the FEBS Journal
MINIREVIEW

Targeted lipidomics – advances in profiling lysophosphocholine and platelet-activating factor second messengers

Hongbin Xu[1,2], Nicolas Valenzuela[2,3], Stephen Fai[3], Daniel Figeys[1,2] and Steffany A. L. Bennett[1,2]

1 Ottawa Institute of Systems Biology, University of Ottawa, Ontario, Canada
2 Neural Regeneration Laboratory, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ontario, Canada
3 Carleton Immersive Media Studio, Azrieli School of Architecture and Urbanism, Carleton University, Ottawa, Ontario, Canada

Keywords
alzheimer disease; lipidomics; lipid second messenger; liquid chromatography; lysophosphatidylcholine; mass spectrometry; neurodegeneration; neurolipidomics; phospholipase; platelet activating factor

Correspondence
S. A. L. Bennett, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada
Fax: +1 613 562 5452
Tel: +1 613 562 5800, ext. 8372
E-mail: sbennet@uottawa.ca
Website: http://137.122.232.177/index.html
D. Figeys, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada
Fax: +1 613 562 5452
Tel: +1 613 562 5800, ext. 8374
E-mail: dfigeys@uottawa.ca
Website: http://www.med.uottawa.ca/associations/oisb/members/member_daniel_figeys.htm
H. Xu, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada
Fax: +1 613 562 5452
Tel: +1 613 562 5800, ext. 4236
E-mail: hxu081@uottawa.ca
Website: http://137.122.232.177/index.html

(Received 27 March 2013, revised 27 June 2013, accepted 1 July 2013)

doi:10.1111/febs.12423

Glycerophosphocholines are the major building blocks of biological membranes. They are also precursors of low-molecular-weight second messengers with mass to charge ratios of 450–600. These messengers include lysophosphatidylcholines (LPCs) and lyso-platelet activating factors (PAFs) that may be further processed into PAFs. Often considered as a single species, LPCs, PAFs and lyso-PAFs are, in fact, families of glycerophosphocholine-derived lipids distinguished by the linkage of their *sn*-1 carbon chains to the phosphoglyceride backbone (ester or ether), their *sn*-1 carbon chain length and degree of unsaturation, and the identity of their *sn*-2 constituents (a hydroxyl or acetyl group). Each LPC and PAF species exhibits a different affinity for its cognate G-protein-coupled receptors, and each species elicits receptor-independent actions that play critical signalling roles. Targeted mass spectrometry-based lipidomic approaches are enabling the molecular identification and quantification of these low-abundance second messengers. Variations between datasets map the temporal landscape of second messengers available for signalling, and provide snapshots of the state of structural membrane compositional remodelling at the time of extraction. Here, we review a number of advances in lipidomic methodologies used to identify LPCs, lyso-PAFs and PAFs, and highlight how these targeted approaches are providing valuable insight into the roles played by the cellular lipidome in cell function and disease susceptibility.

Abbreviations
CA1 and CA3, Cornu ammonis cell fields of the hippocampus; $cPLA_2$, cytosolic phospholipase A_2; GrDG, granule cell layer of the dentate gyrus; $iPLA_2$, calcium-independent phospholipase A_2; LPC, lysophosphatidylcholines; PAF, platelet-activating factors; PAFR, platelet-activating factor receptor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; $sPLA_2$, secreted phospholipase A_2.

Introduction

The discovery that lipids play a critical role in cell signalling is regarded as a major advance in biology in the later part of the past century. In 1968, Albert Lehninger argued that a comprehensive understanding of the membrane at the molecular level would unlock hidden molecular mechanisms of synaptic transmission if we only had the means to identify each type of lipid within complex matrices of several thousand related species [1]. He stated that 'Although considerable information is available on the electrical behaviour of the neuronal membrane, very little is yet known of its molecular composition and structure because of the severe difficulties in obtaining for biochemical study sufficient material uncontaminated by the membranes of other types of brain cells, particularly the glia… Presumably each type of lipid may contribute some specific characteristic to the membrane… [The] study of natural membranes…promise[s] to open the door to a molecular biology of neuronal transmission'. Forty-five years later, new technologies applied to the study of structural phospholipids are enabling their large-scale profiling [2]. This profiling provides new insight into the functional impact of their topographies, geometries and dynamic remodelling into smaller glycerophospholipid second messengers on cellular function. Recent advances in lipid identification, as facilitated by technological development of these lipid analytical tools, have revealed some of the vital roles that small bioactive metabolites of structural membrane lipids (i.e. lipid second messengers) play in various human diseases, including Alzheimer's disease [3–6], cancer [7,8], inflammation and cardiovascular diseases [9], and male infertility [10].

Phospholipids are the major constituents of cellular membranes (Fig. 1A) [11]. They can be divided into 20 distinct molecular classes based on the glycerophospholipid classification system standardized by the LIPID MAPS consortium [12,13]. The polar head group attached to the phosphoglyceride backbone defines each class. As an example, a section through a neuronal plasma membrane is presented in Fig. 1A, depicting various structural phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylinositols (PIs), and phosphatidylserines (PSs) identified at the molecular levels using recent lipidomic approaches [5,14–18]. These lipids have been modelled using VaLID view lipid models [19]. Each structural class (i.e. PC, PE, PI, etc.) is made up of molecular species that differ in their combination of fatty acyl residues (chain length and degree of unsaturation) at the *sn*-1 and *sn*-2 positions and the nature of their *sn*-1 or *sn*-2 chemical linkages (Fig. 1A,B). These differences distinguish individual isoforms and define subgroups within the major sub-classes. For example, a PC (Fig. 1B) consists of a glycerol backbone, a fatty acid carbon chain linked by an acyl ester, alkyl ether or alkenyl vinyl ether at the *sn*-1 position, an acyl ester at the *sn*-2 position (most commonly, although other linkages are also possible), and a polar phosphocholine head group with a phosphodiester linkage at the *sn*-3 position. PCs containing an *sn*-1 alkyl ether linkage are called plasmanyl PCs (or alternatively alkylacyl glycerophosphocholines), whereas those containing an *sn*-1 vinyl ether (alkenyl) bond are known as plasmenyl PCs (or plasmalogens) (Fig. 1B).

Enzymatic processing by cytosolic phospholipase A_2 ($cPLA_2$) or calcium-independent phospholipase A_2 ($iPLA_2$) family members generates powerful sub-classes of smaller intracellular second messengers (~ 450–600 Da) (Fig. 2A). Processing by secreted phospholipase A_2 ($sPLA_2$) enables these metabolites to be released into the extracellular space or re-incorporated at the outer plasma membrane leaflet (Fig. 2A). Two classes of second messengers are produced: (a) oxygenated derivatives of free fatty acids liberated from the *sn*-2 position, such as eicosanoids (e.g. prostanoids and leukotrienes), and (b) bioactive metabolites that retain the phosphoglyceride backbone, such as lysophosphatidylcholines (LPCs) derived from diacyl PCs or platelet-activating factors (PAFs) derived from plasmanyl PCs (Fig. 2A) [20]. This review focuses on advance in lipidomic assessment of these LPCs and PAFs at the molecular level.

LPC and PAF second messengers are not merely transient metabolites of structural glycerophospholipids. They are powerful immediate-response molecules that elicit physiological and pathophysiological responses independently or through activation of specific G-protein-coupled receptors. LPCs act as ligands for a family of G-protein-coupled receptors that regulate cytoskeleton and cellular Ca^{2+} homoeostasis, proliferation, survival, migration and adhesion [21,22]. Further, PAF lipids, a special sub-class of PCs with an alkyl-linked carbon chain at the *sn*-1 position and an ester-linked acetyl group at *sn*-2 position originally named for their platelet-aggregating effects, are now recognized as potent neuromodulators [23] implicated in neurodegeneration [6]. PAFs may be remodelled as part of Lands cycle from the alkyl-lyso-glycerophosphocholine (lyso-PAF) metabolites produced by the actions of group IVa $cPLA_2$ on structural membrane alkylacyl PCs [22,24] (Fig. 2B). These lyso-PAFs are

