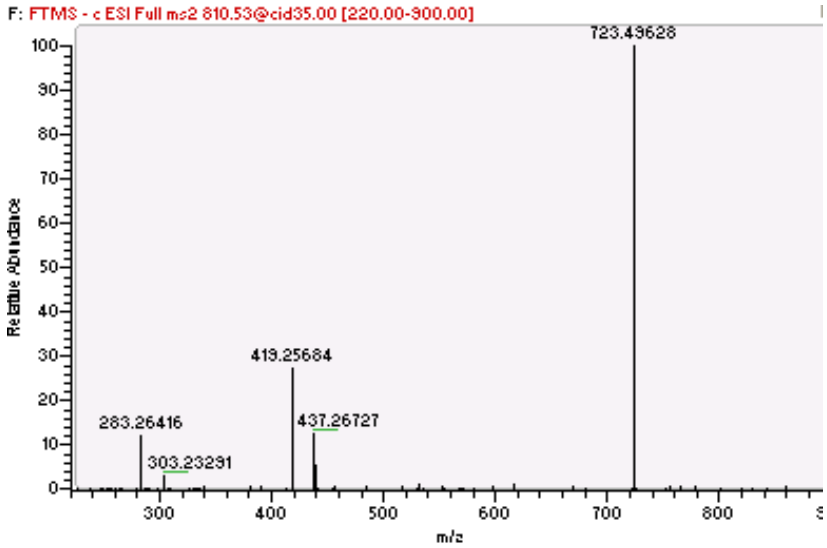


32:0 PS

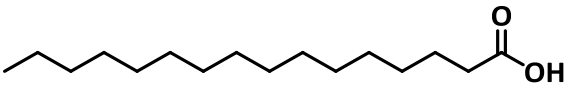




	[M-H]⁻	[FA-H]⁻	[LPA-H₂O-H]⁻	[LPA-H]⁻	[PA-H]⁻
PS 38:4	810.52906	283.26371 303.23241	419.25739	437.26524	723.50076

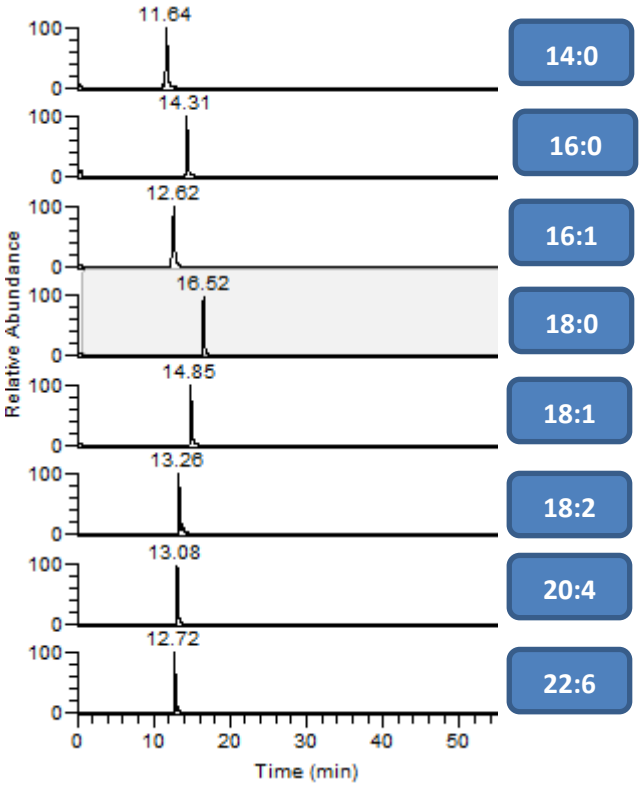


Fatty acids

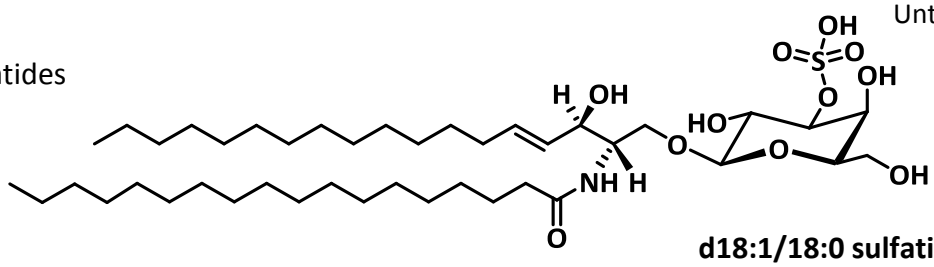


16:0 FA

	FFA
14:0	227.20165
16:0	255.23295
16:1	253.21730
18:0	283.26425
18:1	281.24860
18:2	279.23295
20:4	303.23295
22:6	327.23295

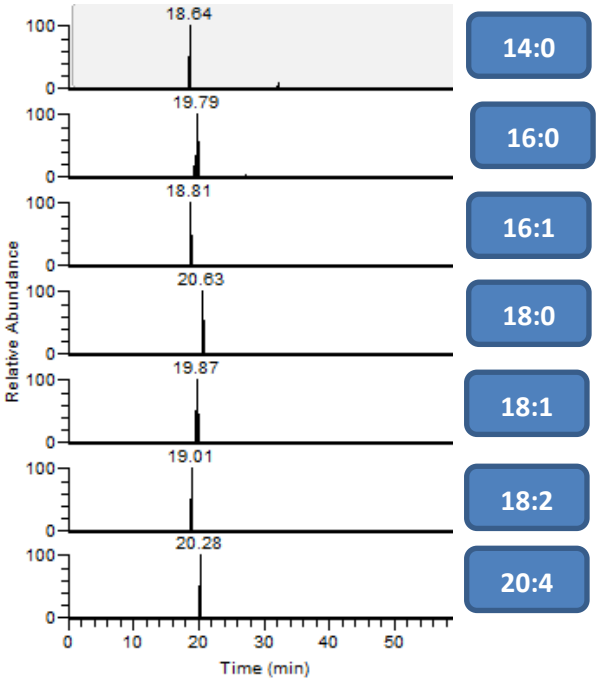


Sulfatides

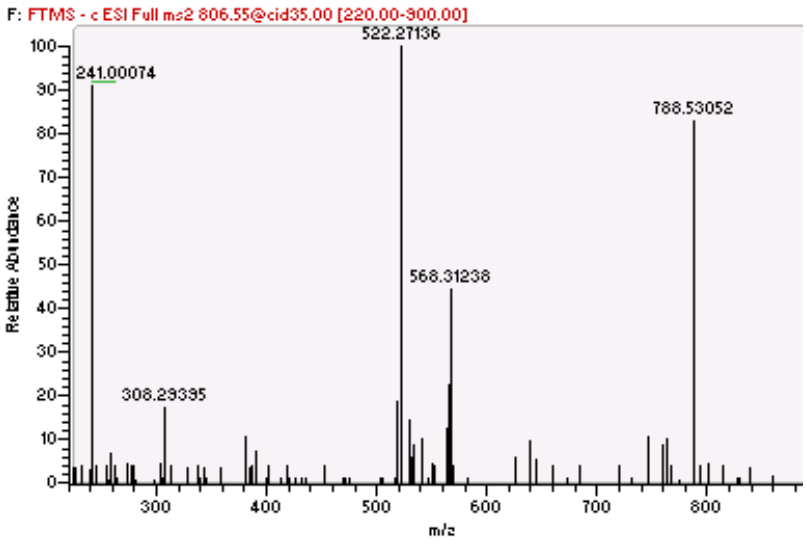


d18:1/18:0 sulfatide

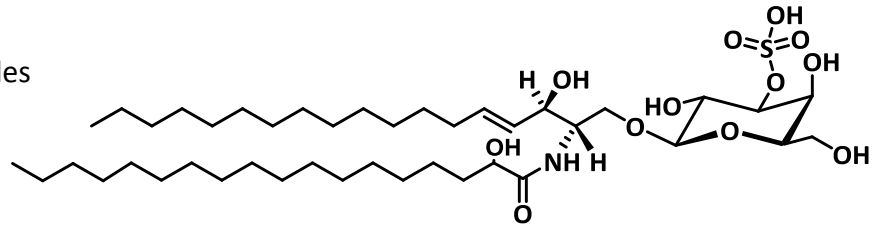
	d18:1 sulfatide
14:0	750.48316
16:0	778.51446
16:1	776.49881
18:0	806.54576
18:1	804.53011
18:2	802.51446
20:4	826.51446
22:6	850.51446



	[M-H]$^-$	[3-sulfogalactosyl-H]$^-$	[sulfatide – d18:1-sulfogalactosyl-H]$^-$	[568 fragment-CO-H_2O]$^-$	See OH-sulfatides	[sulfatide-H_2O-H]$^-$
Sulfatide d18:1/18:0	806.54576	241.00236	308.29589	522.27423	568.27916	788.53519

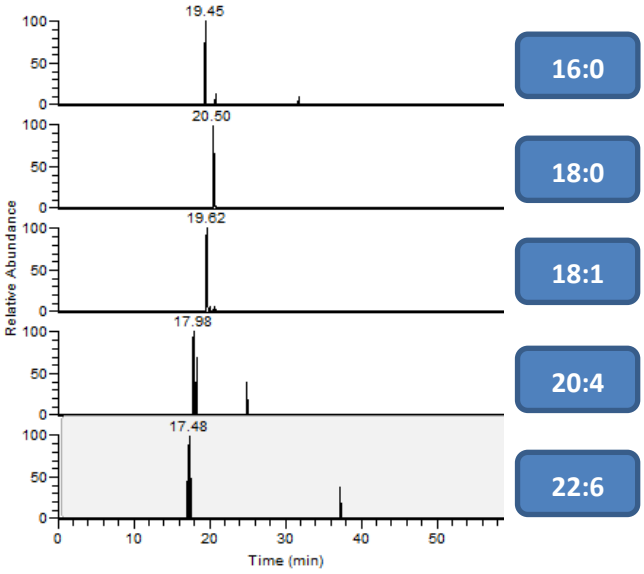


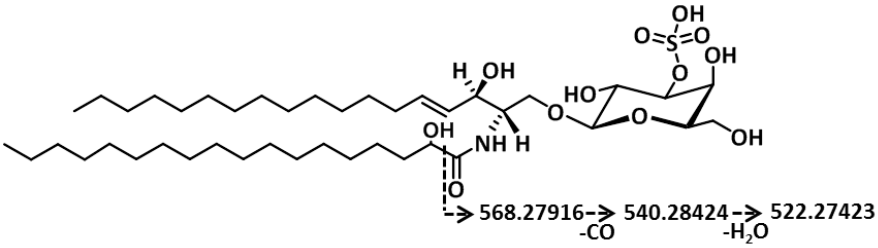
Hydroxylated sulfatides



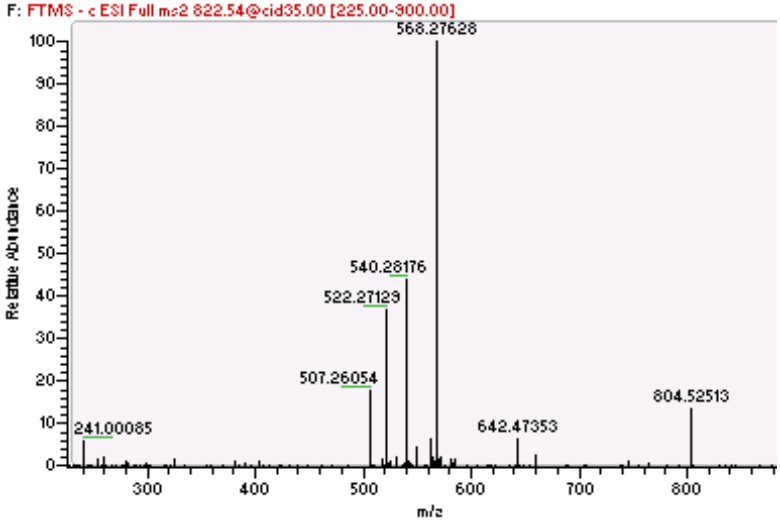
d18:1/18:0 (OH) sulfatide

	d18:1 sulfatide
14:0 (OH)	766.47807
16:0 (OH)	792.50937
16:1 (OH)	792.49372
18:0 (OH)	822.54067
18:1 (OH)	820.52502
18:2 (OH)	818.50937
20:4 (OH)	842.50937
22:6 (OH)	866.50937

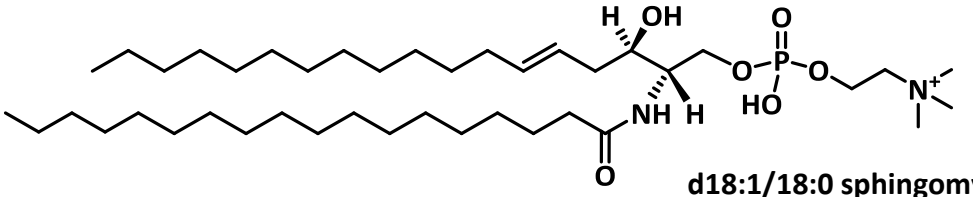




	[M-H]⁻	[3-sulfogalactosyl-H]⁻	[568 fragment-CO-H_2O]⁻	[568 fragment-CO]⁻	Characteristic sulfatide fragment	[sulfatide-H_2O-H]⁻
Sulfatide d18:1/18:0 (OH)	822.54067	241.00236	522.27423	540.28424	568.27916	804.53011

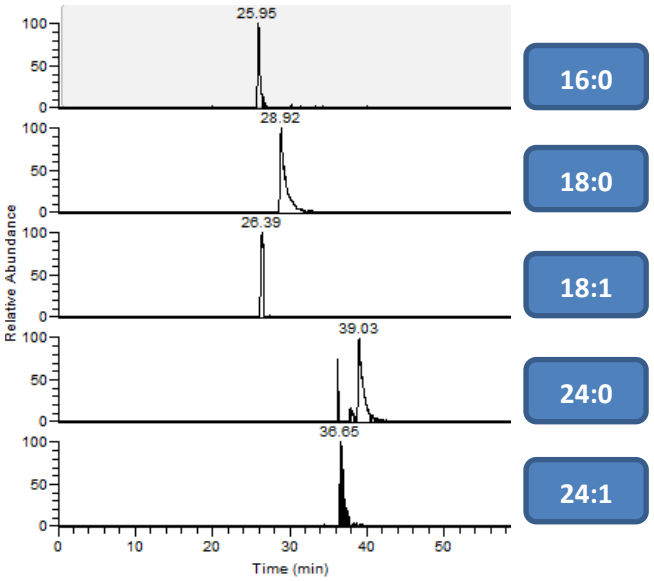


Sphingomyelins

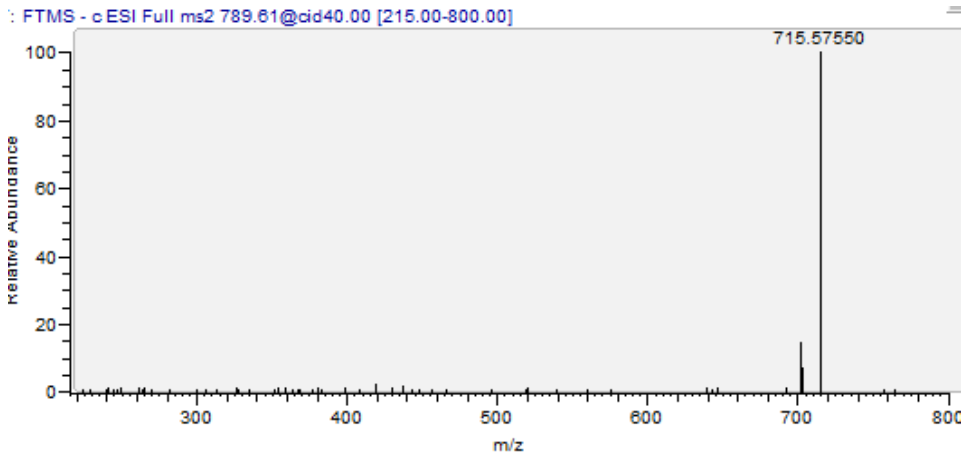


d18:1/18:0 sphingomyelin

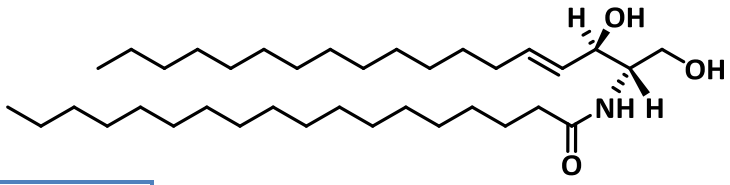
	d18:1 SM
16:0	761.58142
18:0	789.61272
18:1	787.59707
18:2	785.58142
20:4	809.58142
22:6	833.58142
24:0	873.70663
24:1	871.69098



	$[M+CH_3COO^-]^-$	$[M-CH_3]^-$
SM d18:1/18:0	789.61272	715.57540

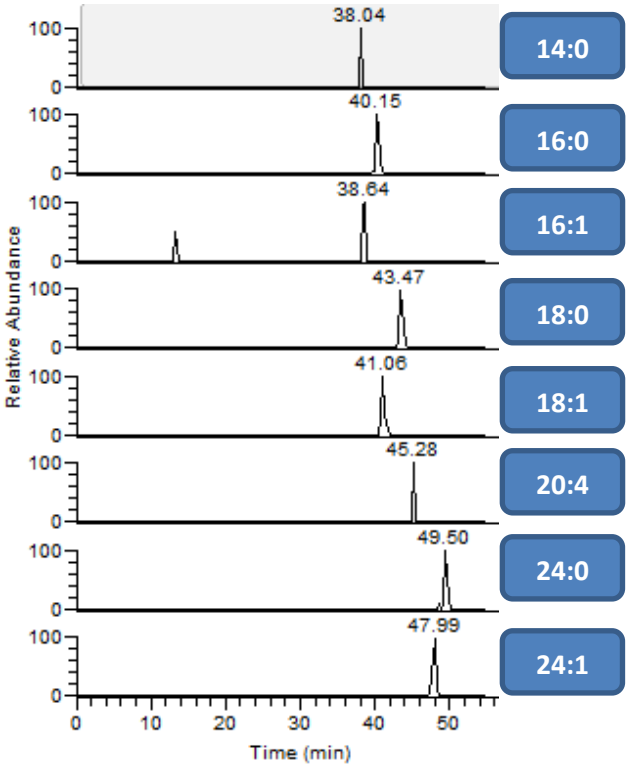


Ceramides

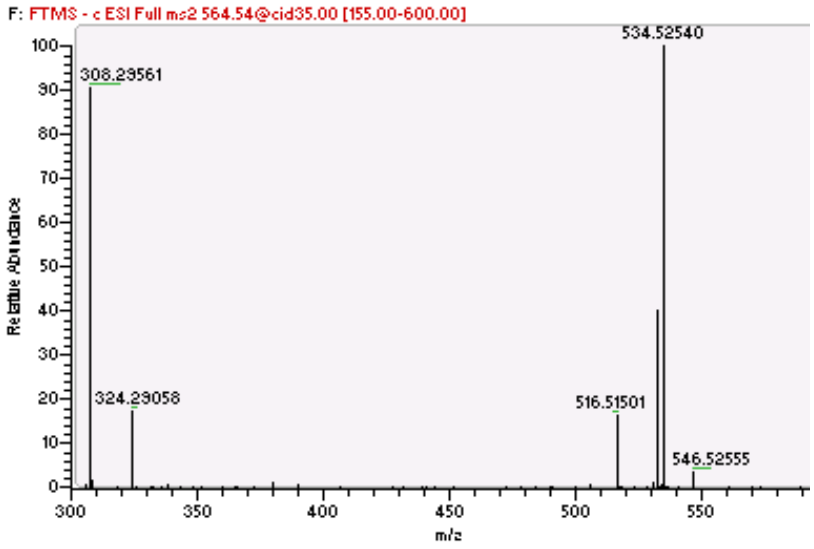


	d18:1 ceramide
14:0	506.45787
16:0	536.50482
18:0	564.53612
18:1	562.52047
20:4	584.50482
22:6	608.50482
24:0	648.63002
24:1	646.61437

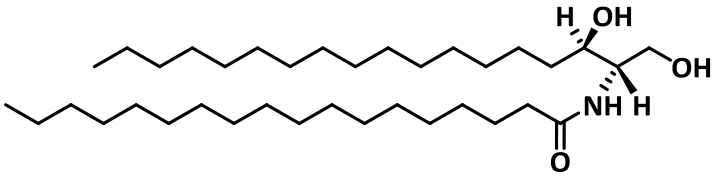
d18:1/18:0 ceramide



	[M-H]$^-$	[M-d18:1-H_2O]$^-$	[M-d18:1-2H]$^-$	[M-HCHO-H_2O-H]$^-$	[M-HCHO-H]$^-$	[M-H_2O-H]$^-$
Ceramide d18:1/18:0	564.53612	308.29589	324.29135	516.51444	534.52501	546.52555

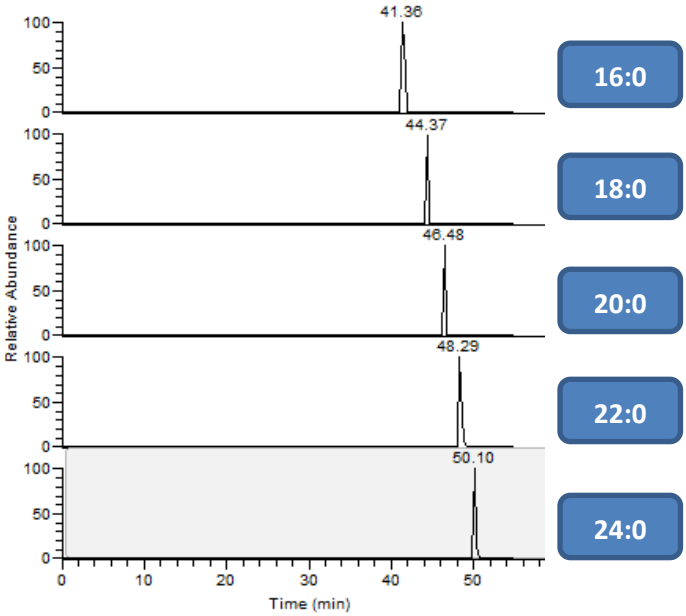


Dihydroceramides

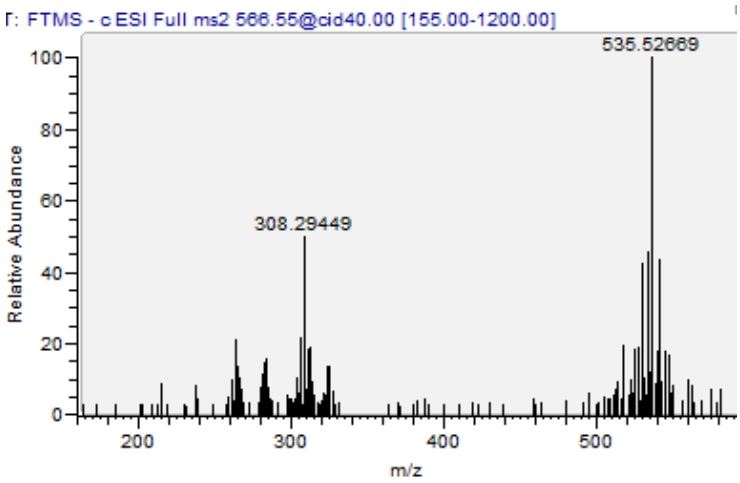


	d18:0 ceramide
14:0	510.48918
16:0	538.52048
18:0	566.55178
20:0	594.58308
22:0	622.61438
24:0	650.64568

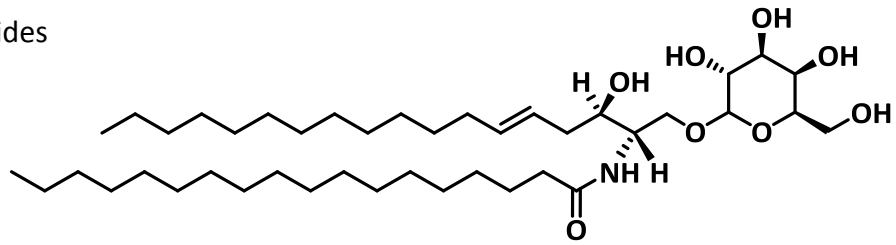
d18:0/18:0 ceramide



	$[M-H]^-$	$[M-d18:0-H_2O]^-$	$[M-HCHO-H]^-$
Ceramide d18:0/18:0	566.55178	308.29589	535.53338

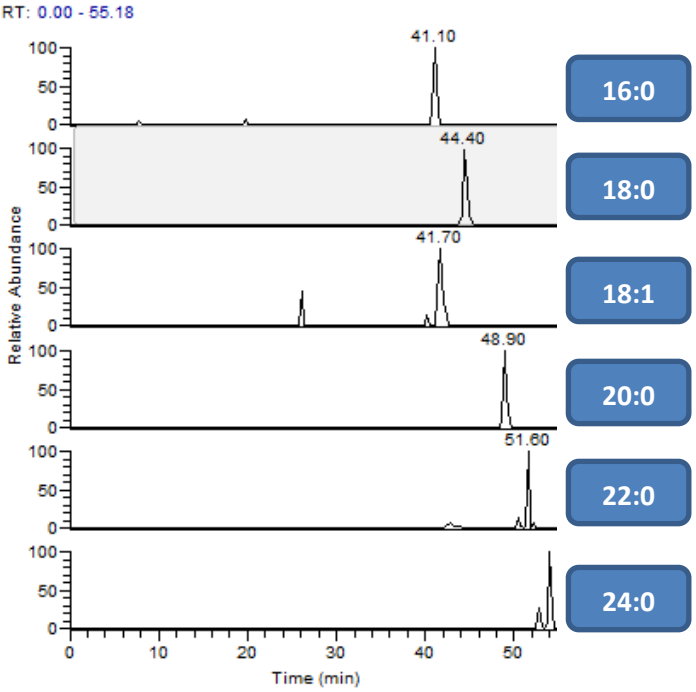


Galactosylceramides

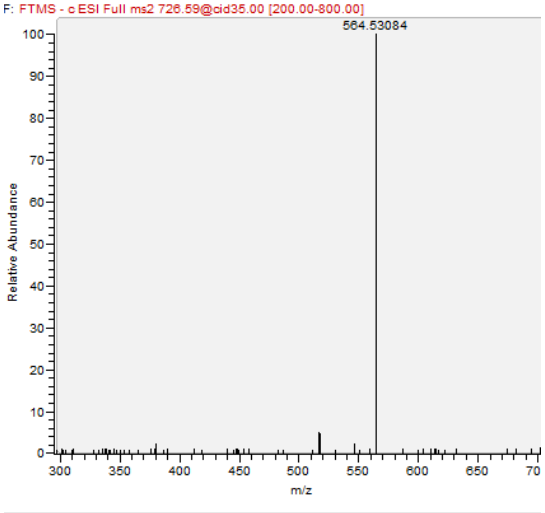


d18:1/18:0 galactosylceramide

	d18:1 galactosylceramide
16:0	698.55764
18:0	726.58894
18:1	724.57329
20:0	754.62024
22:0	782.65154
24:0	810.68284
24:1	808.68719



	[M-H]⁻	[M-galactosyl]⁻
galactosylceramide d18:1/18:0	726.58894	564.53557



III. Application to the study of lipid variation upon LPS-induced J774 macrophage activation

The aim of the following pages is to illustrate what can be done with our untargeted lipidomic method. To do so we used the developed HPLC-ESI-MS method to explore the variation in lipid levels induced by the LPS-induced activation of J774 cells.