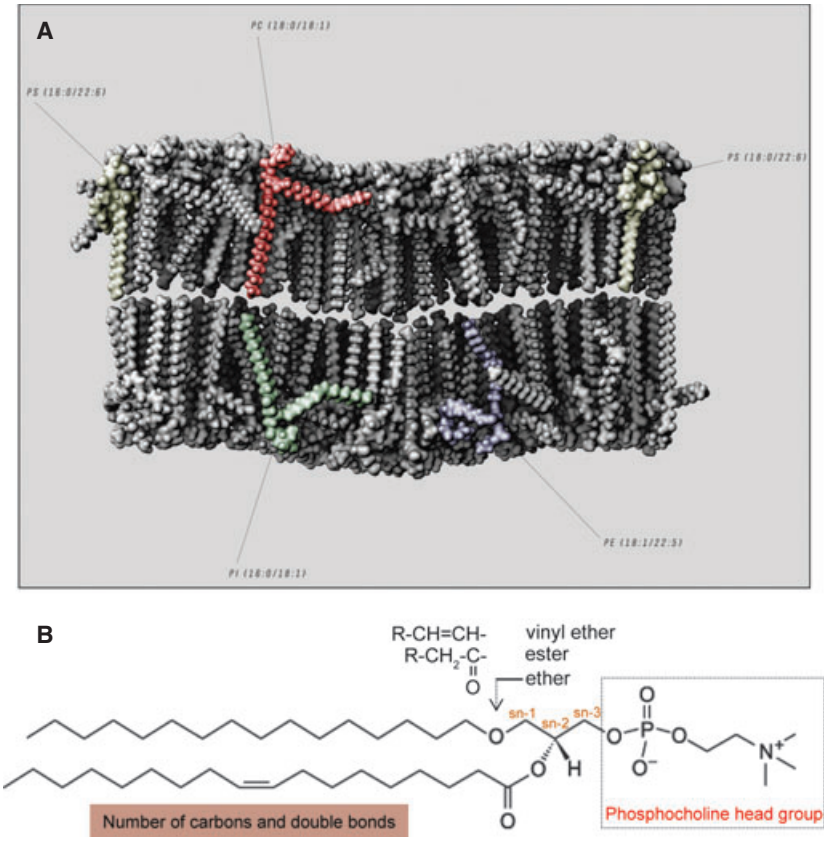

Fig. 1. Membrane phospholipids are the structural building blocks of cell membranes and the source of powerful bioactive second messengers that retain the glycerophospholipid backbone (i.e. lyso-PAFs, PAFs and LPCs). (A) Schematic emphasizing the rich diversity of structural membrane lipid species present in lipid bilayers. Individual PCs, PEs, PSs and PIs are highlighted. Membrane composition was simulated using the following datasets [5,14–18], and lipids were modelled using dynamic VaLID view lipid models fitted using Maya v2012 (Autodesk) with a rig of moveable joints between each atom [19]. Molecular species were identified using the standardized lipid nomenclature that adheres to the LIPID MAPS classification system [12], defining species by their structural determinants. PC(18:0/18:1), for example, defines a lipid species with a phosphocholine polar head group (PC), an ester linkage at the *sn*-1 position, 18 carbons at both the *sn*-1 and *sn*-2 positions, of which the *sn*-1 chain is fully saturated (referred to as :0) whereas the *sn*-2 chain has one double bond (a mono-unsaturated fatty acid, indicated by :1). (B) Structural determinants define individual lipids at the molecular level. Glycerophospholipids are classified according to the common polar head group linked to the *sn*-3 position of the glycerol backbone by a phosphodiester linkage. A PC is depicted. Individual species differ by the number of carbons and double bonds of the two fatty acyl chains at the *sn*-1 and *sn*-2 positions, as well as the type of linkage at the *sn*-1 position (dotted circle). In the standardized nomenclature, vinyl ether (alkenyl) linkages (plasmalogens) are distinguished by P- [i.e. PC(P-16:0/18:0)]; ether (alkyl) linkages are distinguished by O- [i.e. PC(O-18:0:20:4)].

either recycled back into membrane phospholipids by transfer of a long-chain fatty acid to the *sn*-2 position, or further remodelled into PAFs by transfer of an *sn*-2 acetyl group. Both activities are mediated by the lyso-phosphatidylcholine acyltransferases LPCAT1 and 2 (Fig. 2C). PAFs themselves are hydrolysed by one of three PAF acetylhydrolases back to lyso-PAFs (Fig. 2D). PAF acetylhydrolase isoform II in particular has additional transacetylase activity that transfers the PAF *sn*-2 acetyl group to either LPC or sphingosine substrates [25]. Interestingly, not all PAF species exert the same biological effects. We have shown that PC(O-16:0/2:0) and PC(O-18:0/2:0) signal PAF receptor-independent cell death through distinct signalling pathways [26]. In the absence of the G-protein-coupled PAF receptor (PAFR), PC(O-16:0/2:0) activates caspase-7, whereas PC(O-18:0/2:0) triggers caspase-independent death [26]. By contrast, ligand-mediated signalling may be either pro- or anti-apoptotic, depending on the identity of the *sn*-1 fatty acid of the PAF ligand activating PAFR [26–30]. Activation of PAFR by PC(O-16:0/2:0) signals caspase-dependent death in cerebellar granule neurons [26]. Activation of PAFR by PC(O-18:0/2:0) triggers pro-apoptotic signalling [26]. Thus, the identity of the PAF lipid as well as the expression status of the PAFR dictates whether

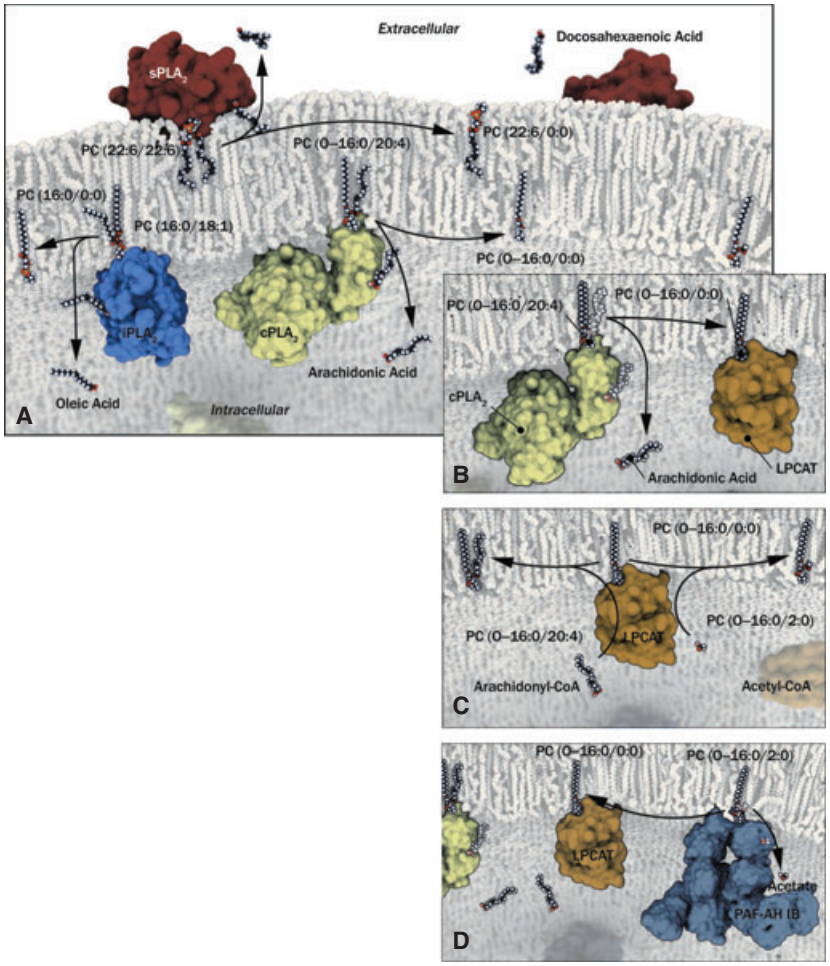

Fig. 2. Glycerophospholipid-derived second messengers are produced by the enzymatic processing of structural membrane lipids. (A) Hydrolysis of the *sn*-2 carbon chain by $cPLA_2$, $iPLA_2$ or $sPLA_2$ generates free fatty acids and small glycerophospholipid-derived second messengers. Various LPCs and lyso-PAFs are shown. (B–D) Lyso-PAFs may be further remodelled to PAFs. (B) In Lands cycle, $cPLA_2$, a group IVa PLA_2 that preferentially hydrolyses arachidonic acid from the *sn*-2 position of 1-*O*-alkyl-2-arachidonoyl and 1-*O*-acyl-2-arachidonoyl glycerophospholipids, generates a lyso-PAF [129]. (C) Addition of an acetyl group to the *sn*-2 position of the lyso-PAF via the actions of lysophosphatidylcholine acyltransferase, generates a PAF. Alternatively, addition of a long-chain fatty acyl group regenerates an alkyl-linked structural membrane lipid. (D) PAFs may be inactivated by hydrolysis of their *sn*-2 acetyl group by one of three PAF acetylhydrolases enzymes (the cytosolic PAF-AH1b trimer (shown here), cytosolic PAF-AHII, or secreted plasma PAF-AH).

PAF second messengers signal apoptosis, neurodegeneration, or neuroprotection. This signalling diversity is just one example of why it is essential to study bioactive lipid second messengers not only as families, but also at the molecular level.

To better understand lipid signaling, we must identify all of the LPC, PAF, and plasmenyl PC second messengers present in a biological system at a defined moment in time, and quantify how these species change in response to various stimuli. Recent advances in MS are enabling comprehensive profiling of lipid compositions at the molecular level [2,31–37]. However, as anticipated by Lehninger in 1968 [1], meaningful lipidomic analyses still face a series of significant challenges, including difficulties associated with the efficiency of extraction of lipids with different linkages and structural determinants, effective separation by HPLC, the differential sensitivity to fragmentation of different structures that complicates high-throughput approaches, and a paucity of bioinformatics tools to identify isobaric lipids with the same mass and charge in large datasets. The remainder of this review describes a number of current lipidomic methodologies used to identify LPCs and PAFs, highlights their strengths and limitations, and emphasizes the need, not only for new 'omic' methodologies, but also

for careful biochemical methodologies that facilitate the separation of membranes and membrane microdomains to address more fully the impact of subcellular membrane metabolism on cellular function.

Methodological considerations underlying lipidomic analysis of glycerophosphocholine-derived second messengers

Lipid extraction

The first step in lipidomics is extraction of lipids from biological samples. The choice of sample dictates the level of biological resolution that may be achieved and the questions that may be addressed by the profiling. For example, use of tissue homogenates from brain and kidney allows for a 'high-level' determination of the lipid landscape across cell type and regions. Such analyses are invaluable for systemic comparison of the dietary impact on lipid composition at the tissue level for example, but cannot be interpreted as representative of the lipid composition specific to functional anatomical and cellular units. Moreover, lipid profiles vary with respect to predominant cell type and membrane microdomain isolated [38]. For example, lipids extracted from dissections of the corpus callosum in the brain are enriched for species produced by myelinating oligodendrocytes and, to a lesser extent, neuronal axons traversing hemispheres [39–41]. Dissections specific to layers II and III of the entorhinal cortex identify neuronal lipids characteristic of cell soma that send afferents to the pyramidal cell layers of the Cornu ammonis cell fields of the hippocampus (CA1 and CA3) and the granule cell layer of the dentate gyrus (GrDG) in the hippocampus [42,43]. However, these profiles also contain glial lipids representative of these particular regions [44,45]. Regional comparisons between lipids harvested from entorhinal cortex and CA3/CA1/GrDG dissections, for example, may be used to characterize differences in second messenger availability within the input circuitry of the entorhinal–hippocampal circuit [42]. Because the predominant cell type alters this regional profile, interpretation of lipidomic datasets must also take into consideration changes in relative regional cell ratios. For example, neuroinflammation results in infiltration of activated astrocytes, microglia and macrophages into injured tissue, and this infiltration will alter the regional lipidomic profile without necessarily indicating a change in resident neurons [46,47]. Such profiles may be compared to 'fingerprints' of the glycerophospholipid landscape specific to various cell types. *In vitro* assessment of pure cultures (i.e. neuronal, oligodendrocyte, astrocytic, microglial, etc.) enables investigators to identify particular lipid compositions under defined activation conditions, and thus these comparisons may be used to predict the source of changes observed at the regional tissue level [6,48,49]. Finally, subcellular fractionation allows precise investigation of membrane microdomains. These approaches have been used to isolate second messengers, for example those that interact with α-synuclein directly at synaptosomal membranes [50] or to profile the composition of a synaptic vesicle [15] or endonuclear compartment [51]. Taken together, these considerations highlight that perhaps the most important aspect of any lipidomic approach is consideration of the biological resolution afforded by the sample under investigation.

For lipidomics, the lipid extraction procedure should quantitatively extract cellular lipids without degradation and contamination with non-lipid components, such as free sugars and amino acids, that may suppress ionization. The effectiveness of the lipid extraction procedure depends to a large extent on the chemical nature of the lipid components and the organic solvent used. PCs are generally extracted using the Folch method or the Bligh and Dyer method [52,53]. These methods are based on chloroform–methanol protocols that include phase partitioning of lipids into the organic layer. For a large variety of physiologically relevant diacyl lipids, the original protocols work well [52,53]. Adaptations described in detail below, notably homogenization in acidified methanol, have been shown to improve recovery of low-abundance lipid second messengers with various chain linkages [54–57]. With all extraction protocols, it is very important to minimize the risk of oxidation of polyunsaturated fatty acids or lipid hydrolysis during extraction. Therefore, extraction should always be performed at low temperature (on ice or at 4°C) immediately after harvesting cultured cells or as soon as possible after tissue dissection. Post-mortem effects on second messenger profiles are a significant problem, as enzymatic (and non-enzymatic) breakdown of structural membrane lipids after death generate artificial lipidomic profiles. The same caveats apply to storage. If dissected tissue is not processed immediately, it should be snap-frozen in liquid nitrogen and stored at −80°C. Following extraction, lipid extracts must be protected from light and dried under a stream of nitrogen to prevent lipid peroxidation. However, care must be taken to avoid reducing the lipid extract to complete dryness or leaving it in this state for extended periods of time. Water cannot be introduced into the final lipid extract, as contamination by water, especially acidified water, results in

hydrolysis of fatty acyl chains, particularly hydrolysis of vinyl ether bonds [58,59]. This is extremely important when analysing low-molecular-weight lipid metabolites of membrane lipids, but may also be exploited experimentally to identify vinyl ether-linked species in parallel MS analyses.

The following is an example of our routine protocol for PC second messengers. Lipids are extracted using a modified Bligh and Dyer method [57]. It has been found that adding acetic acid to the methanol phase in which tissue is homogenized or cells are collected improves recovery of acidic phospholipids (without compromising recovery of neutral phospholipids), extraction efficiency, and the representation of low-abundance second messengers in addition to structural lipids, while enabling simultaneous extraction of second messengers with alkyl and alkenyl linkages in addition to the more abundant acyl-linked lipids [54–57]. At the time of extraction, acidified methanol is added directly to tissue or cells. It is essential to avoid extended exposure of samples to this acid condition, especially in aqueous solution, as this may lead to the hydrolysis of glycerophospholipids to lysolipids [58,59]. As an example, tissue is homogenized or cells are scraped in acidified methanol (2% acetic acid in methanol). Chloroform and 0.1 M sodium acetate are added such that the ratio of acidified methanol/chloroform/0.1 M sodium acetate is 1:0.95:0.8. Samples are vortexed and centrifuged (2 min at 800 ***g***). The lower phase is collected, and the samples are back-extracted three times with 2 mL of chloroform. The collected chloroform phases are pooled and evaporated to near dryness under a stream of nitrogen gas. The dried lipids are reconstituted with 300 μl anhydrous ethanol, and stored as 50 μl aliquots in amber glass vials filled with nitrogen gas at −80°C.

MS analysis

The main advantages of MS are the ability to separate and characterize charged analytes in the gas phase according to their mass to charge ratios (m/z). MS also provides structural information by fragmenting the ionized lipids through collision-induced dissociation. These attributes lead to unparalleled selectivity and sensitivity, and the ability to provide structural information for components in complex mixtures. MS lipidomics has been facilitated by the development of two 'soft' ionization techniques, electrospray ionization (ESI) [60] and matrix-assisted laser desorption/ionization (MALDI) [61]. Both ionization methods were developed to generate molecular ions without fragmentation during the ionization process, thus enabling high-mass and non-volatile compounds (such as lipids) to be amenable to MS analyses. ESI produces gas-phase ions from molecules in solutions, and is easily coupled to liquid chromatography (LC), making this technique the current workhorse in lipidomic research. On the other hand, MALDI allows production of intact gas-phase ions from samples embedded in a dry, crystalline matrix via laser pulses, and has been successfully used in MS imaging applications to map the spatial distribution of lipid molecules in frozen tissue sections at the regional level.

Three main MS-based lipidomics approaches are used for PC second messenger analysis: (a) direct-infusion MS (also known as shotgun lipidomics), (b) LC-MS, and (3) MALDI-MS (e.g. imaging MS). PCs are, for the most part, readily ionizable in positive ion mode, and may be analysed by MS scan, MS-MS, precursor ion scans or neutral loss scans in shotgun and LC-MS applications [6,55,62–66]. In the presence of ammonium-containing salts or weak acids, the fragmentation of protonated molecular ions of glycerophosphocholine (e.g. PC, LPC, PAF, lyso-PAF and related analogues) yields a characteristic peak at m/z 184 [67,68]. A precursor ion scan of 184 from the protonated ions is commonly used for the identification and quantification of choline-containing lipid species [62,69,70]. However, the collision energy needs to be carefully optimized for quantification [67,69]. Also, as with most quantitative methods, it is essential to work in the linear range of the quantification methodology.