Material and methods:

J774 cells ($10*10^6$ cells/dish) were incubated in the presence of LPS (100ng/mL) or vehicle for a 24h period, before analyzing the lipid content following a liquid-liquid extraction[a]. The lipid content was analyzed following HPLC separation using a C18 column (Kinetex, 150 mm x 4,6 mm, 5µm, from Phenomenex) and a water-methanol-CH_3COONH_4 5 mM mobile phase. The ESI probe, operated in negative mode, was used for ionization. The ESI spray voltage was set at 3.5 kV and the capillary temperature at 270 °C while the sheath gas flow was set at 40 arbitrary units. The data represent 14 points/conditions obtained in 4 independent experiments. In this experiment we quantified 6 lysophospholipid families (LPC, LPE, LPI, LPG, LPS, LPE(p)) and the corresponding 6 phospholipid families, 5 sphingolipid families (ceramides, dihydroceramides, sphingomyelins, galactosylceramides, sulfatides, hydroxylated sulfatides,) and the fatty acids.

Note that to avoid lengthy tables we are not presenting the raw data for each lipid but rather illustrating how data can be used to extract potentially meaningful information. Based on the data obtained, we performed a multivariate statistical analysis to explore whether control and LPS-activated cells could be distinguished based on the variations in lipid levels.

Thus to analyze the data, we used a principal component analysis (PCA) as unsupervised clustering or classification method. This is considered as good strategy to reveal the “scores” of data sets, representing the original data in a new coordinate system.[1,2]

Next, based on the PCA results, we performed partial least squares-discriminant analysis (PLS-DA) which is a supervised clustering or classification method used with a prior knowledge on the group labels. For the PLS-DA, the quality of the model obtained is represented by the values of R^2 and Q^2 describing description and prediction parameters,

[a] $CHCl_3$-MeOH-H_2O-HCl 2N (8:4:2:0.3 v/v/v/v)

respectively. Moreover, PLS-DA also gives "variable importance measures" and among them, the "variable importance in projection" (VIP) takes into account the amount of explained *Y*-variance of each component. In layman's terms, this score allows highlighting the compounds most responsible for the separation of the two groups (i.e. vehicle-treated and LPS-treated).

The PCA and the PLS-DA were performed using MetaboAnalyst 3.0, an integrated web-based platform for comprehensive analysis and quantitative metabolomics data.

We performed a PCA analysis for all the lipid families analyzed here. As might be expected, for several lipid families (e.g. FA and PE (**figure 12**)) we obtained no clear cluster or separation between the two data sets.

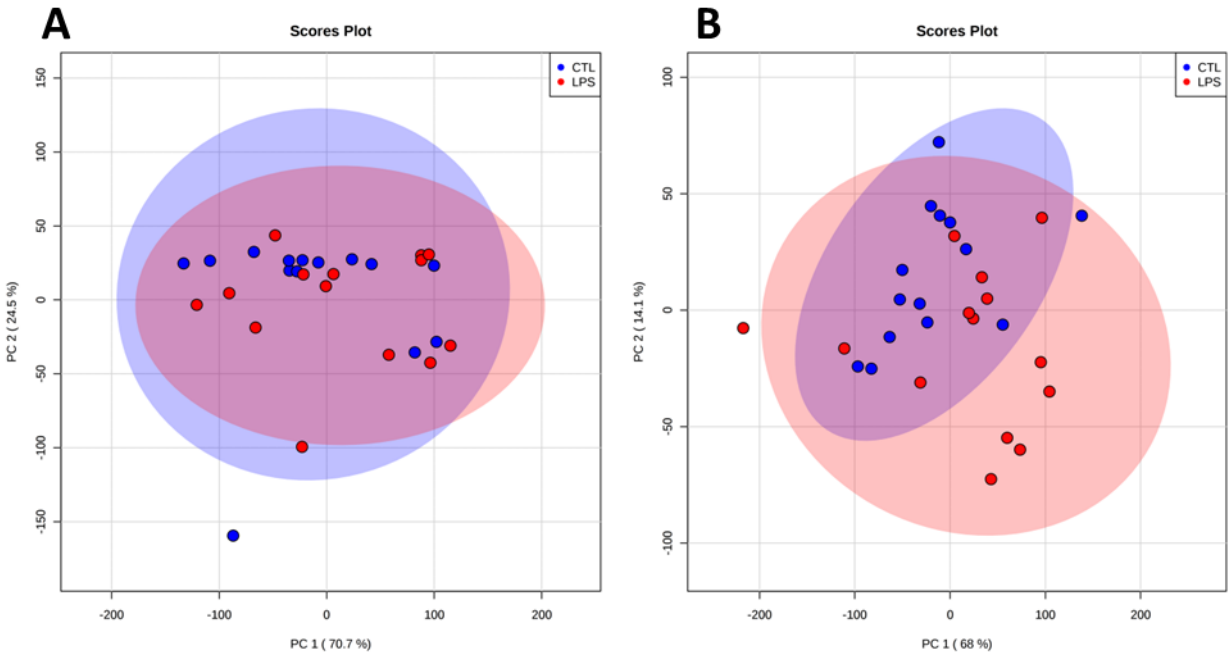


Figure 12. Multivariate analysis of the FA (A) and the PE (B) levels. Results of a PCA obtained based on the FA (A) and PE (B) levels found in control (blue) and 24h LPS treated (red) cells.

However, for some other lipid families, PCA showed clustering between the two groups. This was the case when all the phospholipids were considered (**Figure 14**) or when all the sphingolipids were studied (**Figure 18**). When this was the case, we applied several analyses in order to determine which lipids were responsible for the differentiation between vehicle treated and LPS-treated cells. This is illustrated by **Figure 13**.

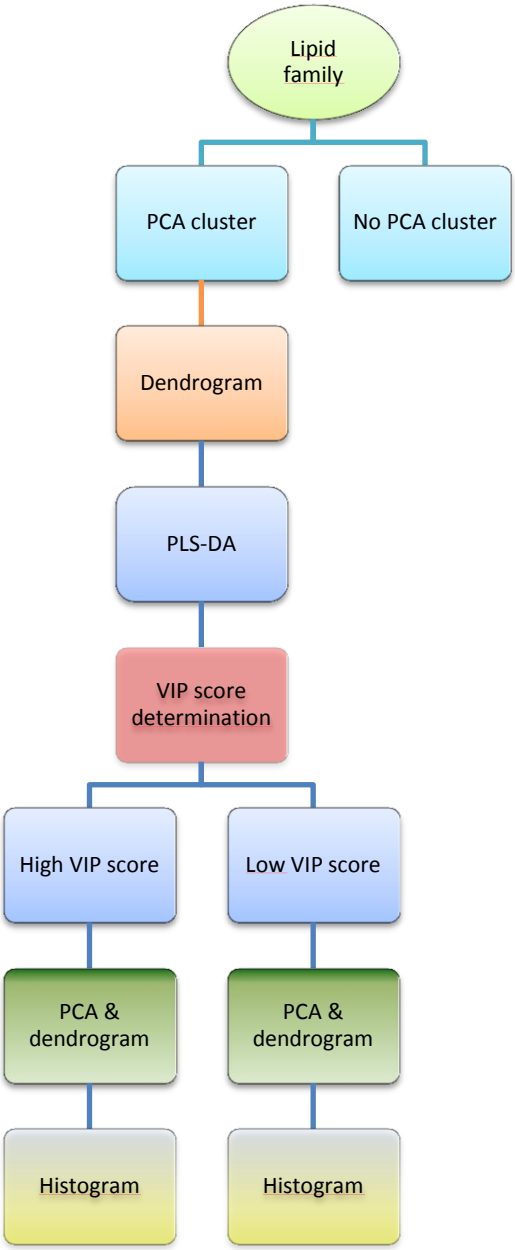


Figure 13. Multivariate analysis strategy. Global pathway of our multivariate analysis.

First, we tested the multivariate statistical analysis using the data obtained for all the phospholipids: PI, PE, PG, PS, PC and PE(p) (**Figure 14**). Here, contrary to what we obtained for FA, a separation is present between the treated and untreated conditions. Moreover, as we can see on the dendrogram, we also achieve a well-structured clustering of the samples. Given the fact that we showed clearly two groups based on the phospholipids study, we performed a PLS-DA to find the lipids most involved in this separation. The quality of this model is supported by the values of R2 (94%) and Q2 (88%).

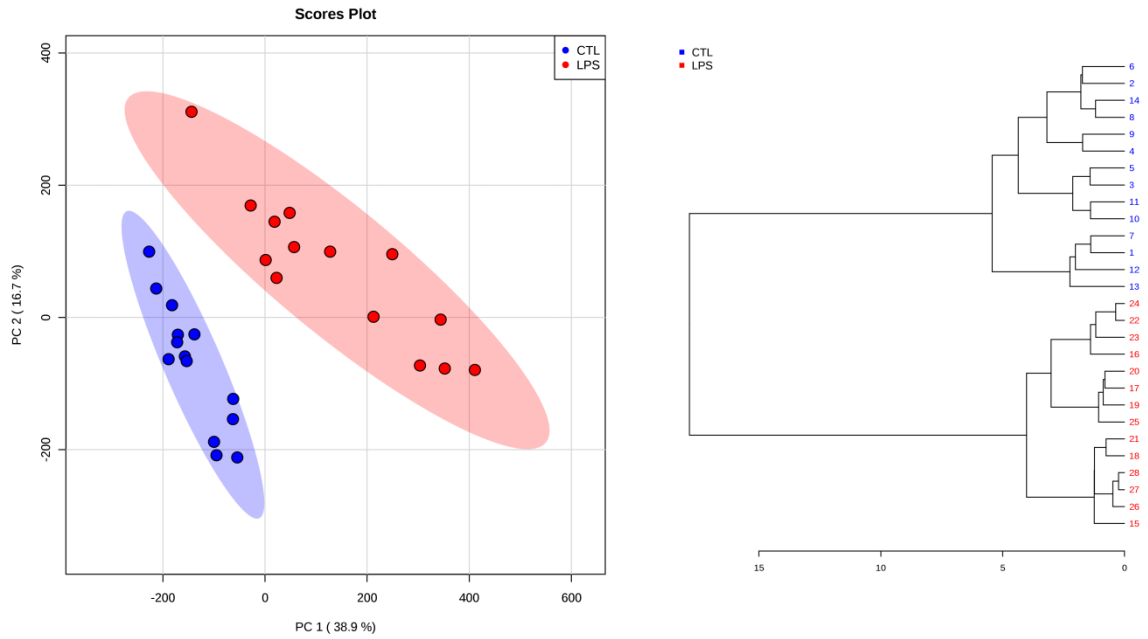


Figure 14. Multivariate analysis of the phospholipids levels. Results of a PCA obtained based on PI, PE, PG, PS, PC and PE(p) levels found in control (blue) and 24h LPS treated (red) cells and dendrogram analysis of control and LPS treated samples (Pearson's distance measure).

Among the highest VIP scores, PI appeared to be the most responsible for the clustering between control and LPS-treated cells (**Table 1**). Moreover, in the same analysis PE(p) appeared as one of the lipid family less responsible for the clustering (**Table 1**).

	VIP score
32:1 PI	5,52
34:1 PI	2,88
38:4 PI	1,97
36:1 PI	1,46
32:0 PI	1,41
38:5 PI	1,38
…	…
…	…
38:5 Pe(p)	0,03
36:1 PS	0,02
38:4 Pe(p)	0,02
34:2 Pe(p)	0,003
34:1 Pe(p)	0,0002

Table 1. Multivariate analysis of the phospholipid levels. Variable importance in projection (VIP) for phospholipids (highest and lowest scores).

Next we performed a PCA analysis with the data obtained for PI (**Figure 15**) and those obtained for PE(p) (**Figure 16**).

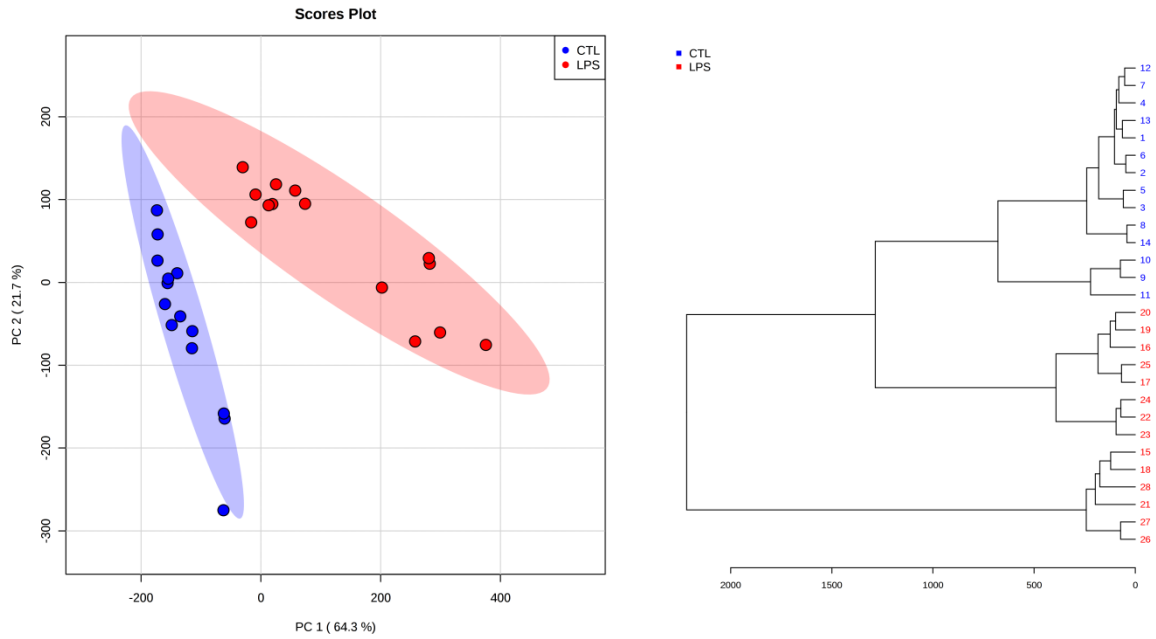


Figure 15. Multivariate analysis of the PI levels. Results of a PCA obtained based on PI levels found in control (blue) and 24h LPS treated (red) cells and dendrogram analysis of control and LPS treated samples (Pearson's distance measure).

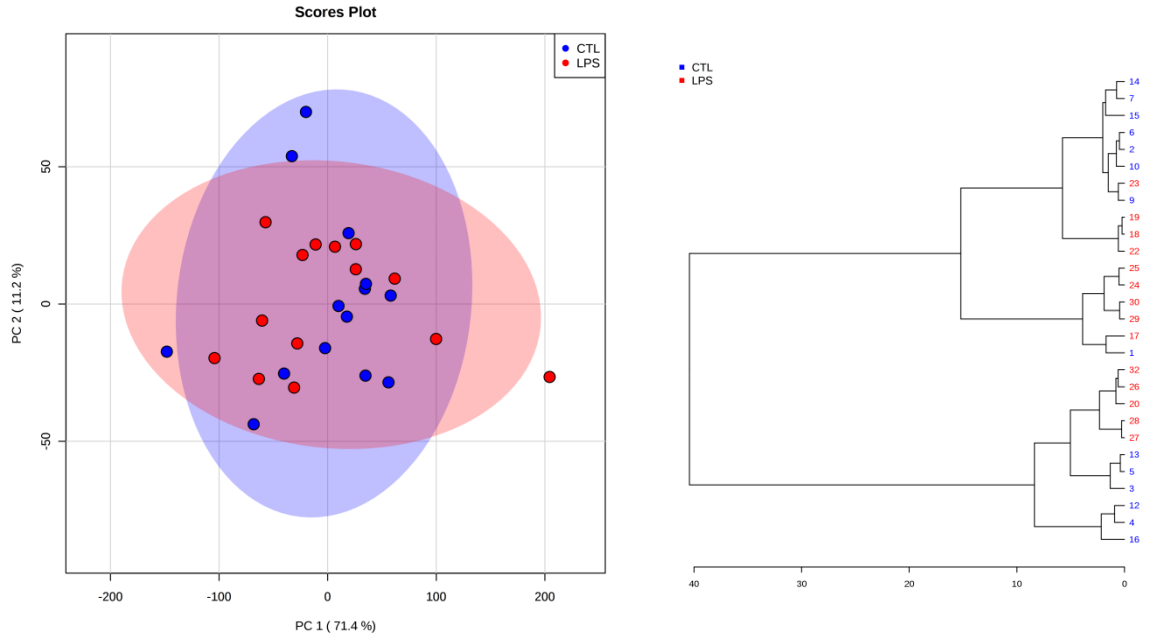


Figure 16. Multivariate analysis of the PE(p) levels. Results of a PCA obtained based on PE(p) levels found in control (blue) and 24h LPS treated (red) cells and dendrogram analysis of control and LPS treated samples (Pearson's distance measure).

As expected based on the VIP scores obtained after phospholipids PLS-DA analysis, we observed a clustering after PCA with PI but not with PE(p). Moreover, dendrograms showed a clear separation between controls and LPS-treated cells for PI but no clear separation for PE(p).

To further support this, we plotted the changes in PI and PE(p) levels resulting from LPS-induced J774 activation (**Figure 17**). The data clearly show that LPS-activation results in extensive changes in cell PI levels, whereas PE(p) levels are barely affected.

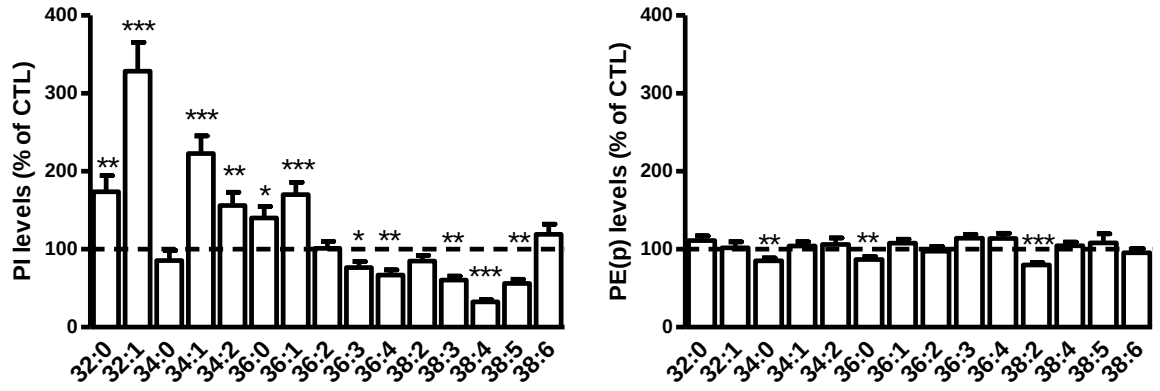


Figure 17. Effect of activation of J774 macrophages on PI and PE(p) levels. PI and PE(p) levels were measured by HPLC-MS following activation of J774 cells with 100ng/mL of lipopolysaccharides (LPS) for 24h. The length of the acyl chains and the degree of insaturation of the individual species are shown on the X-axis. Data are expressed for each individual species in percentage of the control condition (cells incubated with vehicle, dotted line). Values are mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 for LPS activation vs. control cells.

To further confirm the interest of this strategy, we performed a similar analysis with the data obtained for the sphingolipids (i.e. Cer, DihydroCer, GalCer and SM). As for the phospholipids, the PCA analysis resulted in a clear separation in two groups (**Figure 18**). Moreover, as we can see on the dendrogram, we also achieve a well-structured clustering of samples. We next performed a PLS-DA to pinpoint the lipids implicated in this separation based on the VIP scores (**Table 2**). Here again, the quality of this model is well supported by the values of R2 (93%) and Q2 (91%).

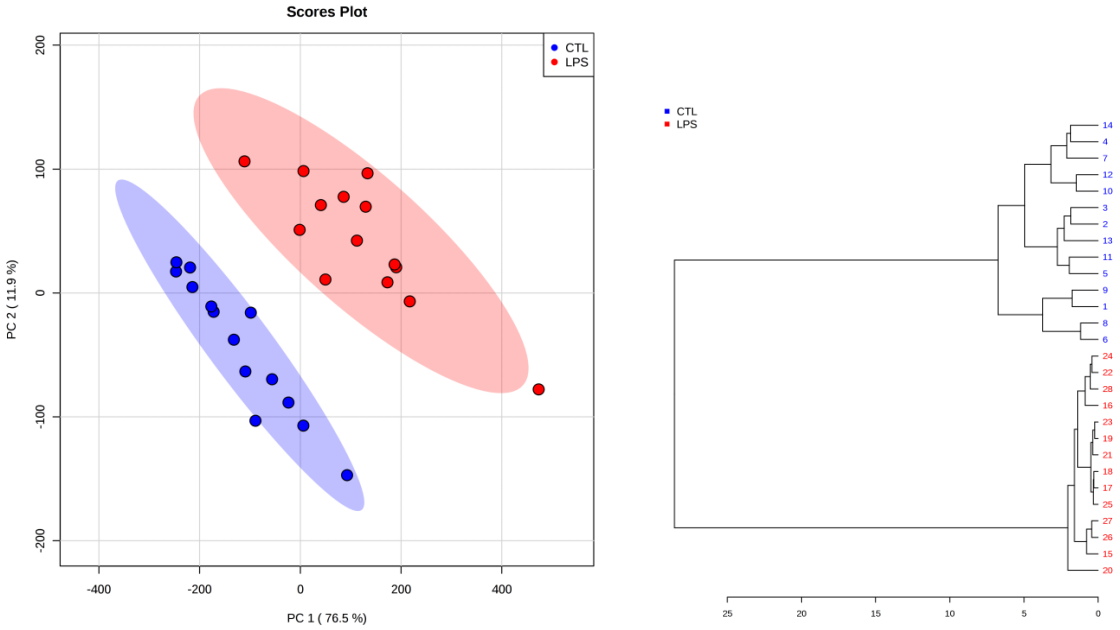


Figure 18. Multivariate analysis of the sphingolipids levels. Results of a PCA obtained based Cer, DihydroCer, Galcer and SM levels found in control (blue) and 24h LPS treated (red) cells and dendrogram analysis of control and LPS treated samples (Pearson's distance measure).

Among the highest VIP scores, GalCer appeared to be the most responsible for the clustering between control and LPS-treated cells. On the opposite, SM look like the less responsible for the separation (**Table 2**), suggesting that their levels are not affected by LPS treatment.

	VIP score
24:0 GalCer	2,34
24:0 Cer	2,21
22:0 GalCer	1,22
16:0 GalCer	0,91
22:0 Cer	0,61
…	…
…	…
24:0 SM	0,3
24:1 SM	0,05
20:0 SM	0,01
16:0 SM	0,002

Table 2. Multivariate analysis of the sphingolipids levels. Variable importance in projection (VIP) for the sphingolipids analyzed here (highest and lowest scores).

Based on these data, we performed another analysis based on the data obtained for GalCer (**Figure 19**) and SM (**Figure 20**).

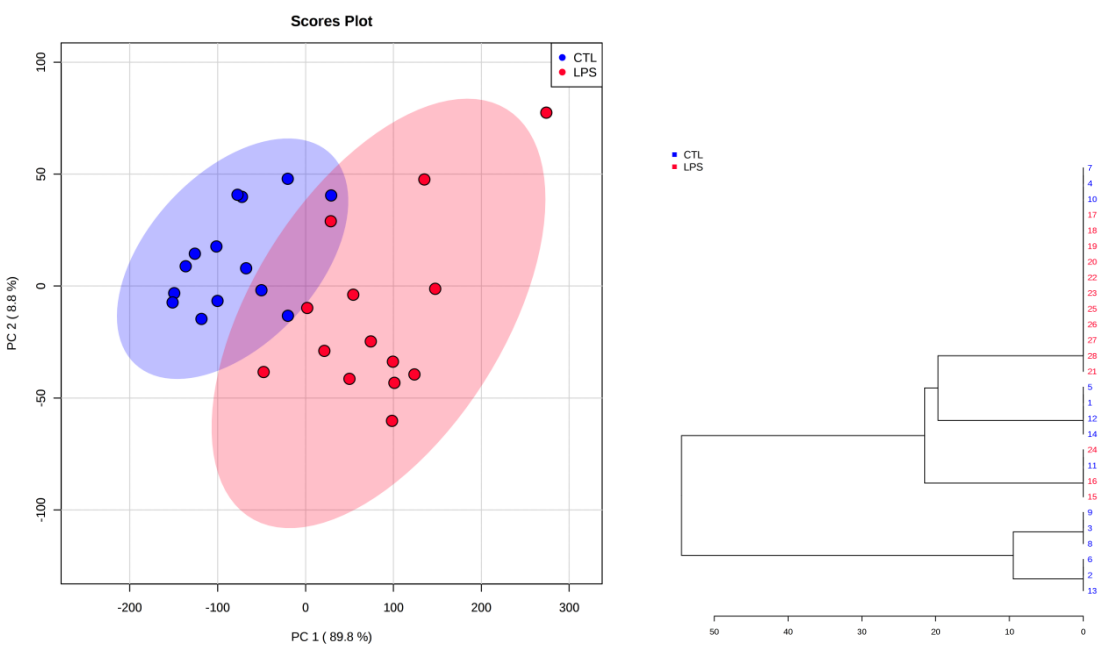


Figure 19. Multivariate analysis of the GalCer levels. Results of a PCA obtained based on GalCer levels found in control (blue) and 24h LPS treated (red) cells and dendrogram analysis of control and LPS treated samples (Pearson's distance measure).

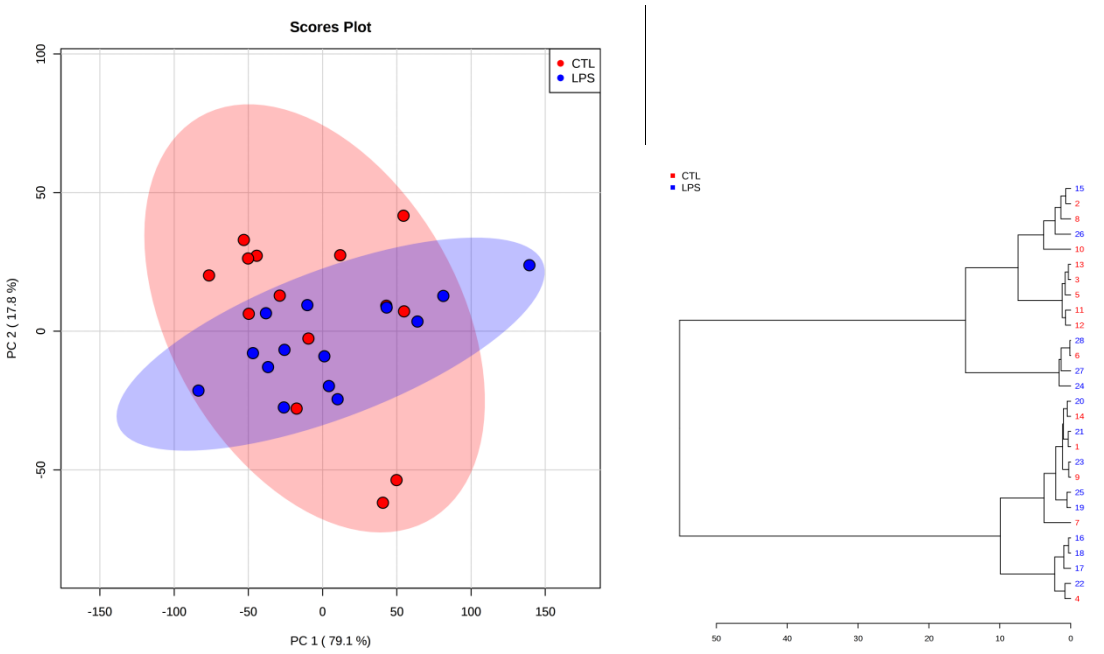


Figure 20. Multivariate analysis of the SM levels. Results of a PCA obtained based on SM levels found in control (blue) and 24h LPS treated (red) cells and dendrogram analysis of control and LPS treated samples (Pearson's distance measure).

As expected depending on the VIP scores obtained after sphingolipids PLS-DA analysis, we observed no clustering for SM after PCA, but unexpectedly also a poor clustering for Galcer

(**Figure 19**). A potential explanation for this is the relatively limited number of species taken into account in the PCA.

Actually, when we look at the graph showing the relative levels of GalCer we can see that LPS-activation induces an increase of GalCer levels, consistently with the higher VIP scores obtained for GalCer compared to SM (**Figure 21**).

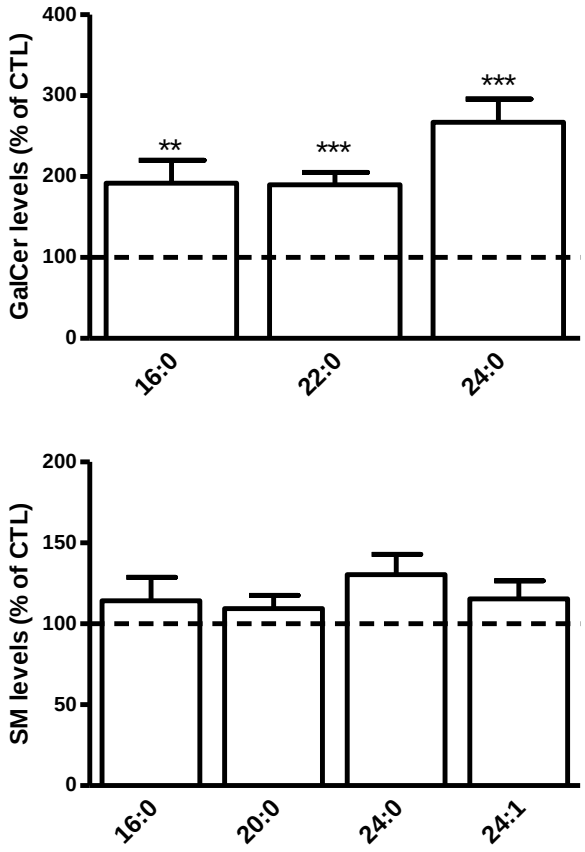


Figure 21. Effect of activation of J774 macrophages on GalCer and SM levels. GalCer and SM levels were measured by HPLC-MS following activation of J774 cells with 100ng/mL of lipopolysaccharides (LPS) for 24h. The length of the acyl chains and the degree of insaturation of the individual species are shown on the x axis. Data are expressed for each individual species in percentage of the control condition (cells not incubated with LPS, dotted line). Values are mean ± SEM. ***P<0.001, **P<0.01 for LPS activation vs. control cells.

Finally, because Cer are also well represented among the high VIP scores for sphingolipids, we performed a PCA and dendrogram, showing a nice cluster between our two groups (**Figure 22**).

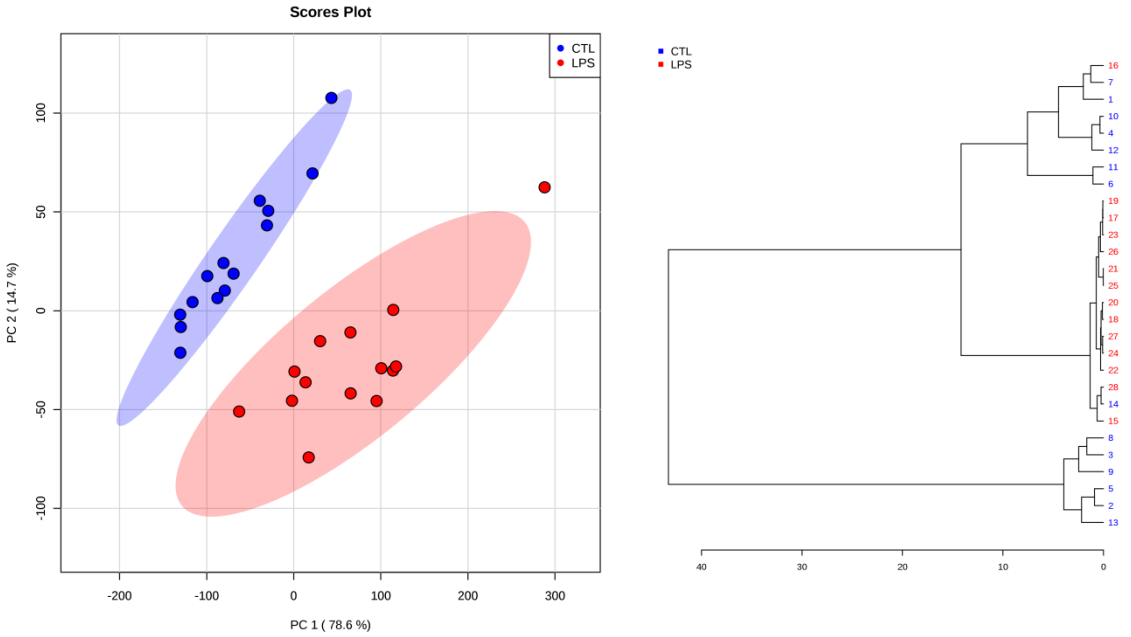


Figure 22. Multivariate analysis of the Cer levels. Results of a PCA obtained based on the Cer levels found in control (blue) and 24h LPS treated (red) cells and dendrogram analysis of control and LPS treated samples (Pearson's distance measure).

Figure 23 shows that LPS-induced activation results in increased levels of 22:0 Cer and 24:0 Cer, which is in agreement with the VIP scores obtained in the PLS-DA analysis (**Table 2**).

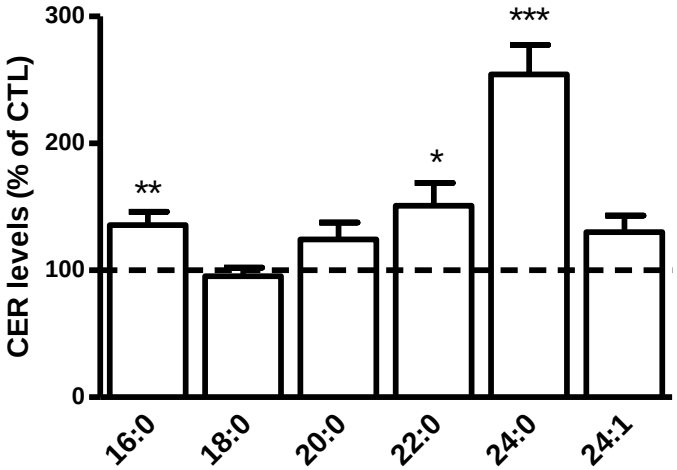


Figure 23. Effect of activation of J774 macrophages on Cer levels. Cer levels were measured by HPLC-MS following activation of J774 cells with 100ng/mL of lipopolysaccharides (LPS) for 24h. The length of the acyl chains and the degree of insaturation of the individual species are shown on the x axis. Data are expressed for each individual species in percentage of the control condition (cells not incubated with LPS, dotted line). Values are mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 for LPS activation vs. control cells.

In conclusion, we developed an untargeted approach for the identification and characterization of lipids by HPLC-ESI-MS allowing us to detect more than 180 lipids in the same chromatographic run. These lipids are mainly species from phospholipids and sphingolipids. Moreover, we applied this analytical method to the study of macrophage activation showing the effect of LPS activation on particular lipid species while others are not affected. We also tried to compare these results to other similar models, since it is not easy to find exactly the same experiment conditions. In RAW macrophages activated by Kdo as TLR4 ligand, Dennis et al. also performed a large scale lipid analysis and showed differences for several lipid families.[3] They showed large variations for PI levels between control and activated macrophages, as we obtained after PLS-DA analysis. However, for other lipid species such as ceramides, results are much different. Actually even though these two models are quite similar, the cells and ligand are not the same which could explain the differences observed in terms of lipid variations.

IV. References

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3 Dennis,E.A. et al. (2010) A mouse macrophage lipidome. J. Biol. Chem. 285, 39976-39985

Part 2. Development and validation of a specific and sensitive HPLC-ESI-MS method for quantification of lysophosphatidylinositols and evaluation of their levels in mice tissues

Part 2. Development and validation of a specific and sensitive HPLC-ESI-MS method for quantification of lysophosphatidylinositols and evaluation of their levels in mice tissues

Adapted from "Development and validation of a specific and sensitive HPLC-ESI-MS method for quantification of lysophosphatidylinositols and evaluation of their levels in mice tissues" by Masquelier J. and Muccioli G. published in J. Pharm. Biomed. Anal. (2016) 15; 126:132-40

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Development and validation of a specific and sensitive HPLC-ESI-MS method for quantification of lysophosphatidylinositols and evaluation of their levels in mice tissues

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I. Abstract

Increasing evidence suggests that lysophosphatidylinositols (LPI), a subspecies of lysophospholipids, are important endogenous mediators. Although LPI long remained among the less studied lysophospholipids, the identification of GPR55 as their molecular target sparked a renewed interest in the study of these bioactive lipids. Furthermore, increasing evidence points towards a role for LPI in cancer development. However, a better understanding of the role and functions of LPI in physiology and disease requires methods that allow for the quantification of LPI levels in cells and tissues. Because dedicated efficient methods for quantifying LPI were missing, we decided to develop and validate an HPLC-ESI-MS method for the quantification of LPI species from tissues. LPI are extracted from tissues by liquid/liquid extraction, pre-purified by solid-phase extraction, and finally analyzed by HPLC-ESI-MS. We determined the method's specificity and selectivity, we established calibration curves, determined the carry over (< 2%), LOD and LLOQ (between 0.116–7.82 and 4.62–92.5 pmol on column, respectively), linearity ($0.988 < R^2 < 0.997$), repeatability (CV < 20%), accuracy (> 80%), intermediate precision (CV < 20%) as well as the recovery from tissues. We then applied the method to determine the relative abundance of the LPI species

in 15 different mouse tissues. Finally, we quantified the absolute LPI levels in six different mouse tissues. We found that while 18:0 LPI represents more than 60% of all the LPI species in the periphery (e.g. liver, gastrointestinal tract, lungs, spleen) it is much less abundant in the central nervous system where the levels of 20:4 LPI are significantly higher. Thus this validated HPLC-ESI-MS method for quantifying LPI represents a powerful tool that will facilitate the comprehension of the pathophysiological roles of LPI.

II. Introduction

Lysophosphatidylinositols (LPI) are endogenous glycerol-based phospholipids in which the glycerol moiety is substituted by a single acyl chain (lysophospholipids) and by a phosphoinositol moiety. LPI are small membrane-derived lipids mainly generated from phosphatidylinositols (PI) by phospholipase A1 (e.g. DDHD1) and phospholipase A2 (e.g. cPLA2).[1-3] LPI can be reacylated into PI via the action of acyltransferases such as MBOAT7 and AGPAT8.[2]

LPI are lysophospholipids, a quite large group of lipids, with some of them already well-studied. For instance, lysophosphatidic acids (LPAs), certainly the most known lysophospholipid, are implicated in many physiological processes such as brain and vascular development, reproduction or immune response, but also in the pathogenesis of many diseases, including atherosclerosis or neuropathic pain or with activities associated in several cancers.[4,5]

Although LPI remain less studied than other lysophospholipids, increasing evidence supports a role for LPI as bioactive lipids. Indeed it is now well recognized that LPI are not merely intermediates in phosphatidylinositols metabolism without any informational function, but bioactive mediators per se. The first reports focusing on the biological activities of LPI suggested that they were involved in stimulation of insulin release from pancreatic islets.[6] It was then suggested that LPI may play a significant role in the regulation of whole body metabolism.[6,7] Other studies suggested an association between LPI levels and the mitogenic activity of cells.[8,9] It was also shown that LPI serum levels were correlated with cancer development.[10-13] More recently, Oka et al. demonstrated that LPI are endogenous ligands for the G protein-coupled receptor (GPCR) GPR55. The demonstration that LPI, and 2-arachidonoyl LPI in particular[14], are the endogenous ligands of the GPR55 receptor allowed

to investigate more mechanistically the previous observations on LPI's effects. Indeed, the identification of a receptor for LPI allows for pharmacological modulation of their effects with the use of agonists or antagonists of the receptor. Moreover, the detection of GPR55 in various human cancer cell lines (breast, brain, skin, pancreas, liver and prostate) and hematological tumors[15-20], brought a new interest on the role of LPI and GPR55 in cancer development.[21-22]

This renewed interest in LPI biological activities and in the effects of GPR55 activation by LPI requires validated analytical methods allowing for the quantification of LPI from biological samples (cells and tissues). Methods for the detection of LPI have been published some time ago and applied to the detection of LPI in platelets[23] or in human peripheral blood neutrophils.[24] However, only few of them gave access to actual levels of LPI in various tissues. For instance, Oka and co-workers reported LPI levels in rat brain[14], while Bollinger et al. measured total LPI levels in mouse serum.[25] However, none of these methods was actually validated. Moreover, most of the previous studies used either less sensitive or less direct methods of quantification. For instance, Oka et al. quantified mouse brain LPI by thin layer chromatography purification of the LPI fraction followed by gas chromatography analysis of the methyl esters derivatives of the LPI's fatty acids.[14]

In this context, the combined use of high pressure liquid chromatography with electrospray ionization mass spectrometry (HPLC-ESI-MS) represents a powerful tool to detect and quantify these analytes. Thus, here we report the development and validation of an HPLC-ESI-MS method allowing for the simultaneous quantification of the major endogenous LPI.

We first determined the optimal conditions to extract LPI from tissues. We then optimized the HPLC-ESI-MS conditions before validating the method. Thus we determined the method's specificity, selectivity, carry over (< 2%), limit of detection and lower limit of quantification (between 0.116-7.82 and 4.62-92.5 pmol, respectively), linearity ($0.988 < R^2 < 0.997$), repeatability (CV < 20%), accuracy (> 80%), intermediate precision (CV < 20%) as well as the recovery from tissues in accordance with the main analytical guidelines.

Finally, we also report here the repartition of the major LPI species in several mouse tissues as well as the absolute LPI levels in the colon, cerebellum, liver, lung, spleen and adipose tissue.

III. Materials and methods

1. Chemicals and materials

The LPI (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoinositol (16:0 LPI); 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphoinositol (18:0 LPI); 1-oleyol-2-hydroxy-*sn*-glycero-3-phosphoinositol (18:1 LPI); 1-arachidonoyl-2-hydroxy-*sn*-glycero-3-phosphoinositol (20:4 LPI)), as well as the internal standard 1-(10Z-heptadecenoyl)-2-hydroxy-*sn*-glycero-3-phospho-(1-myo-inositol) (17:1 LPI), were purchased from Avanti Polar lipids (Alabaster, AL, USA). HPLC grade chloroform and methanol were bought from VWR (Leuven, Belgium). The water used for preparing the mobile phase was obtained in-house by double-distillation while the HPLC–MS grade methanol was purchased from Carl Roth (Karlsruhe, Germany). The ammonium hydroxide solution used as additive for the LC–MS analysis was purchased from Sigma Aldrich (Diegem, Belgium).

2. Instrumentation and HPLC-ESI-MS conditions

The HPLC-ESI-MS system used is a high-resolution and high mass accuracy LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with an electrospray ion source coupled to an Accela HPLC system (ThermoFisher Scientific). Tune Plus® software 2.4 was used for instrument control. Chromatographic separation was achieved using a Kinetex LC-18 column (5 µm, 4.6x150 mm), a core-shell column from Phenomenex, connected to a Phenomenex SecurityGuard C18 guard column in thermostatic compartment at 20 °C after a 20 µL injection. Mobile phases A and B consisted of methanol-H_2O-ammonium hydroxide 50:49.9:0.1 (v/v/v) and methanol- ammonium hydroxide 99.9:0.1 (v/v), respectively. The gradient (0.4 mL/min) was designed as follows: starting at 100% A and reaching linearly 100% B in 30 min, this was followed by 15 min at 100% B before reequilibrating at 100% A. LPI species were analyzed in the negative mode. The ESI spray voltage was set at 5.0 kV and the capillary temperature at 270°C while the sheath gas flow was set at 40 arbitrary units. The mass spectrometer was calibrated for mass accuracy before each series of injections.

For data acquisition and processing, the Xcalibur® software (ThermoFisher Scientific) was used. LPI were integrated as their $[M\text{-}H]^-$ ions after being identified based on their relative retention time (RRT) and fragmentation diagnostic ions (**Table 1**) determined following injection of standards and spiked tissues.

			Detection parameters					
Common name	Molecular formula	Exact mass	$[M\text{-}H]^-$	RRT	$[inositolphosphate\text{-}H_2O\text{-}H]^-$	$[Fatty\ acid\text{-}H]^-$	$[GPI\text{-}H_2O\text{-}H]^-$	$[LPA\text{-}H_2O\text{-}H]^-$
16:0 LPI	$C_{25}H_{49}O_{12}P$	572.2962	571.2889	1.03 ± 0.003	241.0124	255.2324	315.0492	391.226
16:1 LPI	$C_{25}H_{47}O_{12}P$	570.2805	569.2732	0.933 ± 0.004	241.0124	253.2168	315.0492	389.2104
18:0 LPI	$C_{27}H_{53}O_{12}P$	600.3275	599.3202	1.17 ± 0.007	241.0124	283.2637	315.0492	419.2574
18:1 LPI	$C_{27}H_{51}O_{12}P$	598.3118	597.3045	1.07 ± 0.004	241.0124	281.2481	315.0492	417.2417
18:2 LPI	$C_{27}H_{49}O_{12}P$	596.2962	595.2889	0.984 ± 0.001	241.0124	279.2324	315.0492	415.2261
20:4 LPI	$C_{29}H_{49}O_{12}P$	620.2962	619.2889	0.979 ± 0.003	241.0124	303.2324	315.0492	439.2261
17:1 LPI (IS)	$C_{26}H_{49}O_{12}P$	584.2962	583.2889	1	241.0124	267.2324	315.0492	403.2261

Table 1. Detection parameters of the LPI quantified. The table shows the molecular formula for each LPI, as well as the exact mass and the m/z value for the analyzed ions $[M\text{-}H]^-$. The relative retention times (RRT) were calculated using the RT of 17:1 LPI as reference. The RRT values (mean ± s.e.m.) shown here are based on over 40 injections randomly selected over a 6-month period. The m/z values for each diagnostic ions are also reported (four last columns). GPI is glycerolphosphoinositol.

3. Preparation of calibration standards and quality controls standards

For the preparation of stock solutions, LPI (16:0 LPI, 18:0 LPI, 18:1 LPI, 20:4 LPI), as well as the internal standard (17:1 LPI), were dissolved in an appropriate volume of methanol resulting in a concentration of 1 $mmol*L^{-1}$ for each solution. All the solutions were stored at -20 ° C. On the day of the experiment, working solutions were obtained by dilutions of the stock solutions with methanol to yield the required concentrations for the preparation of calibration standards and QC samples. For all the validation process, methanol was used as solvent since it is not possible to use blank matrix due to the endogenous signal for LPI present in all the tissue tested. We controlled that the internal standard (17:1 LPI) used both in the calibration curves and in the tissues to quantify was extracted similarly to the analytes of interest.

4. Analysis of LPI in mice: animals and tissue sample preparation

C57BL/6J mice (8–10 weeks of age) were purchased from Charles River Laboratories (Les Oncins, France) and were housed under standard conditions (12 h light/dark cycle, and controlled 22°C temperature) and supplied with water and food ad libitum. Following sacrifice of the mice, tissues were rapidly recovered and snap-frozen in liquid nitrogen

before storage at −80°C until their analysis. Animal procedures were performed in accordance with the European recommendation 2007/526/CE (which was transformed into the Belgian Law of May 29, 2013), regarding the protection of laboratory animals. The animal experimentation ethics committee from the Université catholique de Louvain approved the protocol of the study (study agreement 2010/UCL/MD/022; lab agreement LA1230314). The day of analysis, tissues were homogenized with an IKA T10 ULTRA-TURRAX® in ice-cold bi-distilled water (2.5 mL) and the complete extraction solution was obtained by adding methanol (5 mL) and chloroform (10 mL) (following an extraction procedure similar to the procedure described by Folch).[26] Based on our results, HCl (2 N, 300 µL) was also added to promote the extraction of LPI. Following the addition of 17:1 LPI (40 pmol/condition) as internal standard, samples were shaken thoroughly and sonicated in ice-cold water. After centrifugation (10 min, 700g at 4°C), the organic layer was recovered and dried under nitrogen to minimize the risk of oxidation. The resulting lipid fraction was then pre-purified by solid-phase extraction (SPE) over silica using methanol as the eluting solvent. Silica is washed with chloroform and methanol and conditioned with chloroform before loading the sample. The eluate obtained was dried under nitrogen and solubilized with chloroform-methanol (1:1; v/v) for the HPLC-ESI-MS analysis.

5. HPLC-ESI-MS method validation

The validation of the method was carried out using the parameters described in different guidelines.[27-30] Thereby, selectivity and specificity were evaluated before a calibration curve was obtained for the four main LPI found in mammalian tissues (16:0 LPI, 18:0 LPI, 18:1 LPI, 20:4 LPI). Several parameters were evaluated such as carry over, recovery, linearity, limit of detection (LOD), limit of quantification (LOQ) and lowest limit of quantification (LLOQ), precision (repeatability and intermediate precision) and accuracy. During the validation procedure four levels (LLOQ, low, middle and high) quality control (QC) solutions were used. Total error was used as decision criterion for the validation process and the acceptance limits were set at $\lambda = \pm 20\%$ as usually used for endogenous mediators.[31-32]

a) Analytical selectivity and specificity

To determine whether this analytical method is able to differentiate the analytes of interest, and the internal standard, from other endogenous components in the matrix, selectivity and

specificity were evaluated using the retention time and the MS/MS spectrum profile of pure standards and of mice tissues (described in **Table 1**). In these profiles, the MS/MS fragmentation of all the parent ions of interest was studied. To confirm our results, both solvent extraction mixture and tissue homogenates were spiked (200 pmol/injection) with the different standards. The intensities and mass spectrum profiles of the compounds were analyzed at the appropriate retention time (see **Table 1**) and compared to the results obtained following the direct injection of the standards and unspiked tissues into the HPLC-ESI-MS.

b) Carry over and recovery

The method's carry over was minimized during the method development. Carry over was assessed by sequential injections of blank samples after a sample containing 3 times the amount of LPI found in the highest points of the calibration curves. Recovery is frequently assessed by measuring analyte levels in unspiked and spiked samples. Here, to evaluate this parameter, we compared the signal obtained with 15 mg of tissues (cerebellum and lung) alone (unspiked sample) to the signal obtained for tissues spiked with the known amount of added LPI (spiked sample). Based on preliminary data from standards and tissues analyses, different levels were chosen depending on the LPI species (45 pmol, 150 pmol, 45 pmol and 75 pmol/condition were used for 16:0 LPI, 18:0 LPI, 18:1 LPI and 20:4 LPI, respectively). Thus, samples containing only tissues and extraction solvents and spiked samples containing tissues, extraction solvents and the set amount of standards were extracted, pre-purified and analyzed by our HPLC-ESI-MS method.

c) Linearity, limit of quantification, limit of detection and lower limit of detection

To establish the calibration curves for 16:0 LPI, 18:0 LPI, 18:1 LPI and 20:4 LPI, we used 7 calibration points per analyte distributed in the range of the expected tissues amounts (based on preliminary experiments). Thus, the calibration curves were constructed by diluting the stock solution of each LPI to levels ranging from 5 to 250 pmol/injection for 16:0 LPI, 18:1 LPI and 20:4 LPI and from 50 to 2500 pmol/injection for 18:0 LPI. Specifically, 50, 100, 200, 500, 1000, 2000 and 2500 pmol/injection and 5, 10, 20, 50, 100, 200 and 250 pmol/injection were used for 18:0 LPI and 16:0 LPI, 18:1 LPI and 20:4 LPI, respectively. The calibration curves were obtained by spiking the extraction solvent mixtures, prior to the extraction, with the internal standard (40 pmol/injection) and the above mentioned

amounts of pure standards. Following the SPE purification and HPLC-ESI-MS analysis, the calibration curves were generated by plotting the amount of each sample (pmol) against the ratio of the AUC of the pure standard and the AUC of the internal standard. Fresh solutions were prepared for each experiment. To determine the content of each LPI in the experimental samples we plotted the peak area ratio between the compound of interest and the internal standard (17:1 LPI) into the equation obtained for each calibration curve. To verify that the matrix has no effect on this ratio we prepared a surrogate matrix containing phospholipids (phosphatidylcholines 0.55 mg/condition; phosphatidylethanolamines 0.55 mg/condition), cholesterol (0.8 mg/condition), sphingomyelins (0.1 mg/condition) and bovine serum albumin (BSA, 1.8 mg/condition) using proportions and amounts mimicking what is found in 15 mg of tissue. This matrix (matrix A) or a 4 fold diluted one (matrix B) were spiked with 16:0 LPI, 18:0 LPI, 18:1 LPI, 20:4 LPI and 17:1 LPI before extraction and HPLC-ESI-MS analysis. The obtained peak area ratios obtained were compared to the values obtained for the HPLC-ESI-MS analysis of reference solution containing the same amounts of 16:0 LPI, 18:0 LPI, 18:1 LPI, 20:4 LPI and 17:1 LPI (**Figure SI1**). Limits of quantification (LOQ) and limits of detection (LOD) were determined by the blank determination (LOD = $X_{bl} + 3S_{bl}$; LOQ = $X_{bl} + 10\ S_{bl}$) following the IHC and AOAC recommendations.[28,33] The lower limit of detection (LLOQ) was defined as the lowest concentration of each analyte for which we obtained an accuracy of 80–120% and precision within our acceptance criteria (CV ≤ 20%).

d) Precision and accuracy

The intra-day (repeatability precision) and inter-day (intermediate precision) precisions were determined using quality control samples (QC) at four concentration levels (corresponding to the LLOQ, and 25%, 50% and 75% of the maximum of the highest value of the calibration ranges) and were obtained by calculating the coefficient of variation (% CV) of the mean. Accuracy was determined using QC solutions of four concentration levels (corresponding to the LLOQ, and 25%, 50% and 75% of the maximum of the highest value of the calibration ranges) with at least 3 different experiments for each concentration level. This parameter is calculated by comparison between the obtained concentration after injection and the true concentration. The concentration of each QC sample was obtained using the calibration curve.