Shotgun lipidomics employs direct-infusion MS without prior chromatographic separation of lipid classes, sub-classes or species [67,71–73]. This method may be used in conjunction with both precursor ion scans and neutral loss scans in positive ion mode for profiling PC second messengers [74]. Three primary approaches are used: tandem MS-based [62], multi-dimension mass spectrometry-based [75], and high-mass-resolution [69,76] shotgun lipidomics. These methods have been comprehensively reviewed [77,78]. Briefly, tandem MS identifies various classes of lipids through detection of the characteristic ion fragment associated with the polar head group (i.e. the characteristic m/z 184 peak for PC discussed above). This methodology has been used with considerable success to profile LPCs in various intracellular organelles, notably when combined with stable isotope labelling to quantify turnover of structural precursors [51,79]. Multi-dimension MS uses the characteristic fragment ions from the head group or neutral loss of the head group following collision-induced dissociation to identify phospholipid class, and precursor ion scans or neutral loss scans to identify fatty acyl carbon chain

composition [67,71]. This methodology has enabled identification and quantification of LPC species including *sn*-1 and *sn*-2 isomers [74]. High-mass-resolution shotgun lipidomics exploits the parallel acquisition (with high mass resolution) available to time of flight (TOF) analysers of product-ion spectrums for each protonated/deprotonated ion following direct infusion [69,76,80]. Targeted precursor ion scans (or neutral loss scans) within a narrow mass range of interest may be extracted and identification can then be reconstructed using commercially available bioinformatic packages [69,81]. Recently, hybrid linear trap/Orbitrap mass spectrometers, notably the newer Orbitrap Velos (ThermoScientific, Waltham, MA, USA), have been used for high-mass-accuracy selection of precursor ions followed by dissociation in a multipole collision cell and fragment ion detection in the mass analyser [78]. The advantages of these methodologies are their high sensitivity for identifying major target species with minimal sample loss and the ability to analyse relatively small sample amounts. However, limitations remain in terms of quantifying species present at relative low abundance, largely due to ion suppression from high-abundance species. Another limitation of direct-infusion MS lies in its inability to resolve isobaric species of the same family with the same exact mass, which in many cases may be important for identifying disease-related changes or distinguishing between species of identical elemental composition but with different linkages (i.e. PAFs versus LPCs). For example, using LC separation of lipid species prior to MS analysis, we demonstrated that the level of a specific PAF species, PC(O-16:0/2:0), was elevated in the temporal cortex of Alzheimer's disease patients and in the TgCRND8 transgenic mouse model [6]. This finding led to further mechanistic insight into how disruptions in lipid metabolism determine the neuronal response to accumulating toxic oligomeric amyloid β_{42} [6,82]. It is important to note that PC(18:0/0:0), the isobaric LPC species of the PAF PC(O-16:0/2:0), was present at much higher abundance in these samples, and it would have been extremely difficult to capture this change without prior knowledge of all of the PAFs and LPCs present in this sample or use of chromatographic separation of PC species prior to MS analysis. We detected multiple isobaric species in various tissue and plasma samples in the range m/z 450 to 600 that were not necessarily expected. Thus, for our projects, direct-infusion MS is not the ideal choice of methodology for discovery purposes, although, once a target lipidome is defined, shotgun lipidomics, notably in multiple reaction monitoring mode, may significantly accelerate analysis of known targeted species, with the caveat that linearity under multiple reaction monitoring mode by infusion of complex brain samples has not been established. Moreover, in instances when no isobaric overlaps within the same lipid family are expected to arise, direct infusion remains an optimal method for quantification with respect to high-throughput simultaneous identification. Conversely, LC-MS significantly reduces the complexity of the lipid mixture presented to the mass spectrometer for analysis. One advantage is that the chromatographic separation allows low-abundance species to be identified with less interference from the high-abundance lipid species. In fact, chromatographic separation before an MS analysis is still a pre-requisite for analysing lipids of very low abundance (picomolar, and, in some cases, attomolar levels) or lipids that require chemical derivatization to enhance ionization [83,84]. Taken together, LC-ESI-MS is currently the gold-standard technique for targeted lipidomics of second messengers when in 'discovery' mode with the research goal of identifying all possible species in a given tissue/sample, whereas shotgun methodologies enable high-throughput identification and quantification of changes in known profiles [6,68,85,86].

The parameters of the chromatographic separation (e.g. column retention time) provide additional information that assist in structural elucidation. Reverse-phase HPLC allows separation of PC species based on their fatty acyl moiety composition. Typically, the order of elution in a sub-class of lipids is related to the length of the fatty acyl chain, such that lipids with shorter fatty acyl chains elute faster than those with longer ones. Further, the higher the number of double bonds, the faster the lipid elutes in comparison to the saturated form. The recent introduction of ultra-high-performance LC, which provides enhanced peak separation capacity, will greatly improve separation of complex lipid samples for better MS results [87]. To avoid interference from other lipid classes present in complex lipid analytes, normal-phase HPLC, capable of separating glycerophospholipids based on their polar head groups, may also be used to separate PCs from others lipids prior to reverse-phase HPLC separation. However, excessive chromatographic separation may cause undesirable sample loss (i.e. oxidation, evaporation, etc.) that will skew final results.

One of the limitations of LC-ESI-MS is that little information pertaining to the assignment of the two fatty acyl constituents may be obtained from the fragmentation pattern. Traditionally, identification of individual lipid species within a complex biological lipid extract is expensive and time-consuming, requiring synthesis and spiking of isotopically labelled stan-

dards for each candidate lipid or parallel runs with and without unlabelled standards. Our group has recently developed a LC-ESI-MS^3 method that allows the rapid elucidation of the atomic connectivity of the fatty acid/alcohol substituent on the *sn*-1 position of LPCs and PAFs. This method utilizes multi-stage fragmentation of sodiated lipid species to reveal the identity of the parent molecule. We have demonstrated the effectiveness of this methodology to unambiguously identify isobaric LPC and PAF species within complex cellular and tissue lipid extracts [88]. There is also evidence that lyso-form fragment ions in MS^2 attributed to neutral loss of fatty acyl moieties may be used to further determine the stereospecificity of structural diacyl glycerophospholipids, although this methodology is not suited to the study of second messengers defined by their pre-existing hydroxyl moieties [89].

Finally, MALDI is another 'soft' ionization technique [61] that has been successfully used for lipid analysis. MALDI-TOF-MS has gained popularity in recent years in lipidomic studies, particularly in imaging MS applications, given its ability to determine the spatial distribution of target lipids on tissue sections [90–98]. MALDI is suitable for generating molecular ions, but may also be used to determine the head group and structural composition of individual fatty acyl chains when coupled to post-source decay fragmentation in a TOF-MS [99,100]. These attributes have made MALDI a popular choice for analysis of glycerophospholipids *in situ* [99–102]. As an example of application of this technique to study bioactive small lipid species, Koizumi *et al.* demonstrated that the amount of PC(16:0/0:0), an LPC, is increased in the area of focal cerebral ischemia in a rat stroke model, with a concomitant decrease in its structural membrane precursor PC(16:0/18:1) in the same area [103]. However, MALDI suffers from limited lateral resolution due to physical limitations and disturbances caused by application of the matrix and high noise at low mass [104]. Further, it is not suitable for large-scale profiling, as the tissue sections are destroyed by repetitive laser desorption/ionization, and it has yet to achieve resolution at the cellular or subcellular level.

Quantifying lipidomic profiles

A comprehensive targeted lipidomic approach involves both qualitative (e.g. lipid profiling and structure elucidation) and quantitative analysis. The latter includes measuring the absolute concentrations of target lipid species or the relative abundance of particular lipid species present relative to an exogenous normalization control in control versus treatment groups, or healthy versus diseased samples [62,65,105–107]. However, true absolute quantification of lipid species, while more challenging and something of a moving target given rapid advances in MS resolution, is essential for mathematical modelling of lipid signalling and metabolic pathways. Relative quantification, whether expressed in picomoles relative to standards or in arbitrary units, may be used to provide essential insight into cellular and tissue responses.

The question of lipid quantification is not trivial. One of the biggest challenges in lipidomics is the design of proper quantification protocols, for which a number of factors need to be taken into account. First, identical lipid species exhibit different behaviours when analysed on different types of mass spectrometers or even when analysed on the same mass spectrometer under different operating conditions [108,109]. Therefore, the relative intensities of signals corresponding to different lipid species in a mass spectrum do not directly reflect their molar abundances. It is also not possible to compare the relative abundances of different phospholipid species between sub-classes (i.e. LPCs versus PAFs or plasmenyl PCs) due to the differences in the ionization efficiencies of their structural determinants [110]. Certainly, absolute abundance in a given sample using a particular methodology may be established for various families following response factor correction using spiked internal standards [106,111]. However, we have found that response factors between PAFs, lyso-PAFs and plasmenyl PCs require correction to their own family of standards and cannot be extrapolated from LPC standards [6,55]. Thus, direct comparison of the calculated abundance of the same lipid species among different samples is possible, provided that the signals of the lipid species are normalized and standardized to appropriate internal standards [108,112]. For all quantitative analyses, non-naturally occurring species, such as those with fatty acid chains comprising odd numbers of carbons, are usually added as internal standards before lipid extraction to compensate for variations in sample preparation and in the ionization efficiency attributable to matrix components or machine instabilities. It is essential to use internal standards with similar instrument responses. For all calculations, the ratio of the peak area (or, in some cases, height) for analyte isoforms to that for the internal standards should be used.

Absolute quantification

Absolute quantification of specific lipid species relies on the availability of pre-existing lipid standards, preferably

as isotopically labelled forms as they exhibit identical responses in mass spectrometers to their natural forms as long as the isotopic labelling involves heavier atoms (usually ^{13}C). These standards may be added and compared to endogenous species in a single run. True absolute quantification requires addition of a reference standard for each lipid present in the analyte matrix, which is not practically feasible. This may be relaxed to use closely related compounds (homologues or simple structural analogues) for comparison to second messengers within sub-classes (i.e. a single spiked PAF to quantify multiple endogenous PAFs), as has been demonstrated for LPC quantification [106]. Care must be taken to ensure comparable ionization efficiency, which requires prior assessment. Liebisch *et al.* have validated this approach for LPCs [106], but such careful analysis has yet to be published for other second messenger families, sub-groups and sub-classes. For example, we have found that PAFs fragment with different efficiencies than lyso-PAFs. Thus, one cannot use PAF standards to assess the abundance of lyso-PAFs in lipid extracts [6].

After normalization of peak area for each species to that for the added internal standard to account for lipid extraction efficiency, the absolute concentration of a lipid species may be determined in comparison with a known amount of isotopically labelled lipid standards spiked at the time of the MS run by comparing the normalized peak areas of the paired labelled and native lipids [113]. It must be emphasized that this strategy is applicable only to a limited number of lipids in complex mixtures, as there are often far more target species than available standards. For example, in our routine LC-ESI-MS analysis of PC second messengers (m/z 450–600) in tissue and plasma, we detect ~ 30–60 species depending on the sample analysed. When analysing structural membrane PCs (m/z 700–900), well over 100 species are detected. Obviously, it is not feasible to quantify each species using corresponding standards as the spectrum is already too dense to accommodate all of the corresponding lipid standards. Thus the absolute concentrations of priority species may be determined using the standard addition method [106]. Here, calibration curves are first established by standard addition of a key unlabelled species to samples before lipid extraction. Linear regression in complex matrices across a concentration gradient of the target species enables back calculation of endogenous levels in the unspiked sample [106], and has been used to successfully quantify absolute levels of PC(O-16:0/0:0) and PC(O-16:0/2:0) in the temporal cortex of the TgCRND8 Alzheimer's disease transgenic mouse model [6].

Relative quantification

For the majority of experiments, absolute lipid levels are not required; instead, targeted lipidomic approaches are used to compare second messenger composition between basal and stimulated or normal and diseased samples [63,112,114]. Here, addition of one or two non-naturally occurring internal standards of the same class or sub-class as the second messenger sub-classes under investigation prior to lipid extraction is sufficient. As in proteomics, isotopic labelling (or derivitization) may be also used for relative quantification of a lipidome or to track the fate of fatty acid chains of phosphoglyceride backbones in order to understand metabolic pathways [115–117] and half lives of defining structural determinants within different cellular microdomains [118]. This method is also useful for producing a comprehensive panel of internal standards for quantitative profiling of phospholipids [114].

Data processing and lipidome characterization

Lipidomic data analysis is a daunting task, as the emerging field does not yet have the same level of bioinformatics support afforded to the more mature disciplines of proteomics and genomics. Targeted lipidomics of lipid second messengers faces three main analytical challenges: (a) a paucity of bioinformatic tools capable of spectral analysis (peak identification and isotopic deconvolution, notably for isobaric species), (b) a pressing need for accurate lipid prediction algorithms before experimenters can proceed to empirical validation, and (c) a requirement for visual and reference tools capable of displaying all theoretically possible lipid conformations in 2D and 3D and referencing existing literature with respect to known effects of individual species. The increasing demand for automated lipidomic data processing is leading to development of a number of software packages, some of which are briefly reviewed here; however, fundamental obstacles remain, such as the impossibility of querying PubMed using the now standard lipid nomenclature that adheres to the LIPID MAPS classification system [12].

Analytical software

LipidView (ABSciex, Framingham, MA, USA) is a commercial software package with a large built-in searchable database, encompassing more than 50 lipid classes and over 25 000 lipid species represented in a lipid fragments database. However, it lacks LC-MS output support, and datasets cannot be fed directly from spectra.

LipidSearch (http://metabo.umin.jp/index-English.htm) is a free online tool that was developed by Mitsui Knowledge Industry (Minato-Ku, Tokyo, Japan) in collaboration with the Taguchi laboratory (Biomolecular Engineering, University of Tokyo, Japan). LipidSearch is designed for identification of glycerophospholipids and sphingolipids. It is capable of analysing both LC-MS and direct-infusion MS spectra. There are three main components in the package: a mass navigator (a visualization and data analysis module), a lipid search tool (the core module for lipid identification), and a lipid database (storing theoretical masses and lipid identification results).

Spectrum Extraction from Chromatographic Data (SECD) and Lipid Mass Spectrum Analysis (LIMSA) are two other open-access software packages designed to perform lipid identification based on MS/MS spectra with LC-MS support [119,120]. SECD works with LC-MS data and can display results in a pseudo-3D map showing retention time, the m/z value and intensity. LIMSA is a dynamic lipid library that can be used alone for batch processing or in combination with other software such as SECD.

MZmine is a Java-based, platform-independent, open-source data processing software with algorithms for spectrum filtering, peak detection, isotopic correction, 2D and 3D map visualization, chromatographic alignment and data normalization [121]. It supports LC-MS output. Data analysis may be fully automated. However, not all identifications have been curated, and users must pay close attention to validating predicted identities.

The limitation of these programs is that the majority rely heavily on polynomial smoothing or subjective straight-line cut-off for background subtraction, which further complicates assessment of low-abundance isobaric second messengers. In summary, there is generally a lack of consensus in the lipidomics field on the choice of software and the optimal method for data processing.

Profiling the lipidomic landscape

In most published lipidomics papers, identified species are commonly reported only with the total number of carbons and unsaturation for each class or sub-class of lipids. This information alone is not sufficient for post-lipidomic experimental verification or assessment of second messenger signalling. Structural elucidation is essential to identify and characterize the biological relevance of various lipid species. It is easier to assign bond linkages, carbon chain positions and species identities to second messengers than it is for structural membrane lipids. Most small signalling lipid species have somewhat simpler and more predictable structures compared to high-molecular-weight membrane glycerophospholipids. For example, LPCs have only one fatty acid chain, which is usually acylated at the *sn*-1 position, while PAF species have a long-chain *sn*-1 alkylated carbon and an acetyl moiety at the *sn*-2 position. However, it must be recognized that structural isomers with reverse *sn*-1 and *sn*-2 chain linkages have been detected. Moreover, structural discrimination between even the canonical isobaric LPCs and PAFs may be difficult. We have recently developed a LC-ESI-MS^3 method that allows rapid elucidation of the atomic connectivity of the fatty acid/alcohol substituent on the *sn*-1 position of PC and PAFs. This method utilizes multi-stage fragmentation of sodiated lipid species to reveal the identity of the parent molecule. We have demonstrated the effectiveness of this methodology to unambiguously identify isobaric LPC and PAF species within complex cellular and tissue lipid extracts [88]. Another challenge is to precisely locate the position of double bonds on the fatty acid chains. A novel approach was recently reported that used ozone electrospray MS to distinguish lipid species that differed only in the sites of double bonds. In this method, the polyunsaturated double bonds are cleaved by ozonolysis during ESI, resulting in a pair of fragments that are diagnostic of the positions of double bonds [122,123].

There have also been advances in online structure prediction tools. The LIPID MAPS consortium hosts a large MS/MS database covering thousands of lipid species [124] that may be used to query expected lipids of a given m/z with particular acyl compositions [125]. Although comprehensive, the LIPID MAPS databases do not contain all theoretically possible lipid species. To address this need, our group has recently developed a Java-based online tool called Visualization and Phospholipid Identification (VaLID, https://www.med.uottawa.ca/lipidomics/resources.html#tools) that includes all theoretically possible phospholipids for any possible m/z value and MS condition [19]. A second recent tool is LipidHome, which generates all theoretically possible lipids currently being implemented with a MS search engine [126]. Together with in-house generated lists of curated species of second messengers, which, in VaLID's case, have been detected in central nervous system samples and validated by enzymatic hydrolysis, commercial standards or LC-ESI-MS^3, these tools are improving the confidence of post-spectral analysis peak assignment. In addition, visualization algorithms are provided to produce multiple chemical structure files for each species [19,126]. These

resources are useful for routine verification of m/z identifications.