6. Statistics

Results are expressed as mean ± s.e.m. All experiments were conducted at least 3 times in triplicates (or at least hexaplicates for validation process). Significance between two distinct groups was determined by unpaired Student t-test. Data analysis was performed using GraphPad® prism5.0 (GraphPad Software Inc., San Diego, CA, USA).

IV. Results and discussion

1. HPLC-ESI-MS method development

We first studied the ionization and fragmentation properties of LPI. Several lysophospholipid families (e.g. LPC, LPG, LPS), including LPI, can be analyzed in both positive and negative modes. We found that in our conditions, the ESI source operated in negative mode resulted in higher signal for LPI compared to the positive mode (data not shown). Based on this, we further characterized LPI MS^2 fragmentation. As shown in **Figure 1**, the negative-ion ESI MS^2 spectra for 16:0 LPI results in four main fragments that can be attributed to the [inositolphosphate-H_2O-H]$^-$, [fatty acid-H]$^-$, [glycerolphosphoinositol-H_2O-H]$^-$, and [LPA-H_2O-H]$^-$ ions (**Table 1** and **Figure 1**). Importantly, the fragmentation pattern is not affected by the nature of the matrix. Indeed, the MS^2 spectra for (i) pure 16:0 LPI, (ii) 16:0 LPI from a soy bean LPI-purified fraction and (iii) 16:0 LPI from mouse brain lipid extract show the same mass spectrum profile (**Figure 1A**-**C**). A similar fragmentation pattern was observed for the other LPI species and for 17:1 LPI used as internal standard in our method (**Table 1** and **Figure 1)**.

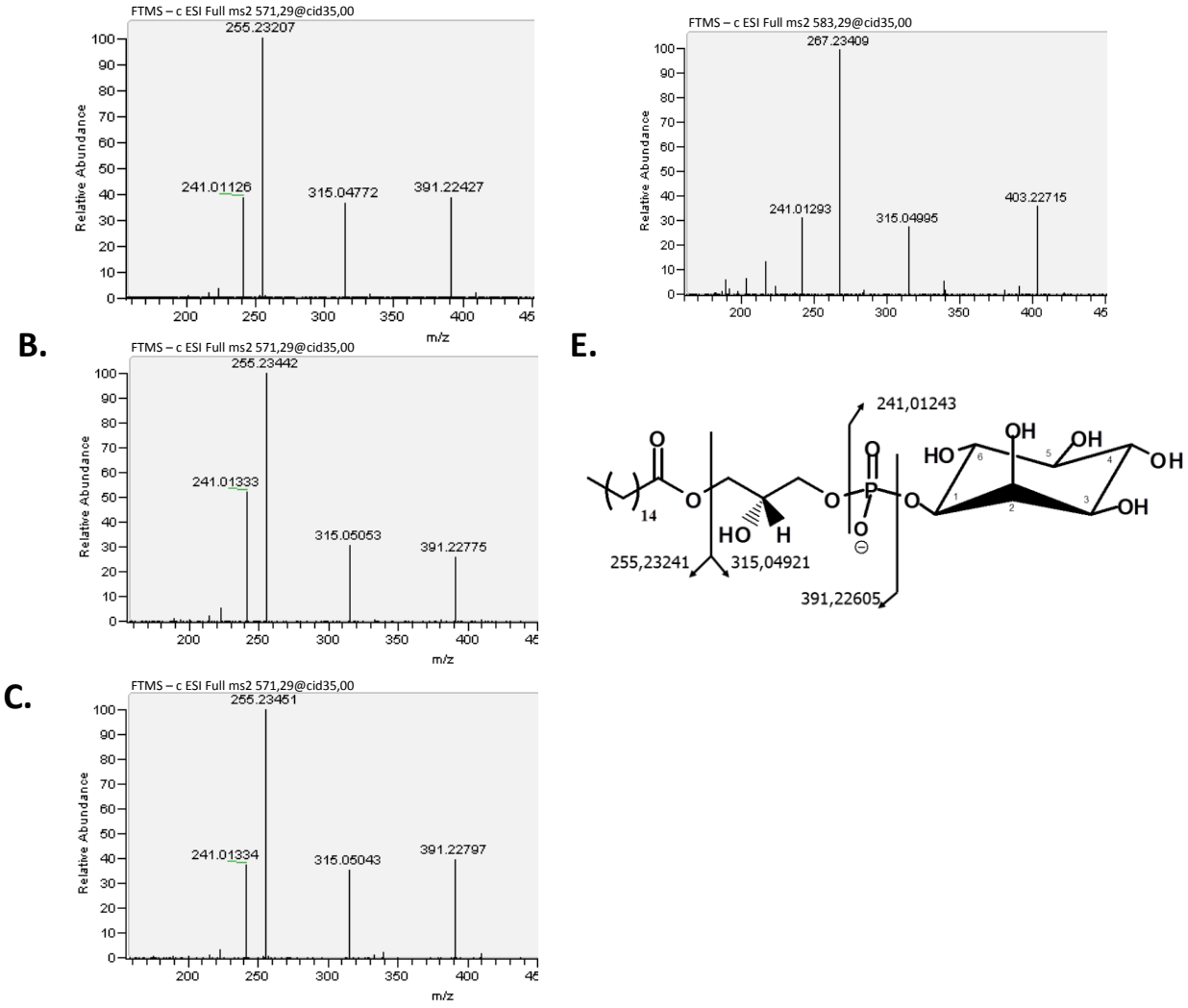

Figure 1. HPLC–ESI–MS/MS fragmentation of 16:0 LPI and of the internal standard 17:1 LPI. Negative ion electrospray ionization MS^2 spectra of 16:0 LPI (m/z = 571.28889) from (A) a pure standard solution, (B) from a soy bean LPI-purified fraction and (C) from a mouse brain purified lipid extract show common MS^2 product ions. As shown on the scheme (E), the MS^2 fragmentation generates four main ions: [Inositolphosphate-H_2O-H]$^-$, [Fatty acid-H]$^-$, [GPI-H_2O-H]$^-$, and [LPA-H_2O-H]$^-$. A similar fragmentation pattern is observed in (D) for 17:1 LPI used as internal standard.

Of note, in our conditions we did not observe product ions resulting from in-source or MS^2 water losses. We then went on to set up the HPLC–ESI–MS method.

To avoid any potential interference with signals arising from phosphatidylinositols (PI), the HPLC chromatographic parameters were chosen in order to achieve a good separation between LPI and the PI. The resulting system is based on a gradient of water and methanol, with NH_4 OH to assist the ionization. As illustrated in **Figure 2**, we found different retention times for the LPI and the PI that could potentially generate upon ionization the LPI analyzed

here. The LPI of interest were identified in complex matrix, such as mice tissues, based on the comparison of their retention time with the retention time obtained when analyzing a soy bean LPI-purified fraction (**Figure 2 A** and **B**).

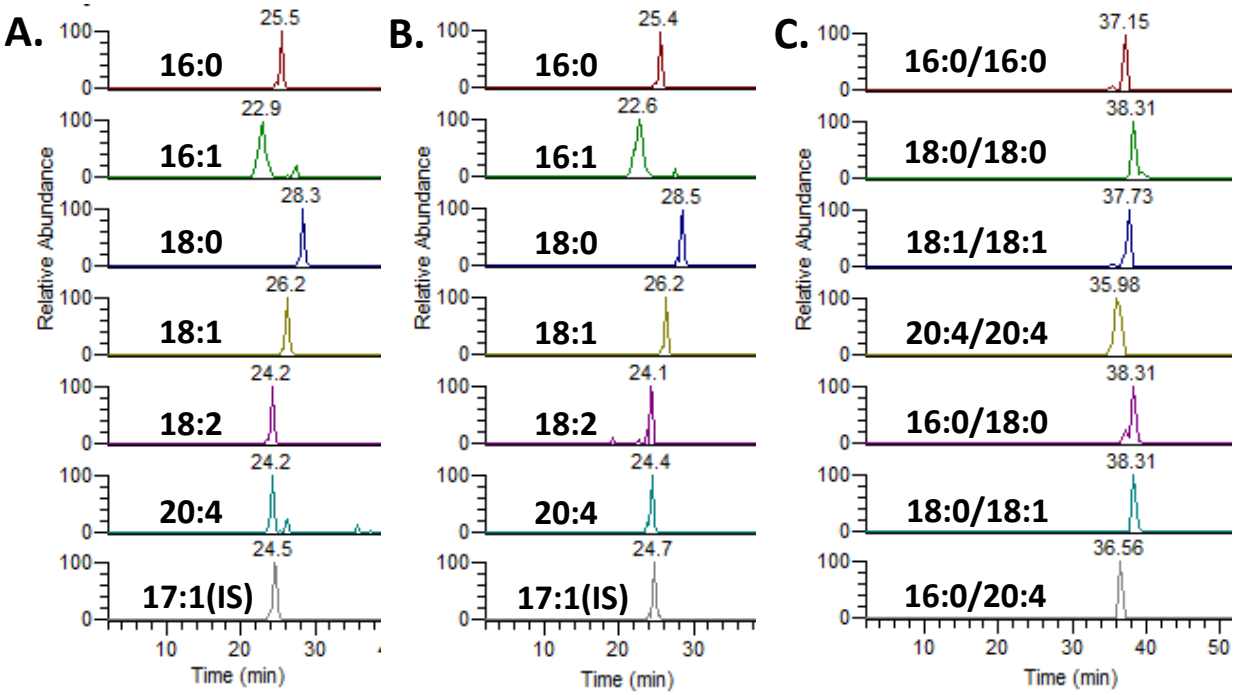

Figure 2. HPLC–ESI–MS extracted ion chromatograms for LPI. HPLC-ESI-MS detection of LPI from (A) soy bean LPI-purified fraction and (B) mouse brain lipid extract. LPI were detected using the orbitrap MS analyzer as singly deprotonated $[M-H]^-$ ions at m/z 571.28889 (16:0 LPI), 569.27324 (16:1 LPI), 599.32019 (18:0 LPI), 597.30454 (18:1 LPI), 595.28889 (18:2 LPI), 619.28889 (20:4 LPI) and 583.28889 (17:1 LPI, internal standard). (C) HPLC-ESI-MS detection of PI from mouse brain lipid extract. PI were detected using the orbitrap MS analyzer as singly deprotonated $[M-H]^-$ ions at m/z 809.51855 (16:0/16:0 PI), 865.58115 (18:0/18:0 PI), 861.54985 (18:1/18:1 PI), 905.51855 (20:4/20:4 PI), 837.54985 (16:0/18:0 PI), 863.56550 (18:0/18:1 PI), 857.51855 (16:0/20:4 PI). Mass accuracy set at 5 ppm.

In our system, LPI were detected as singly deprotonated $[M-H]^-$ ions using the exact mass (mass accuracy was less than 5 ppm). The m/z values of the LPI species analyzed with our method are listed in **Table 1** along with their relative retention time (using the retention time of 17:1 LPI as reference). Lipids are frequently analyzed in tissues following a liquid–liquid extraction step which allows their separation from the proteins and more polar metabolites present in the tissue of interest. Thus, we next aimed at optimizing LPI liquid–liquid extraction. Indeed, it is well known that efficient lysophospholipids, and LPI in particular, extraction can be challenging when working at neutral pH.[25,34] First we tested whether acidification prior to the liquid–liquid extraction would improve the efficiency of the

lipid extraction due to the phosphate protonation. We found that adding 300 µL of HCl 2N to our 17,5 ml solvent mixture allowed for acidifying the extraction mixture even in the presence of the matrix. In these conditions, LPI extraction was significantly improved as shown in **Figure 3A**. Another strategy that could improve LPI recovery is the use of silanized glassware. Usually lipids are analyzed using glass vials and pipettes to reduce non-specific binding and phthalates extraction by the organic solvents. However, due to their structure LPI tend to bind to the glassware thus affecting their recovery.[35-36] Thus silanization of the glassware, by reducing the possibility of polar interactions, should reduce the nonspecific binding of LPI and therefore favor their recovery. Consistent with this hypothesis, we found, as illustrated in **Figure 3B**, that the use of silanized glassware significantly increases the signal obtained for a same amount of LPI.

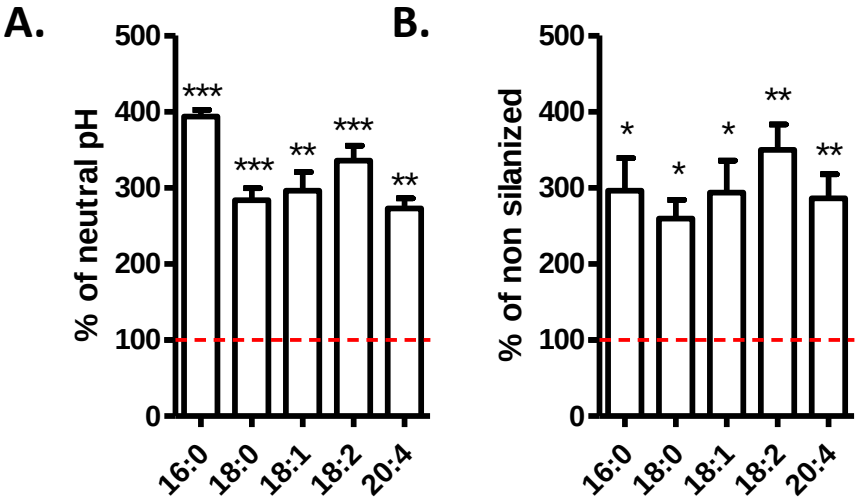

Figure 3. Optimization of the LPI liquid/liquid extraction. (A) Soy bean LPI-purified fraction was extracted by liquid/liquid extraction in neutral (pH = 7.5) or acidified (pH = 1.2) conditions before its analysis by HPLC-ES-MS. Data are expressed for each individual species in percentage of the neutral condition (dashed line). (B) Soy bean LPI-purified fraction was extracted and treated using glass material or using silanized glass material before its analysis by HPLC-ESI-MS. Data are expressed for each individual species in percentage of the non silanized condition (shown as dashed line). Unpaired student t-test, *P < 0.05, **P < 0.01, ***P < 0.001 versus the respective control conditions.

Finally, because of the complexity of the tissue matrix, we decided to add a solid phase extraction (SPE) step to our procedure in order to pre-purify the LPI from the analyzed samples. As for numerous lipid applications, we chose silica as solid phase for our SPE procedure. We next screened several solvent systems and found methanol to be optimal for recovering LPI (data not shown).

Altogether, we developed a new HPLC-ESI-MS method that, after a specific sample clean-up, allows for a highly selective and specific quantification of LPI in complex matrices. We therefore went on to validate this novel method.

2. HPLC-ESI-MS method validation

First, we investigated the presence of carry-over by analyzing blank samples directly after samples containing a high amount of analyte of interest (for instance, we used an amount equivalent to more than 3 times the highest point of the calibration curve for the 16:0 LPI). We also tested the carry over for the internal standard at the commonly used levels (40 pmol/injection). As illustrated in **Figure SI2** we did not detect significant signals for LPI in the blanks following the analysis of the highly concentrated samples.

We then prepared calibration curves for LPI in the range of the expected tissues amounts based on previous experiments (**Figure 4**).

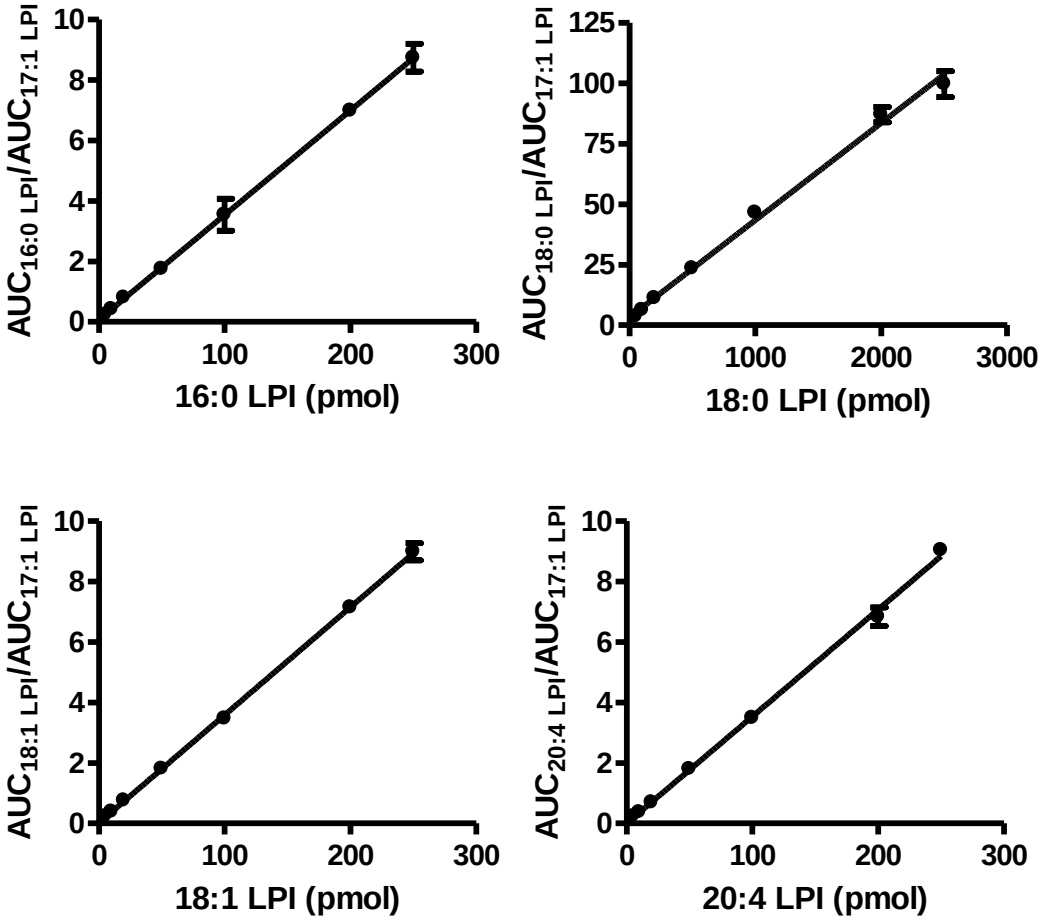

Figure 4. Calibration curves for the four most abundant LPI species. Seven calibration solutions of increasing concentration were extracted, eluted through a silica-gel SPE column, and analyzed by HPLC-ESI-MS. The calibration curves were generated by plotting the amount of each sample (pmol on column) against the ratio of the AUC of the pure standard and the AUC of the internal standard. The parameters of the resulting calibration curves are listed in **Table 2**.

The range, slope, intercept and coefficient of determination (R^2) of the calibration curves are listed in **Table 2**. Because LPI are present in tissue, and as it is done in quantification methods for endogenous bioactive lipids, we used methanol to prepare the calibration curves. However, we confirmed using a surrogate matrix made of lipids and proteins that the matrix was not affecting the peak area ratio between the compounds of interest and the internal standard (17:1 LPI) (**Figure SI2**).

The LOD and LOQ values for each compound were calculated using the blank determination.[28,33] The LOD values are comprised between 0.116 and 7.82 pmol on column (**Table 2**). The determined LLOQ values were found to be between 4.62 and 92.5 pmol on column (**Table 2**).

Calibration curve parameters

	Slope	Intercept	R^2	Calibration range	LOD (pmol)	LOQ (pmol)	LLOQ (pmol)
16:0 LPI	0.035	0.069	0.99	5-250	0.116	0.225	4.62
18:0 LPI	0.040	3,26	0.99	50-2500	7.82	18.53	92.5
18:1 LPI	0.036	0.020	0.99	5-250	0.117	0.344	4.62
20:4 LPI	0.035	0.0012	0.99	5-250	0.228	0.756	4.62

Table 2. Parameters determined for the LPI's calibration curves.

Note that because of the higher abundance of 18:0 LPI in the tissues, it was not considered as a problem that LOD and LLOQ were higher than that of other LPI. The linearity of the curves was evaluated by fitting a linear regression model on the back calculated amount as a function of the introduced amount. The slope and R^2 values were close to 1 for all tested LPI, supporting the linearity of the method (**Table 3**).

We determined the repeatability (intra-day precision) by analyzing 4 QC solutions (LLOQ, low, middle and high, reported in **Table 3**), the same day, in at least six consecutive injections. Furthermore, the same QC solutions were analyzed on three different days to determine the intermediate precision and the accuracy (bias). After analyzing the data, we found the intra-day precision, intermediate precision and bias to be within the acceptance limits, as reported in **Table 3**. The repeatability for all the QC solutions (from LLOQ to 75% of the maximum of the calibration curve) was comprised between 3.72% and 12.69%. The intermediate precision of all QC solution levels was comprised between 8.14% and 19.00%. Furthermore, all the tested QC samples showed accuracy within 80–120%. All the tested precision and accuracy parameters were therefore in accordance with the acceptation criteria (% CV values below 20% for intra- and inter-day precision and accuracy in the range of 100 ± 20%) in the range of amounts tested (four different levels) (**Table 3**).

Used amount (pmol)

	16:0 LPI	18:0 LPI	18:1 LPI	20:4 LPI
LLOQ	4.6	92.5	4.6	4.6
LOW	62.5	625	62.5	62.5
MIDDLE	125	1250	125	125
HIGH	187.5	1875	187.5	187.5

Repeatability precision (intra-day precision) (%CV)

	16:0 LPI	18:0 LPI	18:1 LPI	20:4 LPI
LLOQ	8.5	10.0	3.7	5.2
LOW	3.9	12.7	4.8	3.8
MIDDLE	3.9	5.4	5.2	4.1
HIGH	6.5	7.7	8.9	7.5

Intermediate precision (inter-day precision) (%CV)

	16:0 LPI	18:0 LPI	18:1 LPI	20:4 LPI
LLOQ	15.0	14.3	19.0	13.9
LOW	7.2	8.1	8.2	10.6
MIDDLE	8.9	9.9	9.2	9.7
HIGH	8.5	7.9	10.5	8.7

Accuracy (bias)

	16:0 LPI	18:0 LPI	18:1 LPI	20:4 LPI
LLOQ	-6,0	-8.5	0.1	2.4
LOW	7.2	17.4	7.1	4.7
MIDDLE	8.3	5.8	2.9	2.6
HIGH	1.6	-4.7	0.3	-0.4

Linearity

	16:0 LPI	18:0 LPI	18:1 LPI	20:4 LPI
Slope	0.99	1.03	0.99	0.99
Intercept	0.26	-8.62	0.25	0.29
R^2	0.99	0.99	0.99	0.99
Linearity range	5-250	50-2500	5-250	5-250

Table 3. Validation parameters of the HPLC-ESI-MS method.

To further assess the method, we studied the effect of spiking the tissue (two different tissues were tested) with known amounts of LPI standards during their extraction. As we show in **Figure 5**, the amount obtained after subtracting the amounts obtained for the unspiked matrix (native sample) from the amounts obtained with the spiked sample are not significantly different from the theoretical added quantity, represented by the dashed line. For instance, for 18:0 LPI, the most abundant LPI in cerebellum and in lung, after adding

exactly 150 pmol of standard to the matrix, we were able to detect, with this method, an increase of 164 ± 19 pmol and 141 ± 9 pmol in cerebellum and lung, respectively.

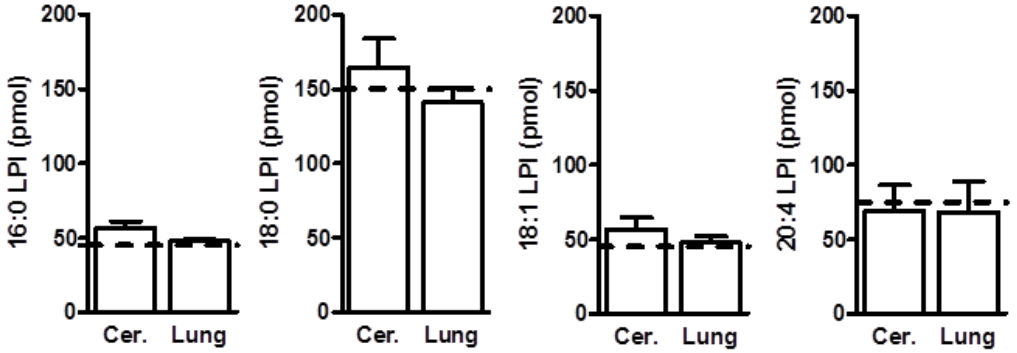

Figure 5. Evaluation of the matrix effect on the quantification of LPI. Cerebellum (cer.) or lung homogenates (15 mg of tissue) were either spiked with known amounts of 16:0 LPI, 18:0 LPI, 18:1 LPI and 20:4 LPI (dashed line) or analyzed as such (unspiked homogenates) using the method described in this paper. The amounts obtained for the unspiked samples were subtracted from the amount obtained in the spiked homogenates. The resulting data thus correspond to the detection of the added amount of LPI standard. Data are expressed as mean ± s.e.m.

3. Quantification of LPI species in mouse tissues

With this newly developed method, we decided to explore the relative abundance of the different LPI species in mouse tissues. Thus, we applied our validated HPLC–ESI–MS method to tissues from the central nervous system (cortex, hypothalamus, brain stem, cerebellum, spinal cord), from the gastrointestinal tract (jejunum, ileum, colon), as well as to other organs such as the liver, lung, or heart. As it is apparent from **Figure 6**, regardless of the analyzed tissue, the main LPI species were 16:0 LPI, 18:0 LPI, 18:1 LPI and 20:4 LPI. Depending on the tissue, were also present in various amounts 16:1 LPI, 18:2 LPI and 22:6 LPI, although at significantly lower levels than the four major LPI species. Our results also clearly show that while 18:0 LPI represents more than 60% of all the LPI species in the periphery, it is much less abundant in the central nervous system. Since the most abundant among the phosphatidylinositols is the 38:4 PI, giving 18:0 LPI and 20:4 LPI, these obtained results suggest that enzyme activities are different between the periphery and the central nervous system explaining the different ratio between LPI.

When taking into account all the analyzed tissues, we found that the abundance of 20:4 LPI is negatively correlated with the abundance of 18:0 LPI (spearman r value of -0.80, $p < 0.0003$).

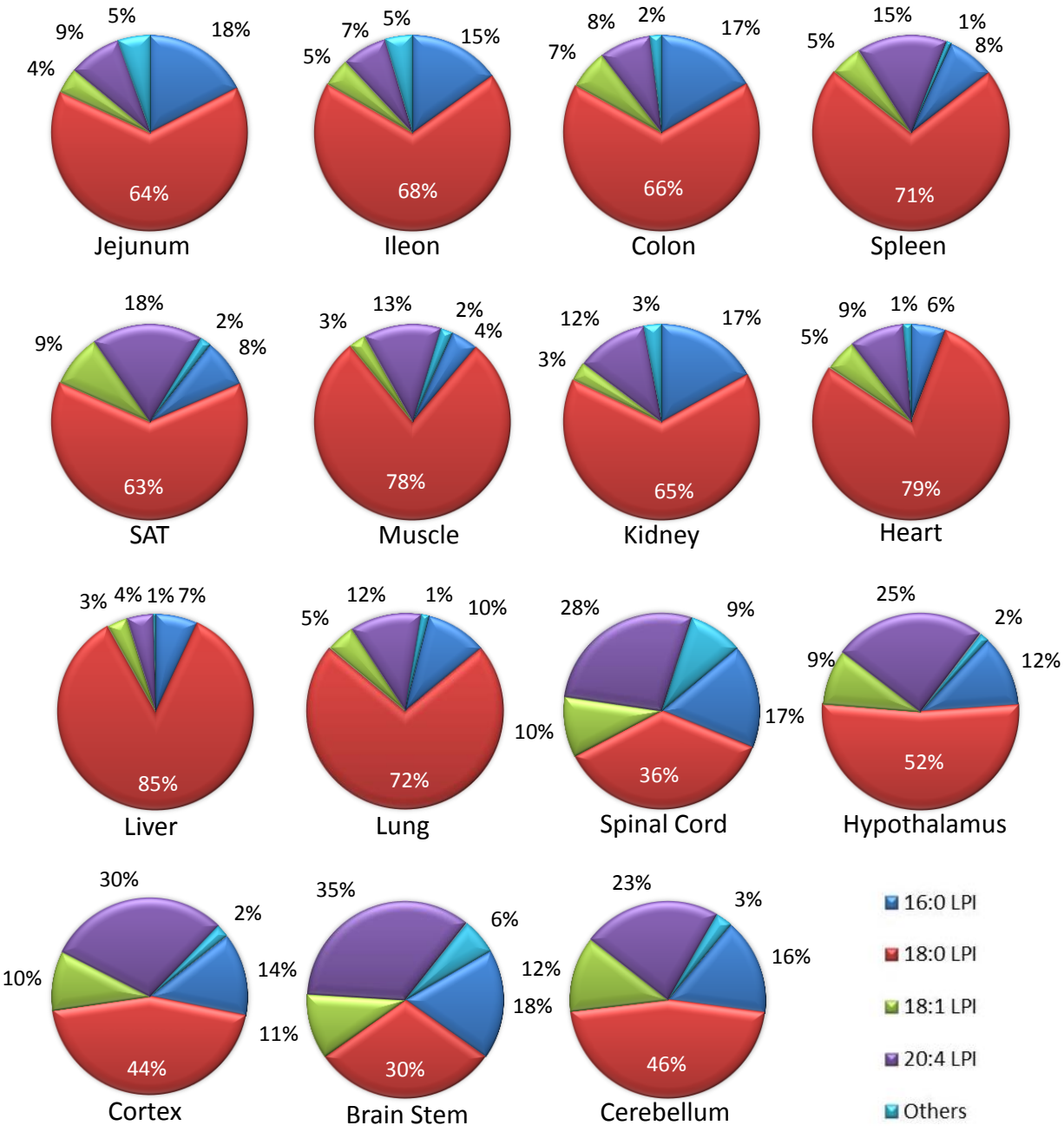

Figure 6. Relative levels of the LPI species in different mouse tissues. Each tissue (15 mg) was analyzed following the HPLC-ESI-MS method described in this paper. The resulting levels of each LPI species are presented as percentage of the total LPI species detected for each tissue. The four main LPI species are shown as individual species, while 18:2 LPI and 22:6 LPI are summed up in the "others". SAT : subcutaneous adipose tissue.

Furthermore, a hierarchical clustering analysis of all the tissues analyzed reveals that the CNS tissues are clustered together and are the less similar to the GI tract tissues (**Figure SI 3**).

Next we selected six tissues – colon, spleen, lung, liver, adipose tissue and cerebellum – in which we quantified the absolute amounts of the major LPI species. The results obtained are reported in **Table 4**. Of note we obtained a similar repartition in terms of fatty acid that what we observed in the previous experiment (**Figure 6**). As for the LPI levels in the brain, they are similar to what was reported previously.[14]

	16:0 LPI	18:0 LPI	18:1 LPI	20:4 LPI	Total
Colon	5.40 ± 1.1	23.84 ± 4.0	3.37 ± 0.4	4.31 ± 0.9	36.92 ± 6.5
Cerebellum	3.34 ± 0.5	9.86 ± 2.7	3.59 ± 0.4	5.56 ± 1.4	22.35 ± 4.9
Liver	4.59 ± 0.5	56.47 ± 6.0	3.67 ± 0.2	4.58 ± 0.4	69.31 ± 7.1
Lung	4.13 ± 0.5	11.84 ± 2.4	1.90 ± 0.2	4.83 ± 1.0	22.70 ± 4.2
Spleen	3.93 ± 0.7	24.78 ± 4.0	2.84 ± 0.5	8.20 ± 1.3	39.75 ± 6.4
SAT	4.32 ± 0.3	31.15 ± 0.5	5.06 ± 0.6	10.36 ± 1.2	50.89 ± 2.7

Table 4. Absolute levels of the four major LPI species in C57Bl/6J mice tissues (nmol/g of tissue). SAT : subcutaneous adipose tissue.

V. Conclusion

To conclude, the present study describes the first detailed validated method for quantification of lysophosphatidylinositols in biological matrix. This method has excellent specificity, selectivity, linearity, recovery, precision and accuracy, thus enabling the accurate measurement of LPI in biological matrix. We applied this method to the relative quantification of LPI species in numerous mouse tissues, and quantified the absolute levels of the four major LPI species in six tissues thus showing the versatility of the method. The validated HPLC-ESI-MS method described here should therefore become a very interesting tool for the study of this family of compounds which is acquiring increasing biological relevance.

VI. Acknowledgments

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Louvain) and from the FRS-FNRS, Belgium (grants CC 1.5.034.10, FRFC 2.4555.08, T.0148.14).

The MASSMET platform is acknowledged for the use of the mass spectrometer.

VII. Appendix A. Supplementary data

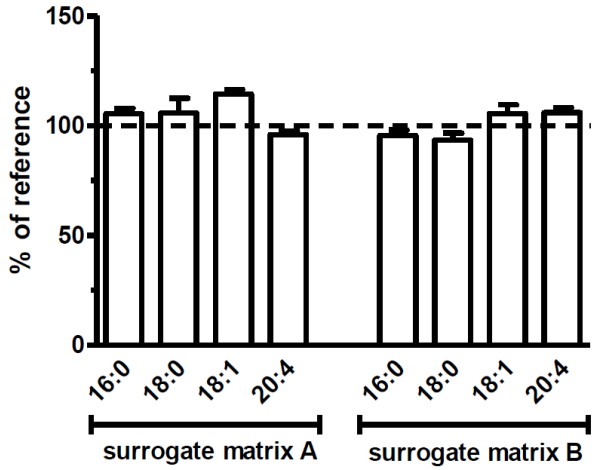

Figure SI 1. Influence of a surrogate matrix on the relative extraction of the studied LPI and of the internal standard (17:1 LPI). Two amounts of surrogate matrix (matrix A and matrix B) containing phospholipids, cholesterol, sphingomyelin and albumin (BSA) were spiked with 16:0 LPI, 18:0 LPI, 18:1 LPI, 20:4 LPI and 17:1 LPI before extraction and analysis by HPLC-MS. For each standard (16:0 LPI, 18:0 LPI, 18:1 LPI, 20:4 LPI) we determined the ratio between its AUC and the AUC of the internal standard (17:1 LPI) and found that it was not different from the ratio obtained when analyzing a reference solution containing the LPI (16:0 LPI, 18:0 LPI, 18:1 LPI, 20:4 LPI) and 17:1 LPI (dashed line, set at 100%).

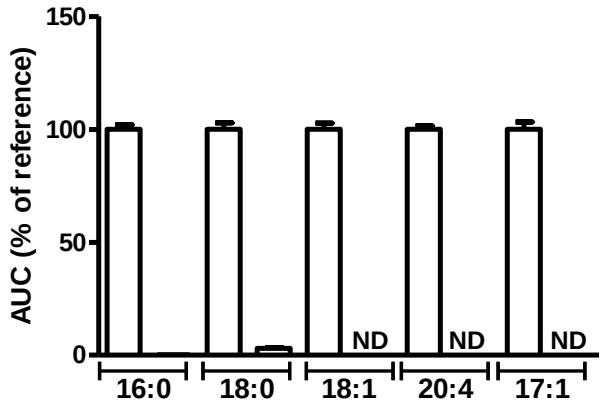

Figure SI 2. Study of carry over for LPI. We measured the residual signal in the blank injection following a standard injection. For each LPI species, the second column represents the signal detected in the blank following the standard injection (set at 100%). The amounts of LPI used are higher than the highest point of calibration curve. Data are expressed in mean + SEM. ND : non detected

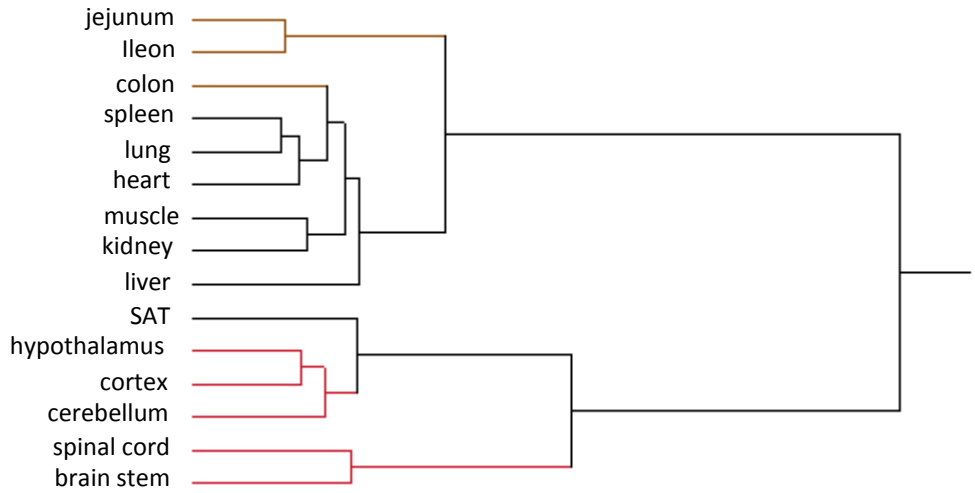

Figure SI 3. Hierarchical cluster analysis of the analyzed tissues. Dendrogram tree diagram representing the hierarchical clustering analysis of the different tissues studied here based on the proportion of the different LPI species. The CNS tissues are shown in red, while the gastrointestinal tract tissues are shown in brown.

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Part 3. Lysophosphatidylinositols in inflammation and macrophage activation: altered levels and anti-inflammatory effects

Part 3. Lysophosphatidylinositols in inflammation and macrophage activation: altered levels and anti-inflammatory effects

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I. Abstract

Lysophosphatidylinositols (LPI) are among the least studied lysoglycerophospholipids, although their first effects were described decades ago. Owing to the relatively recent deorphanization of the GPCR GPR55 as an important molecular target of LPI, these bioactive lipids were put forward with studies on their role in cell proliferation and bone physiology. However, very little is known on the role of LPI or GPR55 in inflammation, therefore we studied the impact of macrophage activation and inflammation on LPI levels. In this study, we show that macrophage activation with lipopolysaccharides (LPS) affects the levels of LPI, but not those of other lysoglycerophospholipids. We also show that LPI decrease macrophage activation in a GPR55-dependent manner, thus highlighting a potential role for LPI in inflammation. Moreover, systemic inflammation induced by LPS in mice also leads to alterations in LPI levels in the lung, liver, spleen and colon. These alterations were different depending on the tissue considered. Furthermore, we observed that LPI levels were altered in two murine models of inflammatory bowel diseases, similarly to what we observed in systemic inflammation. The data presented here point to LPI as a potential player in the pathogenesis of inflammation in general, and colitis in particular.

II. Introduction

Lysoglycerophospholipids are glycerophospholipids in which the glycerol moiety is substituted by a single acyl chain. They are constituted of a constant "polar" moiety linked to the glycerol backbone (defining the family) and, within each family, of a variable acyl chain, on the *sn*2 or *sn*1 position of the glycerol. These lipids, once considered as simple intermediates in the metabolism of phospholipids, are now recognized as bioactive lipids. The most studied lysoglycerophospholipid is certainly lysophosphatidic acid (LPA) which has been implicated in cell proliferation and survival, cancer, inflammation, pain, and the metabolic syndrome.[1,2] Lysophosphatidylcholines (LPC), the most abundant lysoglycerophospholipids, seem to play a role both in the initiation and resolution of inflammation, possibly through a G protein-coupled receptor (GPCR), GPR132.[2,3] Lysophosphatidylserine (LysoPS) production by activated and apoptotic neutrophils is a signal leading to their phagocytosis by macrophages, thus triggering resolution of inflammation.[4] However, much less is known on the properties of other lysoglycerophospholipids such as the lysophosphatidylinositols (LPI) which remain to date the less studied lysoglycerophospholipids. Early studies reported their effects on insulin secretion and on the cell cycle.[5-8] More recently, the GPCR GPR55 was put forth as an important molecular target mediating LPI's effects.[9-11] Identification of this GPCR as the receptor for LPI helped better understand the effects of LPI on cell proliferation, as GPR55 silencing blocks the LPI-stimulating effect on cell growth.[12] Another example of GPR55-mediated effects of LPI is found in bone physiology as LPI affect osteoclast formation through GPR55 activation.[13] As for numerous bioactive lipids, the affinity and efficacy of LPI at the GPR55 receptor differ depending on the acyl chain, with 2-arachidonoyl-sn-glycero-3-phosphoinositol (2-arachidonoyl LPI) having the highest potency.[9]

Regarding the biosynthesis of LPI (see Introduction, Part 3), the key precursors are phosphatidylinositols (PI) which, upon hydrolysis by a phospholipase A1 (PLA1) or a phospholipase A2 (PLA2), will generate 2-acyl LPI and 1-acyl LPI, respectively. Among the PLA1 enzymes, DDHD1 (also known as PA-PLA1) was described for its ability to produce 2-arachidonoyl LPI in vitro and in vivo.[14] PLA2 enzymes are expected to generate LPI, however PLA2 isoforms have been mostly studied in regards to arachidonic acid and eicosanoid synthesis. Among these, cPLA2α is implicated in the biosynthesis of LPI.[15,16] However, while

activation of cPLA2 and the release of arachidonic acid from membrane phospholipids constitute key features of inflammation, little, if any, is known on the effect of inflammation on the cell and tissue levels of LPI.

Complicating the study of the effects of LPI is the intricacy of their degradation. LPI can be reacylated to PI species by acyltransferases.[17,18] They can also be deacylated by lysophospholipases. LPI-specific PLA and PLC activities have been reported although the molecular characterization of these specific lysoPLA and lysoPLC enzymes is still lacking.[17] Of note, cPLA2α has been reported as capable of deacylating LPI into glycerophosphoinositol[19], therefore it could be implicated both in the synthesis and degradation of LPI. Finally, autotaxin, a lysoPLD responsible for LPA biosynthesis, converts LPI to LPA. And more recently, α/β hydrolase domain 6 (ABHD6), a monoacylglycerol hydrolase, was also implicated in lysoglycerophospholipids hydrolysis.[20]

Considering the complexity of the metabolic pathways of LPI and the fact that inflammation can affect several enzymes of these pathways, we decided to study the interplay between LPI and inflammation. Thus, we used an in vitro approach to study the consequences of macrophage activation on LPI levels, as well as the effect of LPI and GPR55 expression on macrophage activation. We then show, in vivo, that inflammation alters the levels of LPI in numerous tissues. Thus this report points to LPI as interesting bioactive lipids in the context of inflammation.

III. Materials and Methods

1. Materials

All lysoglycerophospholipids (LPI, LPE, LPC, LPA, LPG and LysoPS), as well as the internal standards 17:1-LPI and 17:0-LPC, were purchased from Avanti Polar lipids. Lipopolysaccharides (LPS) from E. coli (O55:B5) was purchased from Sigma Aldrich (Bornem, Belgium). Dextran sulphate sodium (DSS) was purchased from TdB Consultancy (Uppsala, Sweden). pDream2.1 vector containing the human GPR55 coding sequence was obtained from GenScript corporation. Lipofectamine LTX plus was purchased from Life Technologies.

2. Animal models

C57BL/6J mice (8-10 weeks of age) were purchased from Charles River Laboratories (Brussels, Belgium) and were housed under standard conditions (12hours light/dark cycle, and controlled 22°C temperature) and supplied with water and food *ad libitum*. For LPS-induced inflammation in mice, LPS (300 µg/kg, in saline containing 0.1% Tween 80) or vehicle were administered i.p and mice were sacrificed by cervical dislocation 4 or 8 hours later (7 mice/group).[21] Two models of colitis were used. *TNBS-induced colitis:* Food (but not water) was withdrawn 24 hours before administration of trinitrobenzene sulfonic acid (TNBS). Mice (10/group) were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (100mg/kg) and xylazine (10mg/kg). TNBS (100mg/kg, in 0.9% NaCl-ethanol, 50:50, v/v) was administered intrarectally (50 µl) into the colon using a cannula inserted 4 cm from the anus. Control mice received 50 µl of a 0.9% NaCl-ethanol (50:50, v/v) solution. To ensure a homogeneous distribution and retention of TNBS (or vehicle) within the colon, mice were held by the tail, in a vertical position, for 1 min after administration.[22] *DSS-induced colitis:* Colitis was induced by adding dextran sulfate sodium (DSS, 5%) to the drinking water of mice for 5 days. Mice (10 mice/group) were sacrificed once the colitis was well established i.e. at day 7.[22] Following sacrifice of the mice, tissues were rapidly recovered and snap-frozen in liquid nitrogen before storage at -80°C until their analysis. All animal care and experimental procedures were conducted in accordance with the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (agreement number LA1230314) and were approved by the local ethics committee (2014/UCL/MD/001).

3. Cell Culture

The murine macrophage cell line J774 (a generous gift from M-P. Mingeot, LDRI, UCL, Belgium) was cultured under standard conditions (37°C in a humidified 5% CO2 incubator) in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. BV2 microglial-like cells (a generous gift from E. Hermans, IONS, UCL, Belgium) were grown in high-glucose DMEM medium with 10% FBS and antibiotics.

Cells were seeded overnight into 24-well plates ($2.5x10^5$ cells per well) for qRT-PCR experiments, and into 35mm dishes ($10x10^6$ cells per dish) for lysoglycerophospholipid quantification, and the experiments performed the next morning. Cells were then incubated

with fresh culture medium containing 100ng/mL of LPS for 8 hours (based on previous experiments.[21] In all experiments, the compounds of interest were added 1 hour prior to LPS. For all experiments, a control condition was performed, where cells were seeded concomitantly but were only incubated with vehicle (DMSO, 0.2%) in the absence of LPS.

4. Primary Peritoneal Macrophage Isolation

Murine peritoneal macrophages were obtained by eliciting an acute peripheral inflammatory reaction with an i.p. injection of thioglycolate as previously described.[21] Briefly, adult NMRI female mice were given an i.p. injection of an aged 4% thioglycolate solution. Three days later, peritoneal cells were recovered in 5 mL PBS, pelleted and resuspended in culture medium (RPMI supplemented with 10% FBS and antibiotics). Three hours after the initial plating, cells were washed with PBS to remove non-adherent cells, growth medium was replenished and cells were seeded overnight. Inflammatory mediator induction experiments were then performed as described.

5. Real-time quantitative PCR

For real-time quantitative PCR (qRT-PCR) experiments from cells, media was removed and TriPure reagent (Roche, Basel, Switzerland) was added to each well of the tissue culture plates, the plates were then stored at -80°C for later assessment. For in vivo experiments, tissue were snap frozen in liquid nitrogen at the time of sacrifice and stored at -80°C for later assessment. TriPure reagent was added on the day RNA extraction was performed. In both cases, the same procedure was performed. Total RNA was extracted using the TriPure reagent according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Promega corporation) from 1 µg of total RNA. qPCR was performed with a STEP one PLUS instrument and software (Applied Biosystems) as previously described.[23] Products were analyzed by performing a melting curve at the end of the PCR reaction. Data are normalized to the 60S ribosomal protein L19 (RPL19) mRNA expression. Primer sequences are given in **Table 1**.

Product	Forward primer (5' to 3')	Reverse primer (5' to 3')
RPL19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT
IL-1β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
COX-2	TGACCCCCAAGGCTCAAATAT	TGAACCCAGGTCCTCGCTTA
IL-6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
MCP-1	GCAGTTAACGCCCCACTCA	CCCAGCCTACTCATTGGGATCA
ABHD6	CTGTCCATAGTGGGGCAAGT	TCAGATGGGTAGTAAGCGGC
AGPAT8	TGGCTTTACCTTTGTGGTGGA	TTGGATGTGGGAAGAGAGTCAG
Autotaxin	AGCACCTGACCAACTGTGT	TGCATCATATTTCGCTTCTGGG
MBOAT7	CGCACCTTTCCGCTCCTAC	TGACTCCAAATAGCCCTCAGCA
PA-PLA1	TAACATTGGCAAAGCAAGCA	TGATGCTGAAGAACGTCCAA
cPLA2α	GTGAGGGGCTTTATTCCACA	ACAGTGCCTTCATCACACCA
mGPR55[a]	CATCATGGGCTTCTGTTCCT	TCCAGATGCAGGCTCTCTTT
hGPR55[b]	TCTACATGATCAACCTGGCAGTCT	CTGGGACAGGACCATCTTGAA

[a] **murine GPR55;** [b] **human GPR55**

Table 1. Primer Sequences

6. Transfection of J774 cells with hGPR55

J774 cells were seeded overnight in 24-well plates ($1.5x10^5$ cells per well) and stable transfection performed the following day using lipofectamine LTX plus and 0.5µg of pDream2.1 plasmid containing the coding sequence for human GPR55. Transfected cells were selected by addition of G418 to the culture medium, then a clonal selection was performed by serial dilution of the initial cell population and several clones with various degrees of hGPR55 mRNA expression obtained. The E1 clone was used for the experiments performed here.

7. Lysoglycerophospholipid quantification

Cells and media were recovered (7mL) and the lipids extracted, in the presence of internal standards (17:1-LPI and 17:0-LPC), by adding 14 ml of chloroform, 7 ml of methanol and 300 µL of HCl 2N. Following vigorous mixing and sonication, the samples were centrifuged and

the organic layer was recovered and then dried under a stream of N_2. For the analysis of LPE, LPG, LPI, LysoPS and LPC, the lipid fraction was pre-purified by solid-phase extraction (SPE) over silica using methanol as the eluting solvent. The resulting lipid fractions were analyzed by HPLC-MS using a LTQ Orbitrap mass spectrometer (ThermoFisher Scientific) coupled to an Accela HPLC system (ThermoFisher Scientific) equipped with an electrospray ionization (ESI) source and operated using the Xcalibur software (Thermo Fisher Scientific). Chromatographic separations were performed using a C18 column (Kinetex, 150 mm x 4,6 mm, 5µm, from Phenomenex). For all lysophospholipids (except LPC), the gradient elution (400 µL/min) consisted of solvent A (MeOH-H_2O-ammonium hydroxide (50:49,9:0,1 v/v/v)) and solvent B (MeOH-ammonium hydroxide (99,9:0,1 v/v)), starting at 100% A and reaching linearly 100% B in 30 min., this was followed by 15 min. at 100% B before reequilibrating at 100% A. For LPC analysis the gradient elution (250 µL/min) consisted of solvent A (MeOH-H_2O-ammonium acetate (85:14,9:0,1 v/v/v)) and solvent B (MeOH-ammonium acetate (99,9:0,1 v/v)), starting at 100% A, for 2 minutes, followed by a linear gradient to 100% B in 13 min., with a final 45 min at 100% B before reequilibration at 100% A. LPE, LPG, LPI and LysoPS were analyzed in the negative mode, while LPC were analyzed in the positive mode. For both modes, the ESI spray voltage was set at 5000V and the capillary temperature at 270°C. The sheath gaz flow was 40 arbitrary units.

8. Statistical Analysis

Results are expressed as mean ± SEM. All experiments were conducted at least 3 times in triplicates. Significance between two distinct groups was determined by unpaired Student t test and comparisons between various groups were assessed by one-way anova followed by a Dunett post-test for comparisons against a “control” condition or by a Bonferroni post-test for comparisons between different conditions. Data were analyzed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

IV. Results

1. LPI levels, but not the levels of the other lysoglycerophospholipids, are altered upon J774 macrophage activation

Upon incubation with lipopolysaccharides (LPS, 100ng/mL, 8h) macrophages become activated and express pro-inflammatory markers such as interleukin (IL)-6 and IL-1β (**Figure SI 1**).[21,24] We used this model to investigate the effect of LPS-induced J774 macrophage activation on lysoglycerophospholipid levels. Among the lysophospholipids investigated here, we found that the levels of LPI were significantly modified, while LPE, LPG, LysoPS and LPC levels were not affected (**Figure 1**). Interestingly, cell activation has a different effect depending on the LPI species, as 20:4 LPI levels were decreased whereas 16:0, 18:0, 18:1 and 18:2 LPI levels were increased.

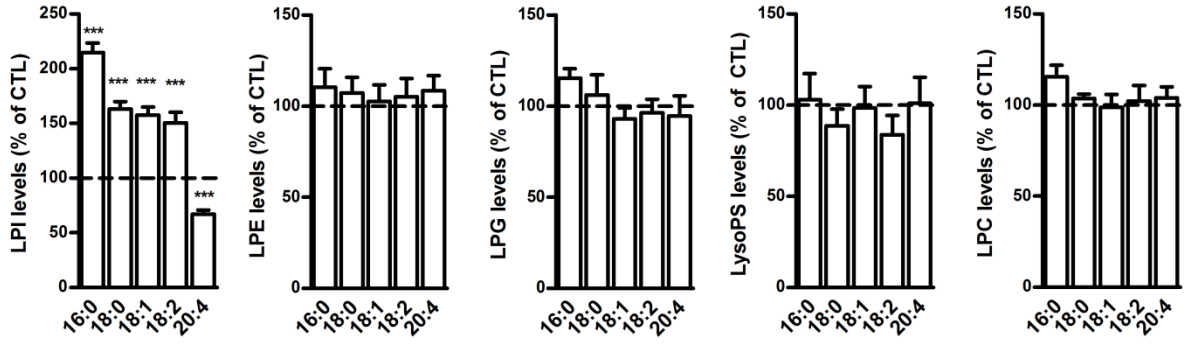

Figure 1. Effect of activation of J774 macrophages on lysophospholipid levels. Lysophospholipid levels were measured by HPLC-MS following activation of J774 cells with 100ng/mL of lipopolysaccharides (LPS) for 8h. LPI: lysophosphatidylinositols, LPE: lysophosphatidylethanolamines, LPG: lysophosphatidylglycerols, LysoPS: lysophosphatidylserines, LPC: lysophosphatidylcholines. The length of the acyl chain and the degree of insaturation of the individual species are shown on the x axis. Data are expressed for each individual species in percentage of the control condition (cells not incubated with LPS, dotted line). Values are mean ± SEM. ***P<0.001 for LPS activation vs. control cells.