Finally, to facilitate lipid-related pathway discovery, the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) maintains one of the most commonly used reference databases for lipid pathways. This database includes information on fatty acid biosynthesis and degradation, as well as information on sterols and phospholipids. One of its collections, KEGG Brite (http://www.genome.jp/kegg/brite.html), includes a option where users can select a lipid of interest and view reactions and pathways associated with that lipid.

Summary

Disturbances in lipid metabolism are now recognized to be primary determinants in the pathogenesis of many common diseases, such as diabetes, neurodegenerative diseases, cancer and atherosclerosis. The signalling pathways triggered by second messenger metabolites of structural membrane lipids may work in concert or opposition to protect or damage target cells and tissue. It is only with the development of new MS tools capable of distinguishing lipid identities at the molecular level that a true appreciation of the diversity (and importance) of the glycerophospholipid second messenger landscape has emerged. However, it must be emphasized that the field of lipidomics in general, and specifically the application of a targeted approach to second messenger profiling, encompasses not only identification and measurement of individual lipid isoforms within the target 'landscape' but also study of the mRNA and protein expression status of the metabolic enzymes, transporters, effectors and protein targets that affect regional lipid second messenger identity and downstream signalling. Further, a thorough lipidomic analysis also includes an unbiased assessment of lipid function ranging from the physicochemical basis of lipid behaviour to lipid–protein and lipid–lipid interactions, as well as the impact of dynamic lipid metabolism on cellular responses to intrinsic and extrinsic stimuli. Lipidomics is not simply about cataloguing lipids present in a biological system at a particular point in time. Advances in genomics have identified genetic determinants of neurodegenerative disease. Direct biochemical investigations have elucidated multiple signalling pathways altered by these genetic determinants leading to cognitive deterioration [127,128]. The combination of genomics with proteomics is being used to map the temporal changes in gene and protein expression that occur during transition from pre-symptomatic to symptomatic disease states. We argue that the next major advance in rational therapeutic design will result from tying the dynamics of the susceptible cellular metabolome into these genomic and proteomic maps of disease. Such insight represents a novel, potentially transformative, approach to treatment of neurodegenerative disease, for example harnessing endogenous mechanisms of resistance to prevent synaptic dysfunction. The emerging field of lipidomics seeks to address these important questions, identifying patterns of membrane disruption that render cells and tissue susceptible to genetic and environmental determinants of disease. Future studies must consider how to integrate these datasets into a framework of cellular metabolism to achieve these goals.

Acknowledgements

This study was supported by funding from the Canadian Institutes of Health Research (MOP-89999 to D.F. and S.B., and TGF-96121 to S.B., D.F. and S.F.). H.X. and N.V. received post-doctoral and post-professional fellowships, respectively, from the Canadian Institutes of Health Research Training Program in Neurodegenerative Lipidomics and the Canadian Institutes of Health Research Institute of Aging. H.X. received additional post-doctoral funding from the Mathematics of Information Technology and Complex Systems (MITACS).

References

1 Lehninger AL (1968) The neuronal membrane. *Proc Natl Acad Sci USA* **60**, 1069–1080.
2 Brown HA & Murphy RC (2009) Working towards an exegesis for lipids in biology. *Nat Chem Biol* **5**, 602–606.
3 Sagin FG & Sozmen EY (2008) Lipids as key players in Alzheimer disease: alterations in metabolism and genetics. *Curr Alzheimer Res* **5**, 4–14.
4 Sanchez-Mejia RO, Newman JW, Toh S, Yu GQ, Zhou Y, Halabisky B, Cisse M, Scearce-Levie K, Cheng IH, Gan L *et al.* (2008) Phospholipase A_2 reduction ameliorates cognitive deficits in a mouse model of Alzheimer's disease. *Nat Neurosci* **11**, 1311–1318.
5 Chan RB, Oliveira TG, Cortes EP, Honig LS, Duff KE, Small SA, Wenk MR, Shui G & Di Paolo G (2012) Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. *J Biol Chem* **287**, 2678–2688.
6 Ryan SD, Whitehead SN, Swayne LA, Moffat TC, Hou W, Ethier M, Bourgeois AJG, Rashidian J, Blanchard AP, Fraser PE, *et al.* (2009) Amyloid-β42 signals tau hyperphosphorylation and compromises

neuronal viability by disrupting alkylacylglycerophosphocholine metabolism. *Proc Natl Acad Sci USA* **106**, 20936–20941.
7 Denizot Y, Desplat V, Drouet M, Bertin F & Melloni B (2001) Is there a role of platelet-activating factor in human lung cancer? *Lung Cancer* **33**, 195–202.
8 Montrucchio G, Sapino A, Bussolati B, Ghisolfi G, Rizea-Savu S, Silvestro L, Lupia E & Camussi G (1998) Potential angiogenic role of platelet-activating factor in human breast cancer. *Am J Pathol* **153**, 1589–1596.
9 Tselepis AD & John Chapman M (2002) Inflammation, bioactive lipids and atherosclerosis: potential roles of a lipoprotein-associated phospholipase A2, platelet activating factor-acetylhydrolase. *Atheroscler Suppl* **3**, 57–68.
10 Roudebush WE, Massey JB, Elsner CW, Shapiro DB, Mitchell-Leef D & Kort HI (2005) The significance of platelet-activating factor and fertility in the male primate: a review. *J Med Primatol* **34**, 20–24.
11 Pereto J, Lopez-Garcia P & Moreira D (2004) Ancestral lipid biosynthesis and early membrane evolution. *Trends Biochem Sci* **29**, 469–477.
12 Fahy E, Cotter D, Sud M & Subramaniam S (2011) Lipid classification, structures and tools. *Biochim Biophys Acta* **1811**, 637–647.
13 LIPID Metabolites and Pathways Strategy (LIPID MAPS) (2013) Lipid classification system: glycerophospholipids [WWW document]. URL http://www.lipidmaps.org/data/classification/LM_classification_exp.php.
14 Igbavboa U, Hamilton J, Kim HY, Sun GY & Wood WG (2002) A new role for apolipoprotein E: modulating transport of polyunsaturated phospholipid molecular species in synaptic plasma membranes. *J Neurochem* **80**, 255–261.
15 Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P *et al.* (2006) Molecular anatomy of a trafficking organelle. *Cell* **127**, 831–846.
16 Lohmann C, Schachmann E, Dandekar T, Villmann C & Becker CM (2010) Developmental profiling by mass spectrometry of phosphocholine containing phospholipids in the rat nervous system reveals temporo-spatial gradients. *J Neurochem* **114**, 1119–1134.
17 Brand A, Crawford MA & Yavin E (2010) Retailoring docosahexaenoic acid-containing phospholipid species during impaired neurogenesis following omega-3 α-linolenic acid deprivation. *J Neurochem* **114**, 1393–1404.
18 Axelsen PH & Murphy RC (2010) Quantitative analysis of phospholipids containing arachidonate and docosahexaenoate chains in microdissected regions of mouse brain. *J Lipid Res* **51**, 660–671.
19 Blanchard AP, McDowell GS, Valenzuela N, Xu H, Gelbard S, Bertrand M, Slater GW, Figeys D, Fai S & Bennett SAL (2013) Visualization and Phospholipid Identification (VaLID): online integrated search engine capable of identifying and visualizing glycerophospholipids with given mass. *Bioinformatics* **29**, 284–285.
20 Farooqui AA & Horrocks LA (2006) Phospholipase A2-generated lipid mediators in the brain: the good, the bad, and the ugly. *Neuroscientist* **12**, 245–260.
21 Meyer zu Heringdorf D & Jakobs KH (2007) Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. *Biochim Biophys Acta* **1768**, 923–940.
22 Shimizu T, Ohto T & Kita Y (2006) Cytosolic phospholipase A2: biochemical properties and physiological roles. *IUBMB Life* **58**, 328–333.
23 Chen C & Bazan NG (2005) Lipid signaling: sleep, synaptic plasticity, and neuroprotection. *Prostaglandins Other Lipid Mediat* **77**, 65–76.
24 Lands WE (1958) Metabolism of glycerolipides; a comparison of lecithin and triglyceride synthesis. *J Biol Chem* **231**, 883–888.
25 Bae K, Longobardi L, Karasawa K, Malone B, Inoue T, Aoki J, Arai H, Inoue K & Lee T (2000) Platelet-activating factor (PAF)-dependent transacetylase and its relationship with PAF acetylhydrolases. *J Biol Chem* **275**, 26704–26709.
26 Ryan SD, Harris CS, Carswell CL, Baenziger JE & Bennett SAL (2008) Heterogeneity in the *sn*-1 carbon chain of platelet-activating factor glycerophospholipids determines pro- or anti-apoptotic signaling in primary neurons. *J Lipid Res* **49**, 2250–2258.
27 Brewer C, Bonin F, Bullock B, Nault M-C, Morin J, Imbeault S, Shen TY, Franks DJ & Bennett SAL (2002) Platelet activating factor-induced apoptosis is inhibited by ectopic expression of the platelet activating factor G-protein coupled receptor. *J Neurochem* **82**, 1502–1511.
28 Ryan SD, Harris CS, Mo F, Lee H, Hou ST, Bazan NG, Haddad PS, Arnason JT & Bennett SA (2007) Platelet activating factor-induced neuronal apoptosis is initiated independently of its G-protein coupled PAF receptor and is inhibited by the benzoate orsellinic acid. *J Neurochem* **103**, 88–97.
29 Southall MD, Isenberg JS, Nakshatri H, Yi Q, Pei Y, Spandau DF & Travers JB (2001) The platelet-activating factor receptor protects epidermal cells from tumor necrosis factor (TNF) α and TNF-related apoptosis-inducing ligand-induced apoptosis through an NF-κB-dependent process. *J Biol Chem* **276**, 45548–45554.
30 Li T, Southall MD, Yi Q, Pei Y, Lewis D, Al-Hassani M, Spandau D & Travers JB (2003) The epidermal