2. PI levels are altered upon activation of J774 macrophage while FA levels are not affected

Since LPI levels are differently affected after cell activation, we tried to show a link between LPI levels and PI as well as FA levels variations in J774. We found that the levels of PI were significantly modified. Actually two groups of PI species can be distinguished. The first group is composed by PI that cannot be metabolized into arachidonic acid, and the second one is

composed of PI species with at least 4 insaturations. Interestingly, PI levels with 4 and more insaturations (36:4 PI, 36:5 PI, 38:4 PI, 38:5 PI and 38:6 PI) were decreased (**Figure 2A**) while in the same time, PI levels with not more than 3 insaturations were stable or increased (**Figure 2B**). We also tried to determine whether fatty acids levels and arachidonic acid levels in particular could be altered, but in these conditions, we observed no variations, possibly due to the metabolism in other compounds such as prostaglandins (**Figure 2C**).

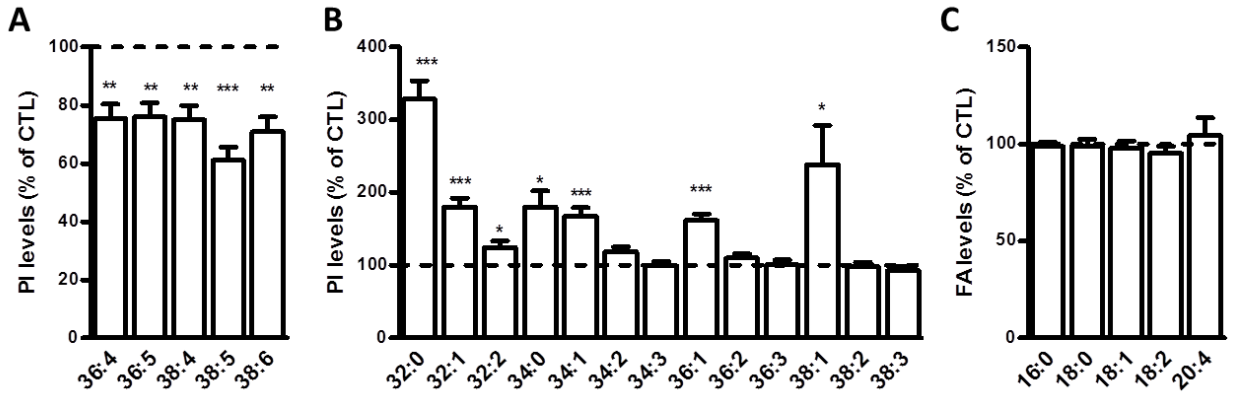

Figure 2. Effect of activation of J774 macrophages on PI and FA levels. Phosphatidylinositol (A-B) and fatty acids (C) levels were measured by HPLC-MS following activation of J774 cells with 100ng/mL of lipopolysaccharides (LPS) for 8h. PI: phosphatidylinositols, FA: fatty acids. The length of the acyl chain(s) and the degree of insaturation of the individual species are shown on the x axis. Data are expressed for each individual species in percentage of the control condition (cells not incubated with LPS, dotted line). Values are mean ± SEM. ***P<0.001, **P<0.01 , *P<0.05 for LPS activation vs. control cells.

3. LPI levels are altered upon activation of BV2 microglial cells and primary peritoneal macrophages

To determine whether alteration of LPI levels could be considered as a common trait of macrophage activation we used, in addition to J774 cells, the microglial BV2 cell line as well as thioglycolate-elicited peritoneal macrophages (TEM). Similarly to J774, LPS incubation induces a strong increase in IL-1β and IL-6 mRNA expression in both BV2 cells and TEM (**Figure SI 1**). LPS activation of BV2 cells induces changes in LPI levels similar to those observed in J774 cells, with a decrease in 20:4 LPI and increased levels of 16:0, 18:0 and 18:1 LPI (**Figure 3A**). The picture is somewhat different in TEM as 20:4 LPI levels are not decreased contrary to what is found in J774 and BV2 cells. Actually, LPI levels are globally

increased upon TEM activation, although only 16:0 and 18:1 reach statistical significance (**Figure 3B**).

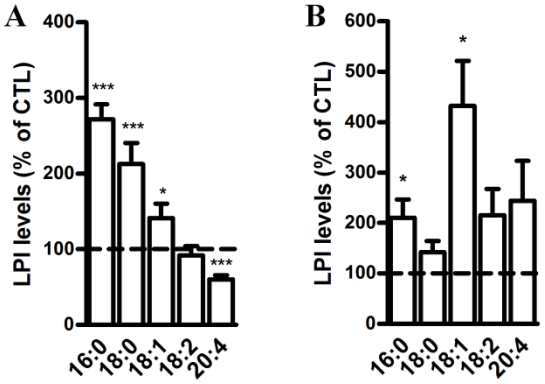

Figure 3. Effect of activation of BV2 cells and primary peritoneal macrophages on LPI levels. Lysophosphatidylinositol levels were measured by HPLC-MS following activation of (**A**) BV2 microglial-like cells and (**B**) thioglycolate-elicited peritoneal macrophages with 100ng/mL of lipopolysaccharides (LPS) for 8h. LPI: lysophosphatidylinositols. The length of the acyl chain and the degree of insaturation of the individual species are shown on the x axis. Data are expressed for each individual species in percentage of the control condition (cells not incubated with LPS, dotted line). Values are mean ± SEM. **P*<0.05, ****P*<0.001 for LPS activation vs. control cells.

4. Determination of the expression profiles of enzymes implicated in LPI metabolism

In order to generate hypotheses on the mechanisms responsible for the changes in LPI levels, we determined the effect of LPS activation on mRNA expression of the enzymes involved in LPI metabolism. These enzymes include cPLA2, PA-PLA1, ABHD6, Autotaxin and the acyltransferases AGPAT8 and MBOAT7.[14,15,20] Activation of J774 cells with LPS results in decreased cPLA2 and PA-PLA1 mRNA expression, with no variation in the expression of ABHD6, AGPAT8 and MBOAT7, while Autotaxin was not expressed by J774 cells. Conversely, in BV2 cells, LPS incubation increased the expression of the two phospholipases (**Figure 4A-B**) as well as the expression of Autotaxin, which was not expressed in the control condition. In TEM it appears that the expression of cPLA2 and PA-PLA1 is differentially affected by LPS-induced cell activation, as the expression of the former is strongly induced while PA-PLA1 expression is reduced by 50% (**Figure 4C**). Moreover, contrary to the two cell lines in culture,

ABHD6 and MBOAT7 expression is also altered in TEM, with the former increasing and the latter decreasing.

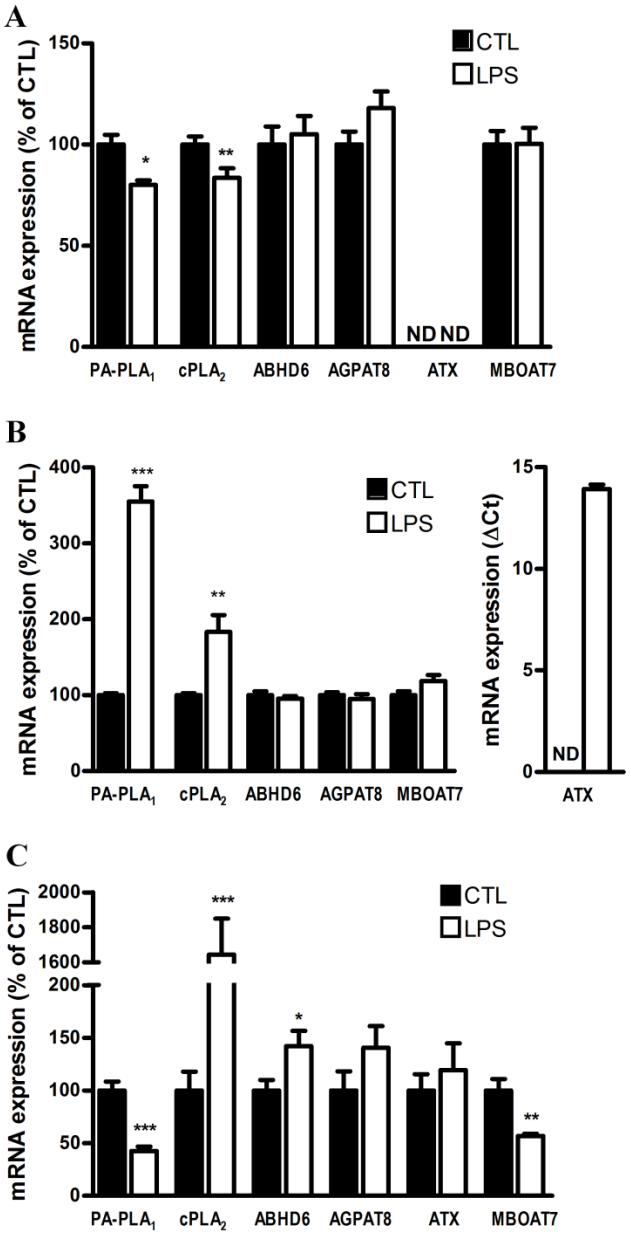

Figure 4. Effect of lipopolysaccharides-induced activation of macrophages on the metabolic pathways of LPI. mRNA expression of PA-PLA1, cPLA2, ABHD6, AGPAT8, Autotaxin (ATX) and MBOAT7 was measured by RT-qPCR in (**A**) J774 cells, (**B**) BV2 cells and (**C)** thioglycolate-elicited peritoneal macrophages activated by incubation with 100ng/mL of lipopolysaccharide (LPS) for 8h. RPL19 was used as housekeeping gene. Data are expressed in percentage of the control condition with the exception of ATX in (B) which is expressed in comparison to the housekeeping gene due to the lack of expression in the control condition. Values are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 for LPS activation vs. control cells.

5. LPI decrease LPS-induced macrophage activation

Bioactive lipids could serve as autocrine signaling molecules regulating macrophage activation.[21,25,26] Thus we asked what would be the effect of the different lysoglycerophospholipids studied here on LPS-induced J774 cells activation. LPI decreased several parameters of macrophage activation, such as LPS-induced mRNA expression of IL-1β, IL-6 and COX-2 (**Figure 5**). LPA significantly enhanced LPS-induced IL-1β mRNA expression, without affecting IL-6 or COX-2 expression. LPC decreased IL-1β and IL-6 expression, while LysoPS only decreased IL-6 expression (**Figure 5**). LPE and LPG had no effect on LPS-induced activation of J774 cells (**Figure 5**).

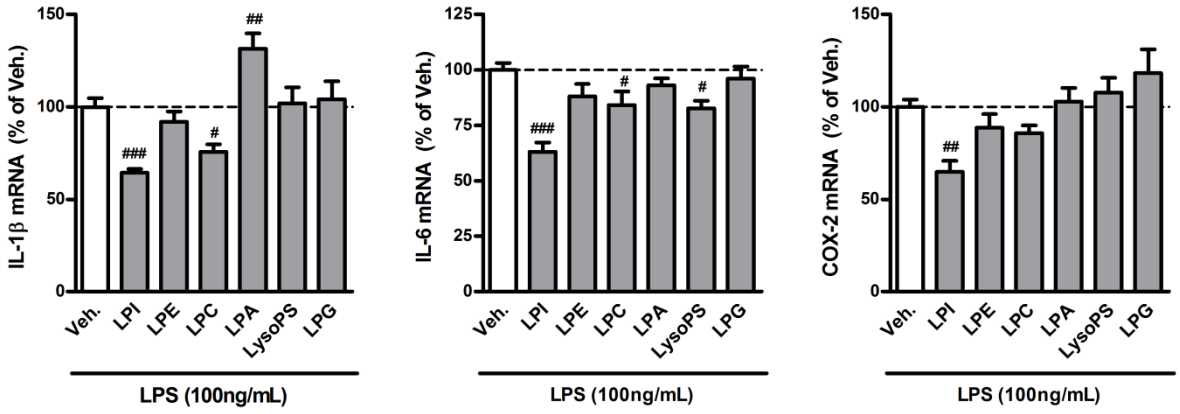

Figure 5. Effect of lysophospholipids on lipopolysaccharides-induced activation of J774 cells. J774 cells were activated with 100ng/mL of lipopolysaccharides (LPS) for 8h. Lysophospholipids were added at 10µM one hour prior to incubation of cells with LPS. mRNA expression of pro-inflammatory markers (IL-1β, IL-6, COX-2) was measured by RT-qPCR with RPL19 as housekeeping gene. Data are expressed in percentage of the LPS-induced mRNA expression of each marker (Veh.). Values are mean ± SEM. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 for lysophospholipid treatment vs. Veh. LPI: lysophosphatidylinositols, LPE: lysophosphatidylethanolamines, LPG: lysophosphatidylglycerols, LysoPS: lysophosphatidylserines, LPC: lysophosphatidylcholines.

6. LPI actions on LPS-induced macrophage activation are mediated by GPR55

LPI bind and activate GPR55 receptors, however, although some reports show that GPR55 is involved in the effects of LPI on cancer cell proliferation or osteoclasts formation, little is known on the involvement of GPR55 in inflammation and macrophage activation.[17,27,28] Thus we asked whether the effect of LPI on LPS-induced macrophage activation was mediated by this receptor. First we noted that the magnitude of effect of LPI on macrophage activation was negatively correlated with the expression of GPR55 mRNA (**Figure 6A**). Indeed, we had

different populations of J774 cells and we noticed that in the cells where ΔCt of GPR55 (compared to the housekeeping gene RPL19) was higher, i.e. where GPR55 expression was lower, LPI had less effect on LPS-induced expression of IL-1β (**Figure 6A**). Second, to clearly establish the role of GPR55, we selected J774 cells showing low GPR55 expression and little effect of LPI in reducing LPS-induced macrophage activation (**Figure 6B-C**, WT columns). Using these cells we then generated, by stable transfection, clones of J774 cells expressing human GPR55 (hGPR55) and tested the effect of LPI. hGPR55 mRNA was well expressed in transfected J774 cells and at a more constant level compared to murine GPR55 (**Figure SI 2**). As illustrated in **Figure 6B-C**, LPI reduce LPS-induced IL-1β and IL-6 mRNA expression in J774-hGPR55 clone E1 but not in J774 WT cells.

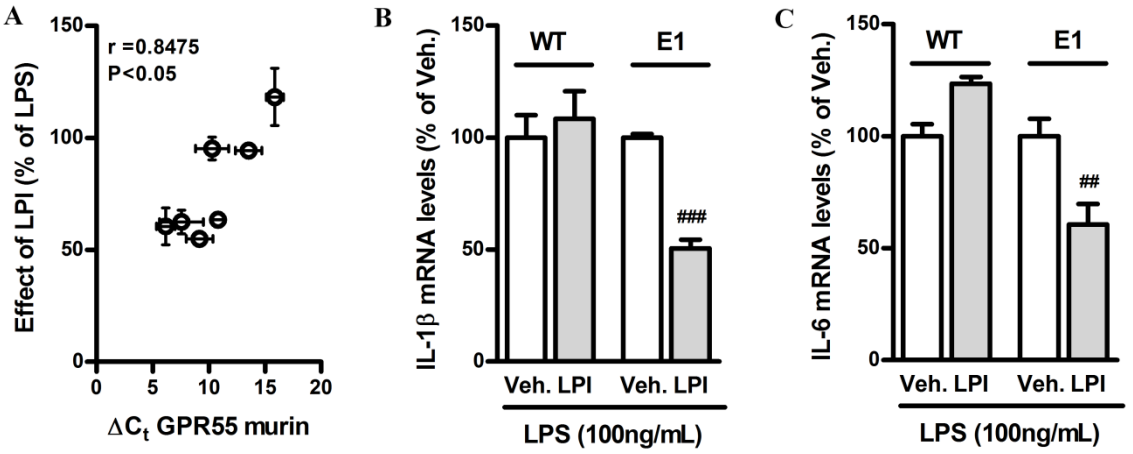

Figure 6. Impact of GPR55 expression on the effects of LPI. J774 cells were activated with 100ng/mL of lipopolysaccharides (LPS) for 8h. Lysophosphatidylinositols (LPI) were added at 10μM one hour prior to incubation of cells with LPS. mRNA expression of pro-inflammatory cytokines (IL-1β and IL-6) was measured by RT-qPCR with RPL19 as housekeeping gene. Data are expressed in percentage of the LPS-induced mRNA expression of each marker (Veh.). (**A**) Correlation between the expression of GPR55 (ΔCt) in different populations of J774 cells and the effect of LPI on LPS-induced IL-1β expression. (**B**,**C**) Effect of LPI on LPS-induced (**B**) IL-1β and (**C**) IL-6 mRNA expression in wild-type J774 cells with low GPR55 expression (WT) and the same population transfected with human GPR55 (E1). Values are mean ± SEM. For B and C, ##*P*<0.01, ###*P*<0.001 for LPI vs Veh.

7. LPI tissue levels are altered by inflammation

Next we wondered whether inflammation in vivo would alter LPI levels. Thus we used a model of LPS-induced inflammation in mice obtained by i.p. injection of a low dose of LPS (300μg/kg) for 4 or 8h.[21] This dose of LPS induces a time-dependent increase in the expression of inflammatory markers in many tissues. Indeed, IL-1β and monocyte

chemoattractant protein-1 (MCP-1) mRNA expression were increased in the colon, liver, spleen, lung and cerebellum at 4h and 8h post-LPS injection (**Figure 7-9**). Of note, their expression was lower in the 8h group compared to the 4h group, although still higher than the control group, in line with a progressive spontaneous resolution of inflammation.

In two tissues, the lung and the cerebellum, LPI levels were generally not affected by inflammation either after 4h or 8h of LPS administration (**Figure 7C-D**), with the exception of 18:2 LPI which is increased in the lung. Note that we did not detect 18:2 LPI in the brain similarly to a previous report by Oka et al.[9] Conversely, all the measured LPI were strongly increased by inflammation in the liver and spleen (**Figure 7C-D**). However this increase was only sustained at 8h in the liver (**Figure 7C**) and proved transient in the spleen, with a return to baseline at 8h (**Figure 7D**). The picture was different in the colon where we found a significant decrease in 16:0, 18:0, 18:2 and 20:4 LPI levels already 4h post LPS injection, and this decrease was sustained at 8h (**Figure 9B**).

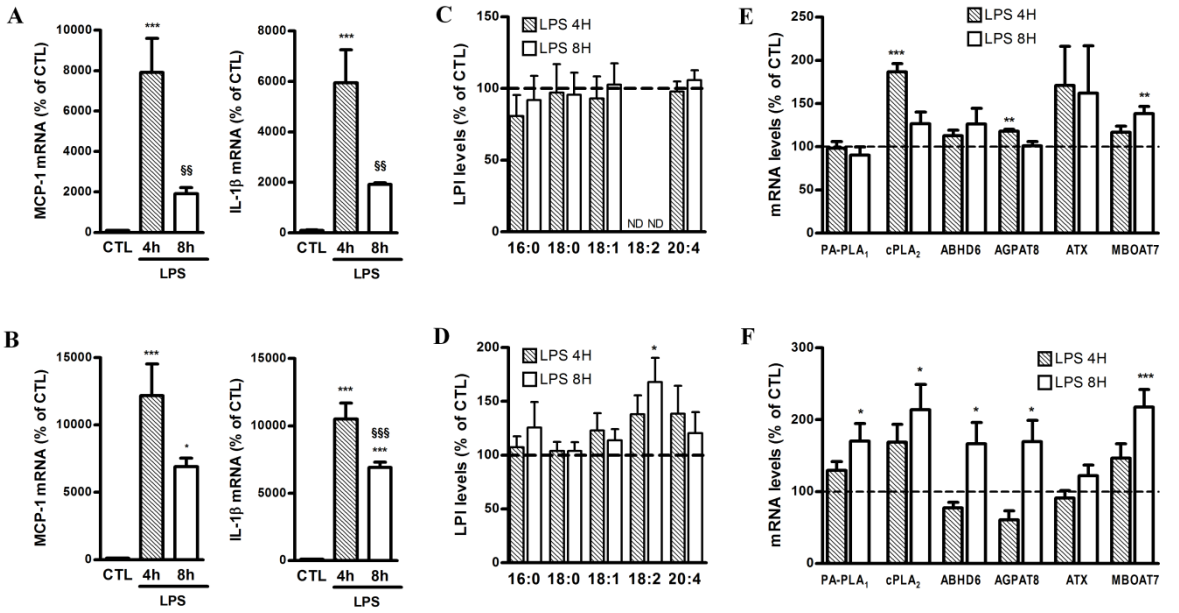

Figure 7. Impact of lipopolysaccharides-induced inflammation on LPI levels and their metabolic pathways in the cerebellum and lung. Systemic inflammation in mice was induced by intraperitoneal administration of 300µg/kg of lipopolysaccharides (LPS) for for 4h and 8h. (**A**,**B**) mRNA expression of pro-inflammatory markers IL-1β and MCP-1 measured by RT-qPCR in (**A**) the cerebellum and (**B**) the lung with RPL19 as housekeeping gene. (**C**,**D**) Lysophosphatidylinositol (LPI) levels measured by HPLC-MS in (**C**) the cerebellum and (**D**) the lung. The length of the acyl chain and the degree of insaturation of the individual species are shown on the x axis. (**E**,**F**) mRNA expression of cPLA2, PA-PLA1, ABHD6, AGPAT8, Autotaxin (ATX) and MBOAT7 measured by RT-qPCR in (**E**) the cerebellum and (**F**) the lung with RPL19 as housekeeping gene. Data are expressed in percentage of the control condition (dotted line in **C**-**F**). Values are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 for mice receiving LPS vs. control mice. $$*P*<0.01, $$$*P*<0.001 for 8 h of LPS vs. 4 h of LPS.

8. LPS-induced inflammation alters the expression of enzymes involved in the metabolism of LPI

Because LPI levels were affected by inflammation in some tissues, we assessed mRNA expression of several enzymes implicated in the metabolism of lysoglycerophospholipids in general and LPI in particular. Similarly to what was done for the cells, cPLA2, PA-PLA1, ABHD6, AGPAT8, autotaxin and MBOAT7 were studied. Changes in mRNA expression of these enzymes in inflammatory settings were quite different depending on the tissue considered. In the cerebellum where LPI levels were not affected, a transient increase of cPLA2 at 4h was observed, accompanied by an increase in the acyltransferases AGPAT8 and MBOAT7 (**Figure 6E**). In the lung, there were no significant differences in enzyme expression at 4h, but all the enzymes, except for autotaxin, were increased at 8h (**Figure 6F**). A similar increase was observed for $cPLA_2$, PA-PLA1, ABHD6 and AGPAT8 in the colon at 8h, while the expression of autotaxin was decreased at both time points (**Figure 9C**). In the liver, expression of PA-PLA_1 and $cPLA_2$ exhibited a trend to increase, which only reached significance for $cPLA_2$ at 8h. Conversely, mRNA expression of ABHD6 and AGPAT8 were decreased in this tissue (**Figure 8E**). In the spleen, PA-PLA1 mRNA expression was increased and AGPAT8 expression was decreased at both time points, while MBOAT7 was increased at 8h (**Figure 8F**).

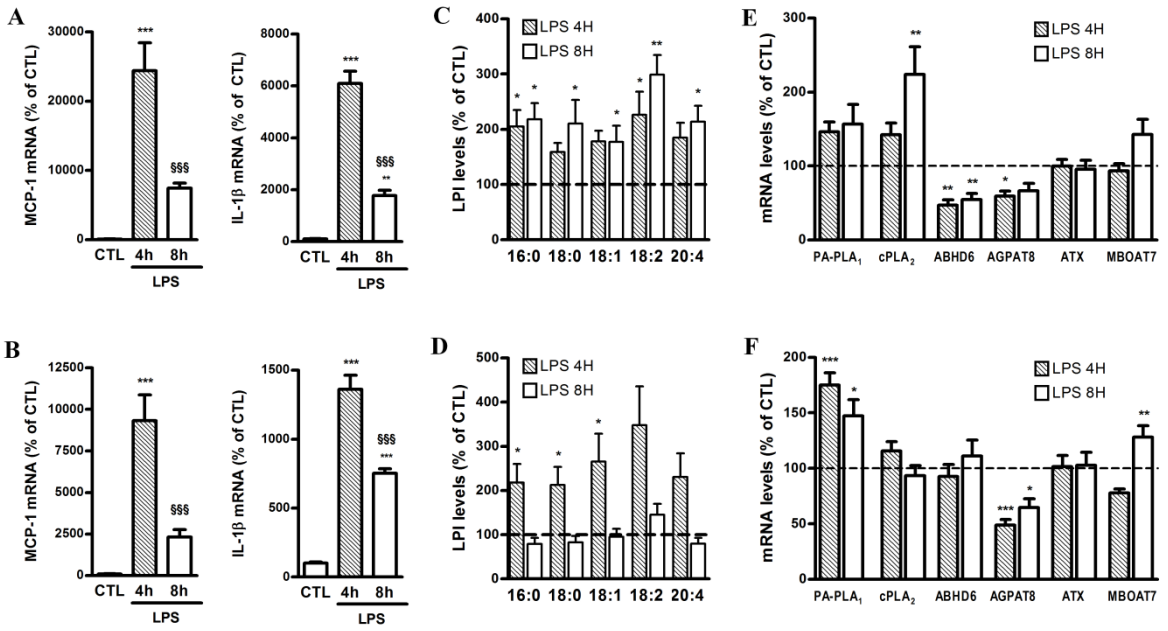

Figure 8. Impact of lipopolysaccharides-induced inflammation on LPI levels and their metabolic pathways in the liver and spleen. (**A**-**C**) Systemic inflammation in mice was induced by intraperitoneal administration of 300µg/kg of lipopolysaccharides (LPS) for 4h and 8h. (**A**,**B**) mRNA expression of pro-inflammatory markers IL-1β and MCP-1 measured by RT-qPCR in (**A**) the liver and (**B**) the spleen with RPL19 as housekeeping gene. (**C**,**D**) Lysophosphatidylinositol (LPI) levels measured by HPLC-MS in (**C**) the liver and (**D**) the spleen. The length of the acyl chain and the degree of insaturation of the individual species are shown on the x axis. (**E**,**F**) mRNA expression of cPLA2, PA-PLA1, ABHD6, AGPAT8, Autotaxin (ATX) and MBOAT7 measured by RT-qPCR in (**E**) the liver and (**F**) the spleen with RPL19 as housekeeping gene. Data are expressed in percentage of the control condition (dotted line in **C**-**F**). Values are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 for mice receiving LPS vs. control mice. $$$*P*<0.001 for 8 h of LPS vs. 4 h of LPS.

9. Intestinal LPI levels are altered in a mouse model of colitis

Because LPI levels were affected by LPS-induced inflammation and were only decreased in the colon (**Figure 9B**), we asked whether these levels would be affected in a similar way in murine models more closely mimicking inflammatory bowel diseases (IBD). Indeed, several bioactive lipids, such as endocannabinoids and sphingolipids have been implicated in the pathogenesis of IBD.[29,30] Therefore we used two well characterized models of IBD, the DSS-induced and TNBS-induced models of colitis (**Figure 9D,F**), in order to determine the impact of colitis on LPI levels.[22,31] Similarly to what we observed in the LPS model, we found that LPI levels, with the exception of 18:0 LPI, were decreased in the colon of mice with DSS-induced colitis (**Figure 9E**). The same trend was observed in the TNBS-induced colitis model (**Figure 9G**).

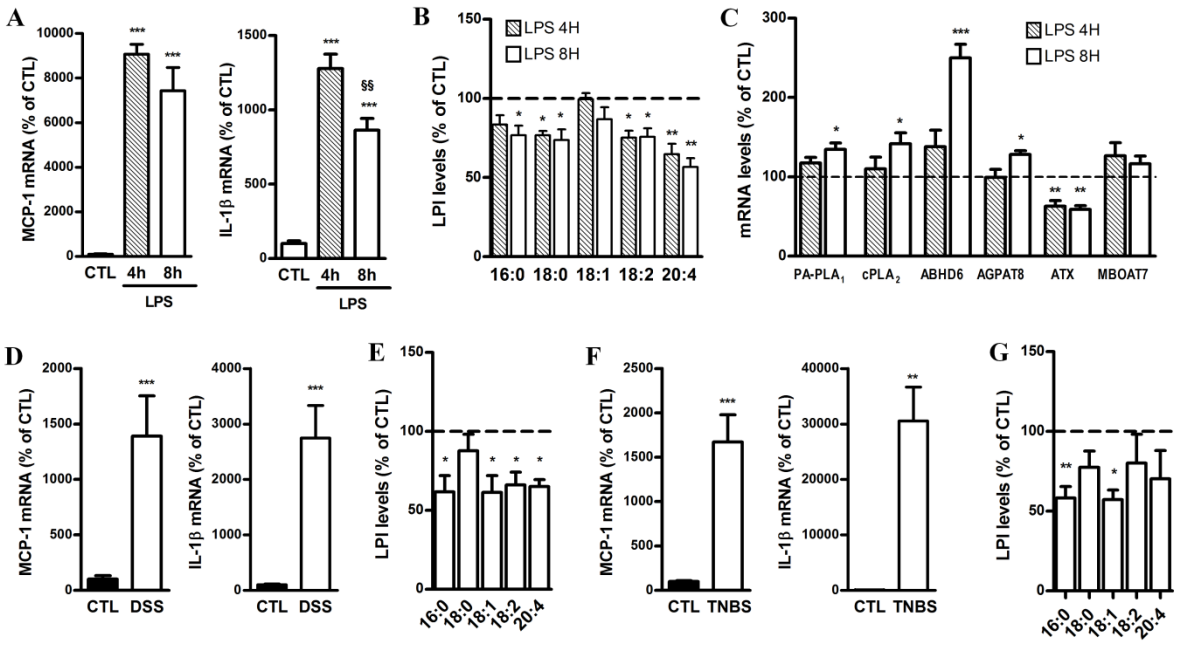

Figure 9. Impact of colon inflammation on LPI levels and their metabolic pathways. Systemic inflammation in mice was induced by intraperitoneal administration of 300µg/kg of lipopolysaccharides (LPS) for 4h and 8h. (**A**) mRNA expression of pro-inflammatory markers IL-1β and MCP-1 measured by RT-qPCR in the colon with RPL19 as housekeeping gene. (**B**) Lysophosphatidylinositol (LPI) levels measured by HPLC-MS in the colon. (**C**) mRNA expression of cPLA2, PA-PLA1, ABHD6, AGPAT8, Autotaxin (ATX) and MBOAT7 measured by RT-qPCR in the colon with RPL19 as housekeeping gene. (**D**,**E**) Colitis was induced by addition of 5% DSS to the drinking water of mice for 5 days. (**D**) mRNA expression of pro-inflammatory markers IL-1β and MCP-1 measured by RT-qPCR in the colon with RPL19 as housekeeping gene. (**E**) LPI levels measured by HPLC-MS in the colon. (**F**,**G**) Colitis was induced by intrarectal administration of TNBS to mice. (**F**) mRNA expression of pro-inflammatory markers IL-1β and MCP-1 measured by RT-qPCR in the colon with RPL19 as housekeeping gene. (**G**) LPI levels measured by HPLC-MS in the colon. Data are expressed in percentage of the control condition (dotted line in **B**, **C, E** and **G**). For **B**, **E** and **G**, the length of the acyl chain and the degree of insaturation of the individual species are shown on the x axis. Values are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 for inflamed mice (LPS or DSS) vs. control mice. $$*P*<0.01 for 8 h of LPS vs. 4 h of LPS.

V. Discussion

Among all lysoglycerophospholipids, LPI are certainly the less studied of the lot. However, there has been a renewed interest in these bioactive lipids, mainly since GPR55, the receptor for LPI has been identified.[9,32] With the exception of some studies showing changes in LPI levels in some cancers (ovarian and peritoneal)[33,34], little is known on the implication of LPI in other pathologies. Therefore, we decided here to study the metabolism of LPI in inflammation, from modification in the expression of their metabolic enzymes to the

alteration of their levels. These studies were conducted in vitro, on activated macrophages, and in vivo.

We started by evaluating the effect of macrophage activation on LPI levels. To do so, we used LPS, a potent macrophage activator. Interestingly, activation of the J774 murine macrophage cell line only led to variations in the levels of LPI, without affecting LPC, LPE, LPG or LysoPS. Actually, while the levels of most of the detected LPI were increased following macrophage activation, the levels of 20:4 LPI were decreased. Because an inflammatory response is often thoroughly regulated, mixing both signals to initiate and terminate the process, we thought that LPI could play a role in macrophage activation. Therefore, we incubated the LPS-activated J774 cells with various lysoglycerophospholipids and we show that LPI incubation decreases the parameters of LPS-induced macrophage activation.

As macrophage activation was affecting LPI levels, we took a closer look at its effects on the expression of metabolic enzymes of LPI. Phosphatidic acid-preferring phospholipase A1, PA-PLA1, also known as DDHD1, is involved in the hydrolysis of fatty acids at the *sn*-1 position of phospholipids, to produce fatty acids and 2-acyl lysophospholipids.[35] Yamashita et al. found that PA-PLA1 was implicated in the formation of 2-arachidonoyl LPI (20:4 LPI) in particular. cPLA2, from the superfamily of phospholipase A2 enzymes, is implicated in the hydrolysis of *sn*-2 fatty acids from phospholipids. Mariggiò et al. reported that cPLA2 may be one of the synthesizing enzymes of 1-stearoyl LPI.[19] These roles for PA-PLA1 and cPLA2 are based on the fact that, among PI, 1-stearoyl-2-arachidonoyl PI is the most abundant. ABHD6 and autotaxin have been reported to hydrolyze LPI, while acyltransferases recycle LPI into PI. We thought of assessing the variations in mRNA enzyme expression and possibly link them with changes in LPI levels. Interestingly, we found different trends for enzyme expression in the J774 and BV2 cell lines, despite similar trends for LPI levels upon activation of these two cell lines, suggesting different metabolic pathways in these cells. For instance, in J774 cells, a decrease in mRNA levels of PA-PLA1 could explain the decrease of 20:4 LPI levels. However, PA-PLA1 is increased in BV2 cells while 20:4 LPI levels are still decreased. Perhaps in this case, this could be explained by the increase in cPLA2 expression. Indeed, this enzyme is not only responsible for LPI biosynthesis, but could also hydrolyze LPI esterified in the sn2 position, such as 20:4 LPI formed through PA-PLA1 activity. However, the metabolic

pathways of LPI are quite complex (see introduction, Part 3), with possible reacylations and subsequent deacylations. Therefore based on our data we cannot link the levels of a particular LPI with the expression of a particular enzyme. This is the case in cell lines in culture, but also in primary peritoneal macrophages, where the levels of all LPI were increased, although PA-PLA1 expression was decreased and ABHD6 expression increased. The latter enzyme was recently implicated in lysophospholipid catabolism with LPI levels increasing in the liver of mice were ABHD6 was knock down.[20] Therefore it seems counter-intuitive that LPI levels will be increased if their catabolic enzyme is increased. However, cPLA2 is greatly increased in these peritoneal macrophages and could perhaps be responsible, following reacylation cycles, of the global increase in LPI levels. However at this point this remains speculative. This reinforces the notion that measuring bioactive lipid levels will offer a global and terminal view of all the changes that might occur in a given metabolic pathway.

As macrophage activation could affect LPI levels, we thought of assessing the impact of a systemic inflammation on LPI levels in various tissues. Therefore we measured LPI levels in the colon, liver, spleen, lung and cerebellum of mice receiving LPS (300 µg/kg) for 4 or 8 hours. This dose of LPS was chosen on the basis of previous studies in the lab to mimic the LPS-induced inflammation present during inflammatory pathologies such as colitis or the metabolic syndrome.[21,23] Here also, it is difficult to link LPI levels with mRNA expression of the metabolic enzymes. The variations in LPI levels are quite different depending on the tissue. In the cerebellum and lung, no major differences are seen except for an increase in 18:2 LPI in the lung. Interestingly, there seems to be an increased turnover of LPI in the lung, as biosynthetic and hydrolytic enzymes are increased, together with MBOAT7 and AGPAT8, which are involved in the recycling of LPI into PI. Two opposite pictures are seen in the liver and spleen, and the colon, with increased LPI levels in the former two and decreased in the latter. Indeed, in the liver, LPI levels are globally increased with an increase in biosynthetic enzymes' expression and a decrease in ABHD6 expression, whereas in the spleen LPI levels are only increased at 4h and are accompanied by an increase in PA-PLA1 mRNA expression. In both tissues, AGPAT8 is decreased, while MBOAT7 exhibits a trend to increase, which is only significant in the spleen. It would seem that in each tissue, variations in LPI levels could be explained by a different cause: a decrease in ABHD6 expression and an increase in $cPLA_2$

in the liver, or an increase in PA-PLA1 in the spleen, both leading to increased LPI levels. In the colon of mice receiving LPS, LPI levels are decreased with a strong increase in ABHD6 expression. Interestingly, this is the only tissue where we observed a decrease in autotaxin levels following LPS administration.

Finally, as we showed in this study that LPI could reduce macrophage activation, we wanted to study the implication of GPR55 in these effects. GPR55 has been recently put forth as the receptor for LPI[17,28] however not much is known on its effects on inflammation or macrophage activation. A serendipitous observation led us to suspect that this receptor could be involved in the effects of LPI on macrophage activation: in cells were GPR55 was well expressed, LPI decreased macrophage activation, whereas in cells where GPR55 was less expressed, the effect of LPI was lost. To confirm our hypothesis, we transfected J774 cells showing low expression of GPR55 and little effect of LPI on macrophage activation with human GPR55 and were able to rescue the effect of LPI on macrophage activation. Our results suggest that LPI could have anti-inflammatory properties when GPR55 is highly expressed.

In conclusion, as LPI levels were altered by macrophage activation, we hypothesized that they could be implicated in the control of macrophage activation. We were able to show that this was indeed the case, through a GPR55-dependent mechanism. Moreover, inflammation also affects LPI levels in vivo. These alterations are tissue dependent and could highlight a potential role for LPI in inflammatory processes.

VI. Acknowledgments

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VII. Supplementary information

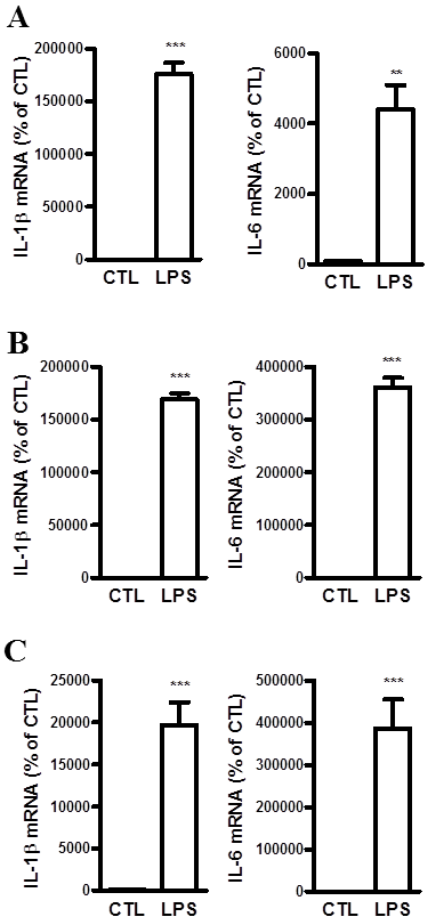

Figure SI 1. Lipopolysaccharides induce macrophage activation. Macrophage cell lines in culture and primary macrophages were activated with 100ng/mL of lipopolysaccharides (LPS) for 8h. mRNA expression of pro-inflammatory markers (IL-1β, IL-6) was measured by RT-qPCR with RPL19 as housekeeping gene in (**A**) J774 cells, (**B**) BV2 cells and (**C**) thioglycolate-elicited peritoneal macrophages (TEM). Data are expressed in percentage of the control condition (cells not incubated with LPS). Values are mean ± SEM. ***P*<0.01, ****P*<0.001 for LPS activation vs. control cells.

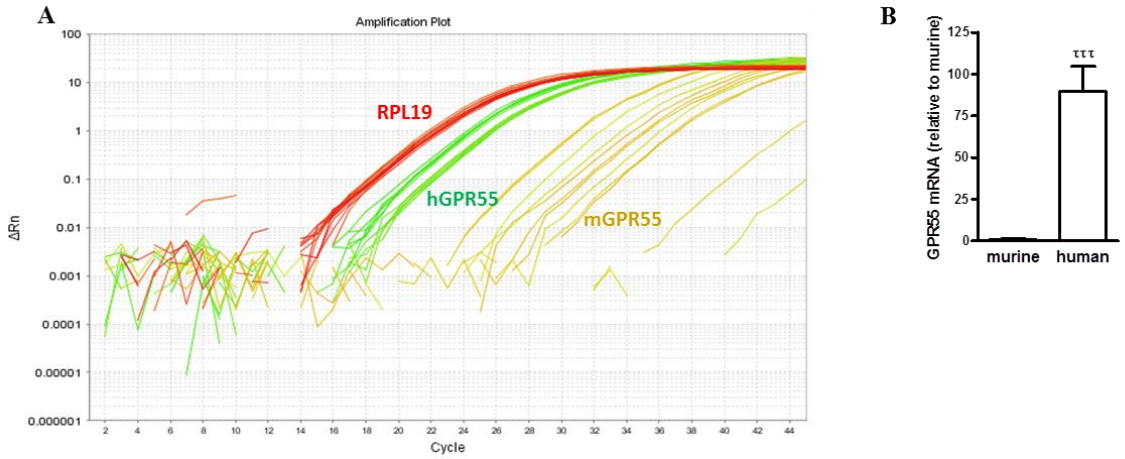

Figure SI 2. Transfection of J774 cells with human GPR55. J774 cells were transfected, using Lipofectamine LTX plus, with pDream2.1 vector containing the coding sequence for human GPR55 (hGPR55). This transfection was done in J774 cells with low expression of murine GPR55 (mGPR55). mRNA expression of mGPR55 and hGPR55 was measured by RT-qPCR with RPL19 as housekeeping gene. (**A**) Amplification plot of RT-qPCR showing the housekeeping gene RPL19 (in red), hGPR55 (in green) and mGPR55 (in yellow). Expression of mGPR55 is low and inconsistent. hGPR55 is more expressed than mGPR55 and in a more stable manner. (**B**) Expression of hGPR55 mRNA is compared to that of mGPR55 set at 1. Values are mean ± SEM. ττττ $P<0.001$ for hGPR55 expression vs mGPR55. ***$P<0.001$ for LPS activation vs. control cells.

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DISCUSSION, PERSPECTIVES and GENERAL CONCLUSION

I. Discussion

Most of the data presented in the three result chapters have been discussed in their respective chapter. Nevertheless at the end of this work, I feel that several topics and concepts deserve some additional discussion.

In the first chapter of the results, we set up a global untargeted method for lipid analysis and we applied it to the detection of a large number of lipids to compare control macrophages and LPS-activated macrophages. In the second part of the results, we focused on a lipid family and we set up a targeted method for lysophosphatidylinositols.

These two different quantification methods lead to an open point: **what is the best choice for the internal standard(s)?**

Even if it is easy to understand that working with internal standards allows to more closely mimic the behavior of the target analyte during the experiment, than perform the quantification via external standard, the selection of the appropriate internal standard remains complicated. Not only because of the poor availability of several compounds (and of the expensive price) but also because of the practical limitation in the number of internal standards usable in an untargeted approach.

For most quantitative analysis, using a stable isotope labeled internal standard (such as deuterated analogs of the targeted analyte) is considered as “the gold standard” and is recommended when feasible. Indeed, in a perfect world, this internal standard should have the same behavior as the analyte of interest, for instance, same extraction efficiency from the biological matrix, chromatographical coelution, and similar mass spectrometry behavior. This type of internal standard can compensate the eventual losses or inefficiencies in the extraction, in the preparation steps of the sample or the potential matrix effect in the MS.[1] However, even using this kind of internal standards, sometimes, they cannot always correctly compensate the matrix effect or the extraction recovery.[2,3] Indeed, even if a compound and its stable isotope labeled analogue will theoretically coelute and it is generally assumed that these two compounds have similar physicochemical properties, when an analyte and its deuterated analogue do not coelute on the column, the internal

standard is not able to compensate for the ion suppression with a different retention time.[4] Moreover, it was already shown that in particular conditions, two coeluting compounds can suppress each other's ionization during ESI ionization.[5] Working with stable labeled isotope also requires to be sure of the purity of the internal standard since any unlabeled impurity could affect the quantification and contribute to artificial higher concentrations of targeted analyte. Nevertheless, even when considering these potential limitations, this type of internal standard remains our first choice whenever possible.

However, for numerous lipid families, and typically for sphingolipids, phospholipids and lysophospholipids, the internal standards most often used are (lyso)phospholipids with fatty acyls chains non endogenously present: either short acyls chains (PI 8:0/8:0, Cer d18:1/12:0, SM d18:1/12:0) or ideally odds numbers of carbons (LPI 17:1, Cer d18:1/17:0, heptadecanoic acid…).

Because a complex lipid mixture (a crude lipid extract from biological sample) can be constituted of thousands or even tens of thousands of lipid molecular species, it is not possible to use hundreds to thousands of internal standards for the analysis. Classically, for complex lipid extracts in untargeted lipidomics analysis, individual lipid molecular species of each lipid family are quantified by using one related internal standard for the general family.[6-9] Indeed, in several studies it has been shown that the ionization efficiency of a general family of lipids (e.g. each phospholipids family) is less dependent on the fatty acyl moieties than on the chemistry of the head group. Moreover, after ESI ionization, the stability of the generated ions is not really dependent of the acyl chain properties (i.e. chain length or unsaturation index).[10,11] However, even it is true for more "polar" lipids, the effects of the acyl chain properties on ion stability, as well as on ionization, should be considered because they become more apparent for lipids with a lower polarity of the head group (e.g. triacylglycerols).

During our untargeted analysis, because of the prices and the poor availability (e.g. glycerophospholipids) of stable isotope labeled internal standards, we mainly used internal standards containing odd carbon number fatty acyl chains, with, as routinely done in lipidomics studies, one internal standard for each lipid family. For some others applications in the laboratory, such as endocannabinoids or oxysterols quantification (not discussed

here), we used deuterated analogues as internal standards. Finally during our targeted analysis for lysophosphatidylinositols quantification, we used the 17:1 lysophosphatidylinositol because it was the only internal standard commercially available for lysophosphatidylinositols when we began this work.

How to further develop our untargeted approach to increase the number of detected lipid species in a single chromatographic run and what are the main limitations?

To enhance the power of our untargeted approach, we could try to detect more and more lipids in our run. However, this is not so easy given the structural diversity of those compounds and by all the potential limitations. First of all, as described below, potential extraction issues leading to the impossibility to detect an analyte can occur. Then, chromatographic issue can limit the extension of the method. We encounter this kind of limitations with LPA (see below) and with lipid families of great interest to our laboratory that contains several isomers (e.g. oxysterols or bile acids) requiring specific chromatographic conditions allowing for a proper separation. Another challenge is constituted by the lower ionization potential of some lipid families. Undeniably the ionization of some lipids (e.g. neutral diacylglycerols) by electrospray ionization is more challenging in comparison with other lipid families which possess ionic head group conjugations. Because ESI-MS is the method of choice in lipidomics analysis, it clearly appears that all lipid subclasses cannot be efficiently ionized by this method and the need for improved or alternative ionization and identification is obvious. A last challenge is constituted by the low abundance of some lipid of high biological interest. Indeed, detecting those compounds within a complete matrix can be complex if not impossible. For those lipids (e.g. some COX-2 metabolites) a first step of purification/concentration is required in order to properly detect and quantify them.

Our HPLC-ESI-MS method allows for the quantification of six families of phospholipids and six families of lysophospholipids. However we are not able to properly detect PA and LPA.

As mentioned in the results section, we tried to develop a method allowing for the detection of a large number of lipids in the same analytical run. This calls for compromises in terms of extraction and chromatographic procedures. Unfortunately, despite our efforts we were not able to obtain a robust and nice chromatographic behavior for PA and LPA. Most of the time

PA and LPA eluted as large bell shaped peaks preventing their quantification (**Figure 1**). Thus here the issue is likely a chromatographic one, rather than a mass spectrometry issue.

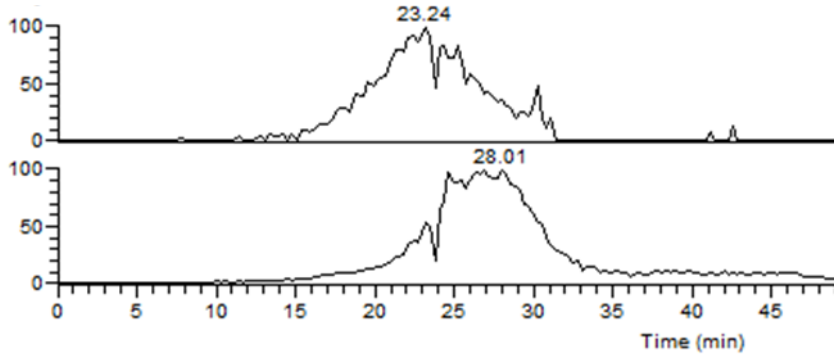

Figure 1. HPLC-ESI-MS extracted ion chromatograms (EIC) for LPA. LC-MS detection of 16:0 LPA (upper trace) and 18:0 LPA (lower trace). Detection as $[M-H]^-$ ions at 409.23606 and 437.26736 for 16:0 and 18:0 LPA, respectively.

We obtained our lipid extracts using a liquid-liquid extraction inspired by the one developed by Folch. While this method is one of the most widely used due to its effectiveness, we could not use it to extract sphingosine-1-phosphate.

Sphingosine-1-phosphate (S1P), the precursors of sphingosine, is a bioactive lipid of great interest in a large number of research fields. It is therefore interesting to quantify its levels in biological matrices. Despite many experiment with this compound (briefly summarized in **Appendix 1**), we were unable to quantify it either using our "global method" or even using more targeted methods based on the literature. Here the issue, as it is illustrated in the appendix, appears to be the extraction from the matrix. S1P is largely more water soluble than most of the other lipids and therefore the methods that will allow its extraction from the matrix will result in lower extraction of the more apolar lipids. Thus probably we will have to develop a targeted method for S1P (and sphingosine) rather than trying to quantify it in an "untargeted approach".

In this thesis, we performed absolute quantification for LPI in our validated targeted method and relative quantification in our untargeted approach. Can we aim towards an absolute quantification for more than 180 species?

The main goal of our untargeted approach is to be able to perform analysis of several lipid families in a same chromatographic run to compare different physio(patho)logical

conditions. Among the many application of this method (or one of its precursor) during our work, we chose here to illustrate its applicability based on the data obtained with LPS-induced macrophage activation. As discussed in the perspectives, we will also use this large scale method for the analysis of tissues from a multiple sclerosis mice model comparing healthy mice with mice with experimental autoimmune encephalomyelitis. In all these analyses, we use representative internal standards to determine area under the curve ratio to compare conditions. That is we did not determine the absolute amount of lipid presents in the samples. Even if it feasible for a limited number of lipid species, it becomes technically tricky to establish calibration curves for all the studied species. Moreover, in the literature only a limited number of the publications give absolute quantification[12] and the main part of the articles use relative quantification showing lipid arrays or % of total of a species.[13-17] Indeed, the point of the "omics" methodologies is to compare a condition to another. A proper quantification is more efficiently reached using dedicated target methods.

In the last chapter of the results, we evaluated the implication of LPI on activated macrophages in vitro and in inflammation in vivo. We evaluated the metabolism of LPI in inflammation, from the changes in the expression of their enzymes implicated in the metabolism to the modifications of their levels. Moreover, we also showed that the LPI effects observed are compatible with the involvement of GPR55. Should we also study PLA_2 activity?

The goal of this study was not to establish all the mechanistic pathways, but rather to show the implication of LPI in these conditions. In this work, all the data for pro-inflammatory markers and for metabolic enzymes presented are from RT-qPCR experiments as routinely performed in our laboratory. The experience that we have with macrophages clearly supports a correlation between changes in cytokines mRNA levels and changes in protein levels. We cannot be as assertive for the enzymes. Indeed, we can imagine that post-translational changes affect enzyme activities thus participating in the changes of LPI levels that we measured. Therefore we are working on a protocol that would allow us to measure the LPI production from PI using J774 cytosolic fractions. Once the protocol set up, we will determine whether LPS incubation affects LPI production in a way that could explain the results obtained in our quantification experiments.

II. Perspectives

At the beginning of this thesis, just after the creation of the BPBL research group, we only had a quantification method for endocannabinoids available. At the end of this thesis, we have largely widened the knowhow of the lab and the number of lipid species that we are able to quantify. Although we are now able to answer to many questions in terms of lipid quantification, this work is only the beginning in terms of lipidomics and new perspectives can be considered. In this part, we will describe some potential interesting perspectives.

Towards an enhanced number of compounds detected in a single run.

The basic principle of an untargeted method is to be able to quantify as much analytes as possible ideally within a single run. As discussed, some lipid families are quite challenging for various, and not always common, reasons. Thus the first perspective should be to add PA and LPA to the global method developed during this work.