platelet-activating factor receptor augments chemotherapy-induced apoptosis in human carcinoma cell lines. *J Biol Chem* **278**, 16614–16621.
31 Piomelli D, Astarita G & Rapaka R (2007) A neuroscientist's guide to lipidomics. *Nat Rev Neurosci* **8**, 743–754.
32 Hou W, Zhou H, Elisma F, Bennett SAL & Figeys D (2008) Technological developments in lipidomics. *Brief Funct Genomics Proteomics* **7**, 395–409.
33 Blanksby SJ & Mitchell TW (2010) Advances in mass spectrometry for lipidomics. *Annu Rev Anal Chem* **3**, 433–465.
34 Bou Khalil M, Hou W, Zhou H, Elisma F, Swayne LA, Blanchard AP, Yao Z, Bennett SAL & Figeys D (2010) Lipidomics era: accomplishments and challenges. *Mass Spectrom Rev* **29**, 877–929.
35 Han X (2010) Multi-dimensional mass spectrometry-based shotgun lipidomics and the altered lipids at the mild cognitive impairment stage of Alzheimer's disease. *Biochim Biophys Acta* **1801**, 774–783.
36 Shevchenko A & Simons K (2010) Lipidomics: coming to grips with lipid diversity. *Nat Rev Mol Cell Biol* **11**, 593–598.
37 Astarita G & Piomelli D (2011) Towards a whole-body systems [multi-organ] lipidomics in Alzheimer's disease. *Prostaglandins Leukot Essent Fatty Acids* **85**, 197–203.
38 Lasiecka ZM, Yap CC, Vakulenko M & Winckler B (2009) Compartmentalizing the neuronal plasma membrane from axon initial segments to synapses. *Int Rev Cell Mol Biol* **272**, 303–389.
39 Mikawa S, Suzuki M, Fujimoto C & Sato K (2009) Imaging of phosphatidylcholines in the adult rat brain using MALDI-TOF MS. *Neurosci Lett* **451**, 45–49.
40 Isaac G, Pernber Z, Gieselmann V, Hansson E, Bergquist J & Mansson JE (2006) Sulfatide with short fatty acid dominates in astrocytes and neurons. *FEBS J* **273**, 1782–1790.
41 van der Knaap LJ & van der Ham IJ (2011) How does the corpus callosum mediate interhemispheric transfer? A review. *Behav Brain Res* **223**, 211–221.
42 van Groen T, Miettinen P & Kadish I (2003) The entorhinal cortex of the mouse: organization of the projection to the hippocampal formation. *Hippocampus* **13**, 133–149.
43 van Strien NM, Cappaert NL & Witter MP (2009) The anatomy of memory: an interactive overview of the parahippocampal–hippocampal network. *Nat Rev Neurosci* **10**, 272–282.
44 Trotter J, Karram K & Nishiyama A (2010) NG2 cells: properties, progeny and origin. *Brain Res Rev* **63**, 72–82.
45 Capogna M (2011) Neurogliaform cells and other interneurons of stratum lacunosum-moleculare gate entorhinal–hippocampal dialogue. *J Physiol* **589**, 1875–1883.
46 Doring A & Yong VW (2011) The good, the bad and the ugly. Macrophages/microglia with a focus on myelin repair. *Front Biosci* **3**, 846–856.
47 Ma MT, Yeo JF, Shui G, Wenk MR & Ong WY (2012) Systems wide analyses of lipids in the brainstem during inflammatory orofacial pain – evidence of increased phospholipase A_2 activity. *Eur J Pain* **16**, 38–48.
48 Gaudin M, Panchal M, Auzeil N, Duyckaerts C, Brunelle A, Laprevote O & Touboul D (2012) Choline-containing phospholipids in microdissected human Alzheimer's disease brain senile plaque versus neuropil. *Bioanalysis* **4**, 2153–2159.
49 Eberlin LS, Norton I, Dill AL, Golby AJ, Ligon KL, Santagata S, Cooks RG & Agar NY (2012) Classifying human brain tumors by lipid imaging with mass spectrometry. *Cancer Res* **72**, 645–654.
50 Wislet-Gendebien S, Visanji NP, Whitehead SN, Marsilio D, Hou W, Figeys D, Fraser PE, Bennett SAL & Tandon A (2008) Differential regulation of wild-type and mutant α-synuclein binding to synaptic membranes by cytosolic factors. *BMC Neurosci* **9**, 92.
51 Hunt AN & Postle AD (2006) Mass spectrometry determination of endonuclear phospholipid composition and dynamics. *Methods* **39**, 104–111.
52 Folch J, Arsove S & Meath JA (1951) Isolation of brain strandin, a new type of large molecule tissue component. *J Biol Chem* **191**, 819–831.
53 Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
54 Weerheim AM, Kolb AM, Sturk A & Nieuwland R (2002) Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. *Anal Biochem* **302**, 191–198.
55 Whitehead SN, Hou W, Ethier M, Smith JC, Bourgeois A, Denis R, Bennett SAL & Figeys D (2007) Rapid identification and quantitation of changes in the platelet activating factor family of glycerophospholipids over the course of neuronal differentiation by high performance liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem* **79**, 8359–8548.
56 Liu ST, Sharon-Friling R, Ivanova P, Milne SB, Myers DS, Rabinowitz JD, Brown HA & Shenk T (2011) Synaptic vesicle-like lipidome of human cytomegalovirus virions reveals a role for SNARE machinery in virion egress. *Proc Natl Acad Sci USA* **108**, 12869–12874.
57 Bonin F, Ryan SD, Migahed L, Mo F, Lallier J, Franks DJ, Arai H & Bennett SAL (2004) Anti-apoptotic actions of the platelet activating factor acetylhydrolase I α_2 catalytic subunit. *J Biol Chem* **279**, 52425–52436.