However, next to this “untargeted” philosophy, an analyst working in a “biological” setting, can also consider “metabolic pathways driven analysis”. Actually one of the main goals, in terms of lipidomics, of the BPBL research group in the next future is to be able to quantify lipid families constituting “whole metabolic pathways”. The global strategy is to study entire pathways such as the “cholesterome” or the “sphingolipidome”. Here, the suffix “ome” as for lipidome could be described as the “entire ensemble of a class of substances metabolically linked for an individual or a species”. For instance, in the lab, we already set up quantification methods for oxysterols and cholesterol.[18,19] This is interesting and useful of course, but does not allow to fully capture the metabolic changes induced by a given event (physiopathological or pharmacological) on a metabolic pathway. Thus we are now trying to set up a quantification method for most of cholesterol’s metabolites encompassing oxysterols, bile acids, sulfated oxysterols, etc to be able to quantify all the compounds involved in this pathway (**Figure 2**).

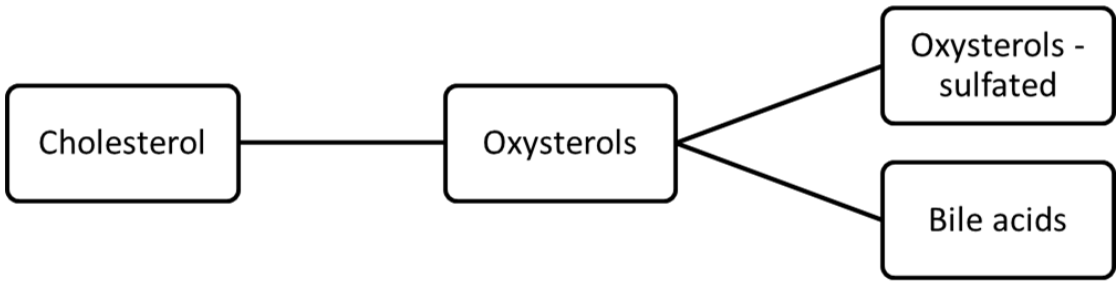

Figure 2. Schematic oxysterols metabolic pathway. "Oxysterome" as studied in our lab.

However, this can be quite complex because of the diversity in structures (and physicochemical properties) of the constituents of a given metabolic pathway. Thus if one want to quantify all the metabolites in a single run this can quickly become challenging.

Indeed, for each parameter, from the biological matrix to the graph showing the results, there will be multiple choices to do. When performing a single targeted analysis the choice of the extraction procedure, the purification, the separation systems, the ionization process etc. are all focused to find the better conditions for a limited number of compounds, often similar between them, chemically speaking. On the contrary, when the goal is to study a large number of analytes, it is not so simple and each step involves compromises to find the best way of analyze for all the needed compounds.

A similar challenge lies within the sphingolipidome. Today our method allows the quantification of ceramides, dihydroceramides, sphingomyelins, galactosylceramides and sulfatides (**Figure 3**).

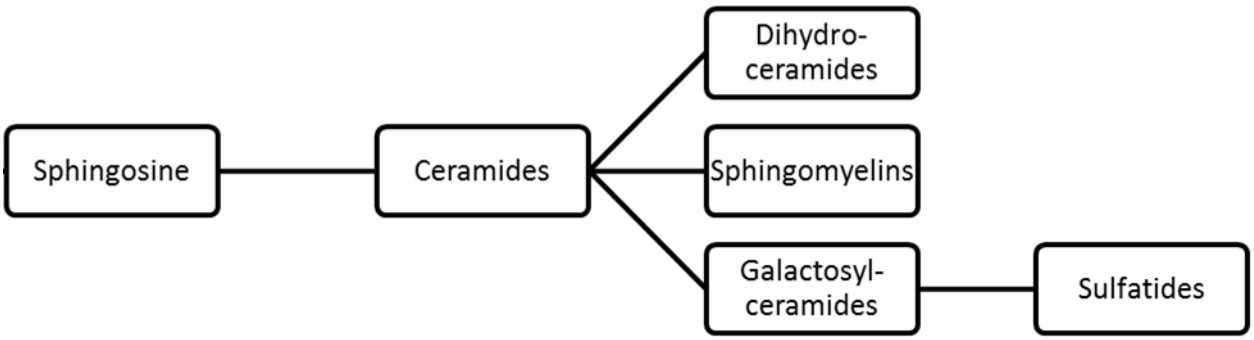

Figure 3. Schematic "sphingolipidome" pathway. "Sphingolipidome" as studied in our lab with the untargeted set up approach.

However, at least in principle, this could be extended to the quantification of other sphingolipids such as ceramide-1-phosphate, sphingosine-1-phosphate and glucosylceramide (**Figure 4**).

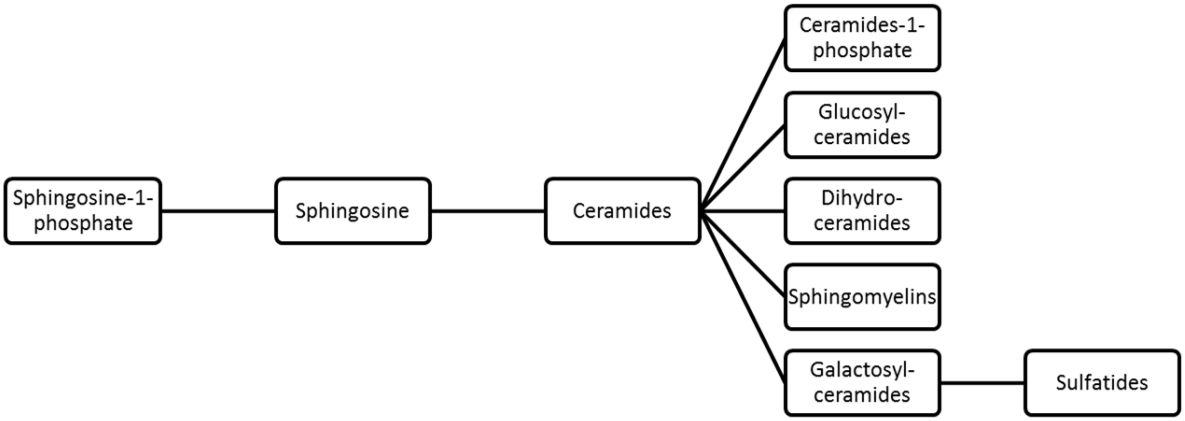

Figure 4. Schematic extended "sphingolipidome" pathway. More extended "Sphingolipidome" with new lipid families such as ceramide-1-phosphate.

Towards a better chromatography and an improved sensitivity…

All the previous results, as already mentioned, were obtained by coupling an HPLC and a LTQ-Orbitrap XL as mass spectrometer. This machine was very useful for the developed lipidomic method thanks to the exact mass but also the fragmentation patterns as presented in the first part of the results. However, even if LTQ-Orbitrap XL present several advantages with its analytical performances such as resolution, mass accuracy or linear dynamic range, this spectrometer showed limitation in terms of sensitivity. Moreover, as a consequence of the sensibility limitation, the amount injected did not allow us to use Ultra Performance Liquid Chromatography (UPLC) columns. That is why the transposition of this developed method to a tandem quadrupole coupled to a UPLC could allow us a gain of time, of solvent consumption without loss of detected species even if using tandem quadrupole does not allow us to process the data a posteriori as it is possible with the LTQ-Orbitrap XL data. As example, we previously performed several tests for detection of LPI after UPLC separation (**Figure 5**).

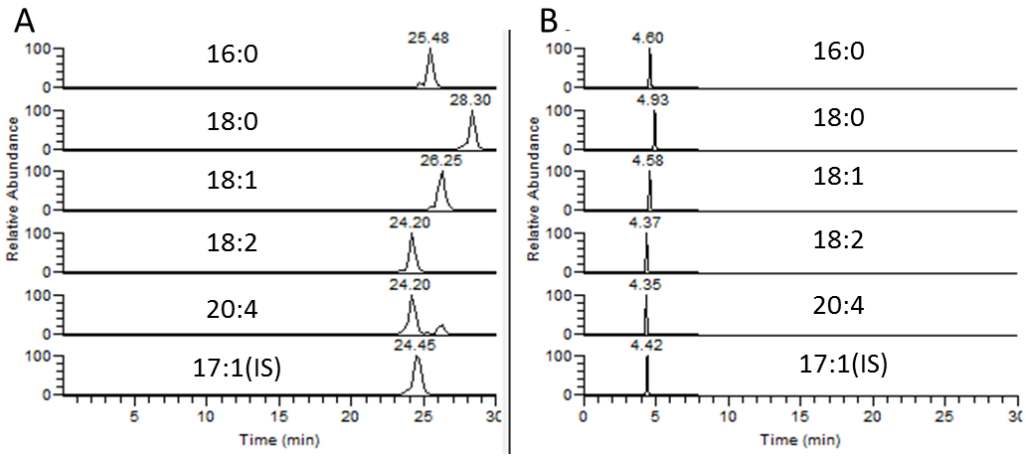

Figure 5. HPLC-ESI-MS (A) and UPLC-ESI-MS (B) chromatograms of standard soy LPI. Liquid chormatography-mass spectrometry (LC-MS) separation of lysophosphatidylinositols in soybean. Detection as signly depronotated $[M-H]^-$ ions at *m/z* 571.28889 (16:0), 599.32019 (18:0), 597.30454 (18:1), 595.28889 (18:2), 619.28889 (20:4) and 583.28889 (17:1). HPLC was achieved with Kinetex LC-18 column (5 µm, 4.6 × 150 mm) after a 20 µL injection. UPLC was achieved with Hypersil GOLD column (1.9µm, 2.1 x 100 mm) after a 2 µL injection. For both systems mobile phases A and B consisted of methanol-H_2O-ammonium hydroxide 50:49.9:0.1 (v/v/v) and methanol- ammonium hydroxide 99.9:0.1 (v/v).

On these figure, we can easily see the gain of time performing analysis in UPLC-MS. This is also linked with a reduced injected volume and a decrease of solvent consumption, allowing a reinjection if needed and money gain. However, even if this can appear easy based on this chromatogram (**Figure 5**), this transfer of method to an another chromatography system and spectrometer for all the compounds in a same run could be difficult for several reasons such as abundance, isobars species, coelution, transitions etc. Moreover, it will be crucial to be able to separate *sn*-1 & *sn*-2 lysophospholipids for the further evaluation of the potential link with their metabolic enzymes.

Towards a better understanding of lipids implication in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis.

First of all, as mentioned in the first part of the results, we developed an untargeted method for lipid analysis. We applied this analytical method to evaluate lipid levels variations *in vitro* after activation of macrophages with LPS. In the context of the study of lipids in inflammation, we implemented in the lab a multiple sclerosis model, the experimental autoimmune encephalomyelitis (EAE), to study the variations in lipid level in this inflammatory disease mouse model. During the first part of my thesis, we performed a first pilot study, with a limited number of animals. In our lab, EAE was induced in C57BL/6 mice

using $rMOG_{35-55}$ (a myelin component widely used for the induction of EAE) emulsified in complete Freund's adjuvant (**Figure 6**). The clinical signs of EAE were evaluated using a clinical scoring scale ranging from 0 to 5 (i.e. from normal mouse with no clinical disease (0) to moribund or dead animals (5)).

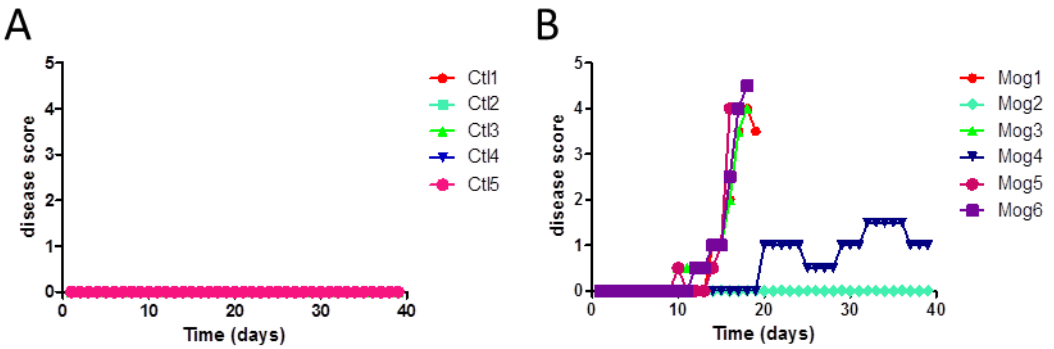

Figure 6. Clinical scorings of EAE mice. Data represent mean clinical scores derived from daily observation for control mice (A) and MOG treated mice (B).

Given the fact we worked with a limited number of mice to implement the mouse model in the lab, we sacrificed the mice at clinical score of 4 (mice cannot move their hind limbs and the first signs of weakness of front limbs are apparent) and therefore only compared control mice and mice at clinical score of 4. We analyzed several tissues with an HPLC-MS method allowing the detection of some lipid family. For instance, we identified lower levels of sulfatides in MOG treated mice than in control mice (**Figure 7**), which was already demonstrated in myelin extracted from human white matter autopsy samples.[20] Moreover, we established correlations between lipids and important marker of neurodegenerative diseases. For instance, 16:0 LPI and ATF3 (activating transcription factor 3) were positively correlated (**Figure 7**).

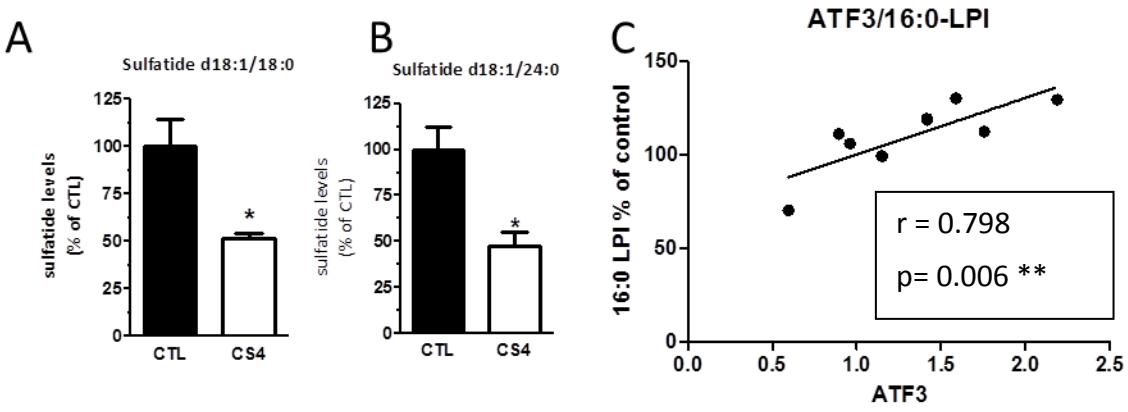

Figure 7. Effect of MOG administration on sulfatide levels (A-B) and correlation between 16:0 LPI and ATF3 (C). Sulfatide levels were measured by HPLC-MS following vehicle administration for CTL and after MOG administration and clinical score of 4 reached for CS4. Data represent d18:1/18:0 (**A**) and d18:1/24:0 (**B**) sulfatide levels. Values are mean ± SEM. *P<0.05 for clinical score 4 EAE mice vs. control cells. ATF3 and 16:0 LPI were positively correlated (**C**).mRNA expression of ATF3 was measured by RT-qPCR and 16:0 LPI level was measured by HPLC-MS.

More recently, we performed a second study with 45 mice to perform a "time-evolution" study. Indeed, this large number of animals allowed us to sacrifice mice at each clinical score (from 1 to 4). For time constraint we were not able to study the lipid composition yet. Nevertheless it would be very interesting, with the developed untargeted lipidomics method, to study mice tissue lipid levels, especially in spinal cord (rostral and caudal) and brain stem allowing the comparison of several stages of the evolution of the pathology. In a second time, after lipid analysis, it could also be very interesting to compare lipid profiles and either to administer these lipids either modulate their levels (**Figure 8**).

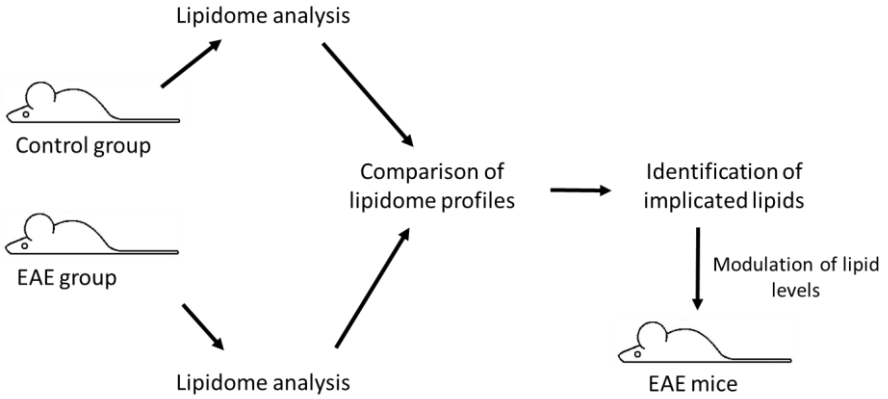

Figure 8. Schematic view of lipid analysis in EAE study. Different steps for lipid analysis and study of their implication in a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE).

III. General conclusion

At the end of this work, several new analytical tools for the quantification of lipids are developed. The analysis of these compounds in complex biological matrices is a key challenge for their evaluation in inflammatory processes therefore several analytical methods for identification and quantification of lipids has been set up. Liquid chromatography coupled with mass spectrometry revealed to be a powerful analytical tool for screening and identification of lipids (**Figure 9**).

- Set up and application of an HPLC-MS/MS method allowing for study of numerous lipid subclasses
- Detection, identification and quantification of LPI: Set up and validation of a quantification method for LPI
- "Cartography" of LPI levels in several tissues (relative and absolute quantification)
- Studies of the effect of inflammation on lysophospholipids levels *in vitro* and *in vivo*
- Studies of the effect of LPI on macrophage activation

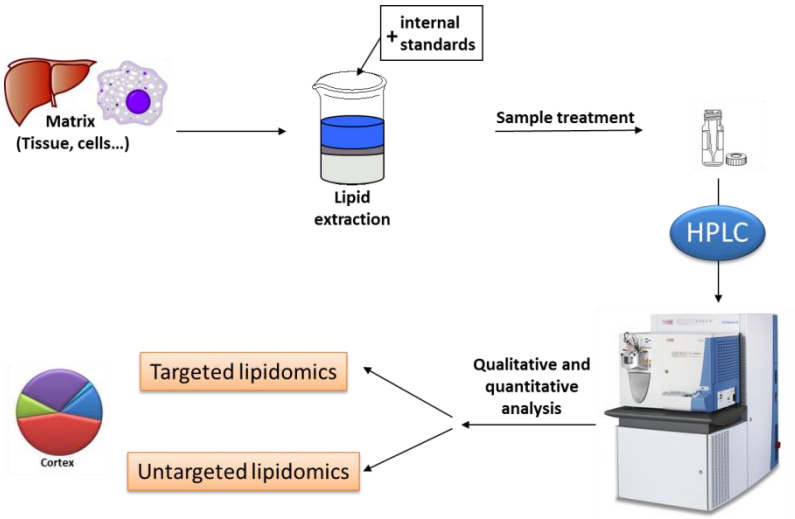

Figure 9. General pathway for lipid analysis. Quantification method from biological matrices extraction to the lipid quantification.

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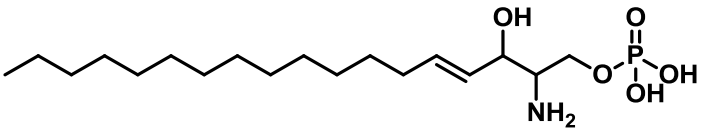
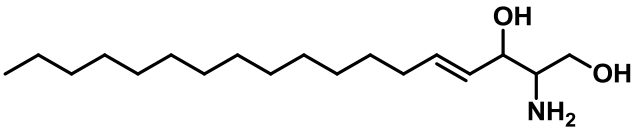
APPENDIX

Sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) (**Figure 1**) is a bioactive sphingolipid metabolite well recognized as key regulator in several physiological and pathophysiological processes such as cancer, diabetes or neuroinflammatory diseases (e.g. multiple sclerosis).[1-3]



sphingosine-1-phosphate



sphingosine

Figure 1. Structures of spingosine-1-phosphate and sphingosine.

Most of the known effects of S1P are mediated by a one of the five G protein-coupled receptors termed S1PR1-5. For example, S1P regulation of immune cells trafficking is mediated by S1PR1.[4] For these reasons and because S1P is a precursor of sphingosine and other sphingolipids, we wanted to be able to quantify it. However, despite several attempts, at the end of this work we are still unable to quantify S1P.

First, as we usually perform for our lipid identification, we tested S1P in various ionization and chromatographic systems by injecting S1P standard solutions onto C18 and HILIC HPLC columns using several mobile phases. In these conditions we obtained a nice ionization and chromatographic elution as illustrated in **Figure 2**.

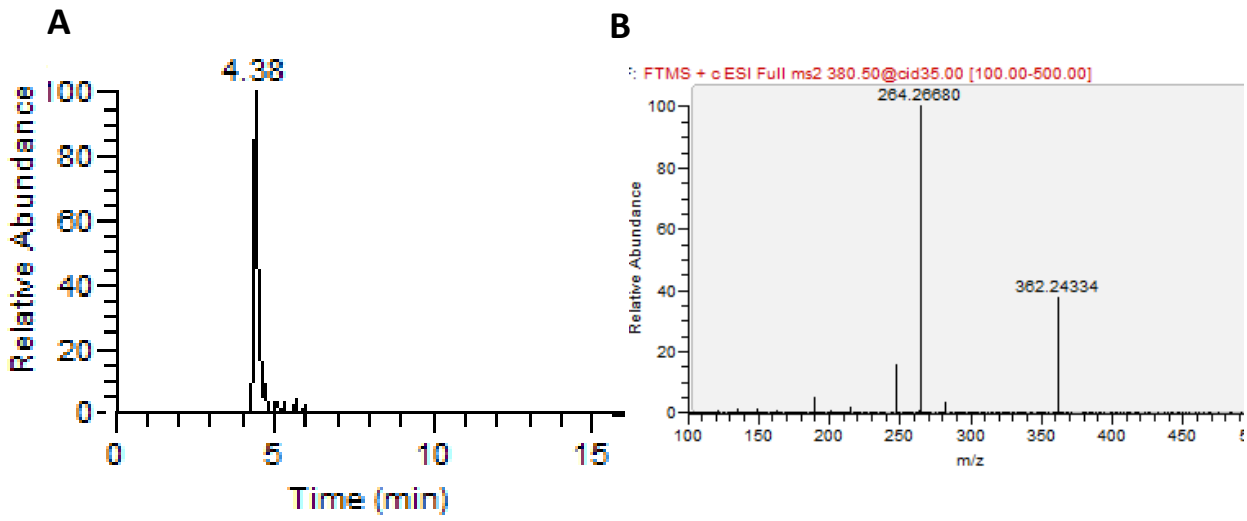


Figure 2. HPLC-ESI-MS extracted ion chromatogram (EIC) of spingosine-1-phosphate (A) and spectra of fragments after MS^2 (B). (**A**) LC-MS of S1P detected as $[M+H]^+$ ions at m/z 380.25604 and (**B**) product ions after fragmentation corresponding of classical ions after fragmentation of S1P (362,24602 & 264,26967).

Second, we went on and tried to detect S1P after extraction from a matrix or aqueous solution using our usual Foch extraction. As might have been expected from the literature, this was unsuccessful. Indeed, as described in the literature, S1P is not easy to extract in classical lipid extraction conditions.

Nevertheless, to test the hypothesis that S1P was adsorbed to the vial, thus potentially explaining its poor recovery, we next tested the recovery efficiency from a vial following evaporation of the solvent. Based on the literature we tested several solvents, such as ethanol, methanol + 1% DMSO, ethyl acetate, isopropanol, methanol-chloroform 1:1, methanol $HCOONH_4$ 1mM 0.2% HCOOH, ACN-H_2O 50:50 $HCOONH_4$ 20 mM pH 4.4, without great improvement of S1P recovery. The best recovery was achieved with methanol $HCOONH_4$ 1mM 0.2% HCOOH allowing for slight signal after injection in the HPLC-MS.

To try to improve the extraction, we also tested different other extraction systems such as a “butanol extraction” and a methanol protein precipitation. Actually based on the literature, S1P seemed to be easily extracted using butanol (and an aqueous phase containing citric acid and disodium hydrogen phosphate, pH 4.0).[5,6] Some authors described S1P extraction for LC-MS analysis with simple methanol precipitation.[7,8] Nevertheless, even in those conditions, we were not able to obtain a decent recovery.

Thus we tried to "improve" the methanol precipitation by adding 1mM $HCOONH_4$ 0.2% HCOOH during the precipitation (**Figure 3**). This allowed us to obtain a slightly better extraction compared to pure methanol.

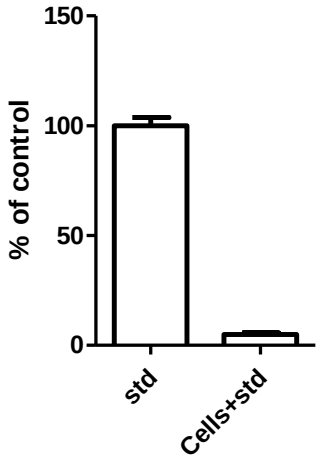


Figure 3. Efficiency of exaction for S1P. The direct injection of S1P (800 pmol) and the same amount of S1P used for spiking J774 cells with a very poor extraction efficiency.

As a positive control, we also used sphingosine (**Figure 1**) during our experiments. Indeed, with this compound we did not get any extraction/recovery issues.

Moreover we tried to perform a standard addition using this protocol but here again our data do not show a proportional extraction (data not shown). The fact that it is indeed an extraction issue is confirmed by **Figure 4** showing a perfect line obtained by spiking wells extracts with S1P post extraction.

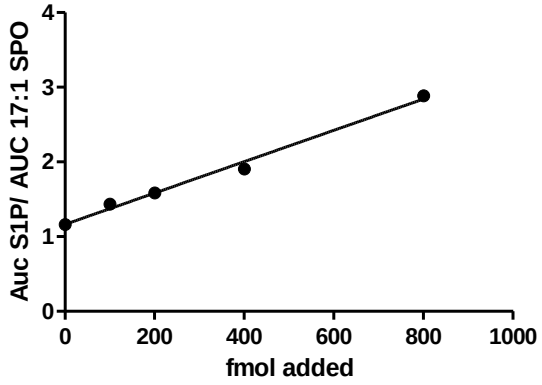


Figure 4. Standard addition of sphingosine-1-phospate. J774 cells were used to perform standard addition with S1P (spiked post-extraction) and 17:1 sphingosine (17:1 SPO) as internal standard.

These data illustrate that some lipids are not extracted by the usual Folch lipid extraction. Nevertheless, S1P seems to be a particularly tricky lipid to extract, despite what is reported in the literature. Additional conditions will have to be tested in order to find an extraction method allowing sufficient recovery for S1P to be detected.

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