58 Ford DA, Rosenbloom KB & Gross RW (1992) The primary determinant of rabbit myocardial ethanolamine phosphotransferase substrate selectivity is the covalent nature of the sn-1 aliphatic group of diradyl glycerol acceptors. *J Biol Chem* **267**, 11222–11228.
59 Kayganich KA & Murphy RC (1992) Fast atom bombardment tandem mass spectrometric identification of diacyl, alkylacyl, and alk-1-enylacyl molecular species of glycerophosphoethanolamine in human polymorphonuclear leukocytes. *Anal Chem* **64**, 2965–2971.
60 Fenn JB, Mann M, Meng CK, Wong SF & Whitehouse CM (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246**, 64–71.
61 Karas M & Hillenkamp F (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem* **60**, 2299–2301.
62 Brugger B, Erben G, Sandhoff R, Wieland FT & Lehmann WD (1997) Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc Natl Acad Sci USA* **94**, 2339–2344.
63 Ivanova PT, Cerda BA, Horn DM, Cohen JS, McLafferty FW & Brown HA (2001) Electrospray ionization mass spectrometry analysis of changes in phospholipids in RBL-2H3 mastocytoma cells during degranulation. *Proc Natl Acad Sci USA* **98**, 7152–7157.
64 Taguchi R, Hayakawa J, Takeuchi Y & Ishida M (2000) Two-dimensional analysis of phospholipids by capillary liquid chromatography/electrospray ionization mass spectrometry. *J Mass Spectrom* **35**, 953–966.
65 Kerwin JL, Tuininga AR & Ericsson LH (1994) Identification of molecular species of glycerophospholipids and sphingomyelin using electrospray mass spectrometry. *J Lipid Res* **35**, 1102–1114.
66 Hsu FF, Turk J, Thukkani AK, Messner MC, Wildsmith KR & Ford DA (2003) Characterization of alkylacyl, alk-1-enylacyl and lyso subclasses of glycerophosphocholine by tandem quadrupole mass spectrometry with electrospray ionization. *J Mass Spectrom* **38**, 752–763.
67 Han X & Gross RW (2005) Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev* **24**, 367–412.
68 Pulfer M & Murphy RC (2003) Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev* **22**, 332–364.
69 Ejsing CS, Duchoslav E, Sampaio J, Simons K, Bonner R, Thiele C, Ekroos K & Shevchenko A (2006) Automated identification and quantification of glycerophospholipid molecular species by multiple precursor ion scanning. *Anal Chem* **78**, 6202–6214.
70 Welti R, Wang X & Williams TD (2003) Electrospray ionization tandem mass spectrometry scan modes for plant chloroplast lipids. *Anal Biochem* **314**, 149–152.
71 Han X & Gross RW (2005) Shotgun lipidomics: multidimensional MS analysis of cellular lipidomes. *Expert Rev Proteomics* **2**, 253–264.
72 Han X, Yang J, Cheng H, Ye H & Gross RW (2004) Toward fingerprinting cellular lipidomes directly from biological samples by two-dimensional electrospray ionization mass spectrometry. *Anal Biochem* **330**, 317–331.
73 Han X, Yang K & Gross RW (2008) Microfluidics-based electrospray ionization enhances the intra separation of lipid classes and extends identification of individual molecular species through multi-dimensional mass spectrometry: development of an automated high-throughput platform for shotgun lipidomics. *Rapid Commun Mass Spectrom* **22**, 2115–2124.
74 Yang K, Zhao Z, Gross RW & Han X (2009) Systematic analysis of choline-containing phospholipids using multi-dimensional mass spectrometry-based shotgun lipidomics. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**, 2924–2936.
75 Yang K, Cheng H, Gross RW & Han X (2009) Automated lipid identification and quantification by multidimensional mass spectrometry-based shotgun lipidomics. *Anal Chem* **81**, 4356–4368.
76 Schwudke D, Oegema J, Burton L, Entchev E, Hannich JT, Ejsing CS, Kurzchalia T & Shevchenko A (2006) Lipid profiling by multiple precursor and neutral loss scanning driven by the data-dependent acquisition. *Anal Chem* **78**, 585–595.
77 Han X, Yang K & Gross RW (2012) Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. *Mass Spectrom Rev* **31**, 134–178.
78 Schuhmann K, Herzog R, Schwudke D, Metelmann-Strupat W, Bornstein SR & Shevchenko A (2011) Bottom-up shotgun lipidomics by higher energy collisional dissociation on LTQ Orbitrap mass spectrometers. *Anal Chem* **83**, 5480–5487.
79 Postle AD & Hunt AN (2009) Dynamic lipidomics with stable isotope labelling. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**, 2716–2721.
80 Stahlman M, Ejsing CS, Tarasov K, Perman J, Boren J & Ekroos K (2009) High-throughput shotgun lipidomics by quadrupole time-of-flight mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**, 2664–2672.
81 Herzog R, Schuhmann K, Schwudke D, Sampaio JL, Bornstein SR, Schroeder M & Shevchenko A (2012)

LipidXplorer: a software for consensual cross-platform lipidomics. *PLoS ONE* **7**, e29851.
82 Kennedy MA, Kabani N, Lambert J-P, Swayne LA, Ahmed F, Figeys D, Bennett SAL, Bryan J & Baetz K (2011) Srf1 is a novel regulator of phospholipase D activity and is essential to buffer the toxic effects of C16:0 platelet activating factor. *PLoS Genet* **7**, e1001299.
83 Woo HK, Go EP, Hoang L, Trauger SA, Bowen B, Siuzdak G & Northen TR (2009) Phosphonium labeling for increasing metabolomic coverage of neutral lipids using electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* **23**, 1849–1855.
84 Chen S, Belikova NA & Subbaiah PV (2012) Structural elucidation of molecular species of pacific oyster ether amino phospholipids by normal-phase liquid chromatography/negative-ion electrospray ionization and quadrupole/multiple-stage linear ion-trap mass spectrometry. *Anal Chim Acta* **735**, 76–89.
85 Milne S, Ivanova P, Forrester J & Alex Brown H (2006) Lipidomics: an analysis of cellular lipids by ESI-MS. *Methods* **39**, 92–103.
86 Wenk MR (2005) The emerging field of lipidomics. *Nat Rev Drug Discov* **4**, 594–610.
87 Rainville PD, Stumpf CL, Shockcor JP, Plumb RS & Nicholson JK (2007) Novel application of reversed-phase UPLC-oaTOF-MS for lipid analysis in complex biological mixtures: a new tool for lipidomics. *J Proteome Res* **6**, 552–558.
88 Smith JC, Hou W, Whitehead SN, Ethier M, Bennett SA & Figeys D (2008) Identification of lysophosphatidylcholine (LPC) and platelet activating factor (PAF) from PC12 cells and mouse cortex using liquid chromatography/multi-stage mass spectrometry (LC/MS3). *Rapid Commun Mass Spectrom* **22**, 3579–3587.
89 Hou W, Zhou H, Khalil MB, Seebun D, Bennett SA & Figeys D (2011) Lyso-form fragment ions facilitate the determination of stereospecificity of diacyl glycerophospholipids. *Rapid Commun Mass Spectrom* **25**, 205–217.
90 Rujoi M, Estrada R & Yappert MC (2004) *In situ* MALDI-TOF MS regional analysis of neutral phospholipids in lens tissue. *Anal Chem* **76**, 1657–1663.
91 Jackson SN, Wang HY & Woods AS (2005) *In situ* structural characterization of phosphatidylcholines in brain tissue using MALDI-MS/MS. *J Am Soc Mass Spectrom* **16**, 2052–2056.
92 Cornett DS, Reyzer ML, Chaurand P & Caprioli RM (2007) MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. *Nat Methods* **4**, 828–833.
93 Isaac G, Jeannotte R, Esch SW & Welti R (2007) New mass-spectrometry-based strategies for lipids. *Genet Eng* **28**, 129–157.
94 McDonnell LA & Heeren RM (2007) Imaging mass spectrometry. *Mass Spectrom Rev* **26**, 606–643.
95 Schiller J, Suss R, Fuchs B, Muller M, Zschornig O & Arnold K (2007) MALDI-TOF MS in lipidomics. *Front Biosci* **12**, 2568–2579.
96 Chen J, Hsieh Y, Knemeyer I, Crossman L & Korfmacher WA (2008) Visualization of first-pass drug metabolism of terfenadine by MALDI-imaging mass spectrometry. *Drug Metab Lett* **2**, 1–4.
97 Whitehead SN, Chan KH, Gangaraju S, Slinn J, Li J & Hou ST (2011) Imaging mass spectrometry detection of gangliosides species in the mouse brain following transient focal cerebral ischemia and long-term recovery. *PLoS ONE* **6**, e20808.
98 Xu H, Kongmanas K, Kadunganattil S, Smith CE, Rupar T, Goto-Inoue N, Hermo L, Faull KF & Tanphaichitr N (2011) Arylsulfatase A deficiency causes seminolipid accumulation and a lysosomal storage disorder in Sertoli cells. *J Lipid Res* **52**, 2187–2197.
99 Fuchs B, Schober C, Richter G, Suss R & Schiller J (2007) MALDI-TOF MS of phosphatidylethanolamines: different adducts cause different post source decay (PSD) fragment ion spectra. *J Biochem Biophys Methods* **70**, 689–692.
100 Stubiger G, Pittenauer E & Allmaier G (2008) MALDI seamless postsource decay fragment ion analysis of sodiated and lithiated phospholipids. *Anal Chem* **80**, 1664–1678.
101 Li YL, Gross ML & Hsu FF (2005) Ionic-liquid matrices for improved analysis of phospholipids by MALDI-TOF mass spectrometry. *J Am Soc Mass Spectrom* **16**, 679–682.
102 Hayasaka T, Goto-Inoue N, Sugiura Y, Zaima N, Nakanishi H, Ohishi K, Nakanishi S, Naito T, Taguchi R & Setou M (2008) Matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight (MALDI-QIT-TOF)-based imaging mass spectrometry reveals a layered distribution of phospholipid molecular species in the mouse retina. *Rapid Commun Mass Spectrom* **22**, 3415–3426.
103 Koizumi S, Yamamoto S, Hayasaka T, Konishi Y, Yamaguchi-Okada M, Goto-Inoue N, Sugiura Y, Setou M & Namba H (2010) Imaging mass spectrometry revealed the production of lyso-phosphatidylcholine in the injured ischemic rat brain. *Neuroscience* **168**, 219–225.
104 Carado A, Passarelli MK, Kozole J, Wingate JE, Winograd N & Loboda AV (2008) C60 secondary ion mass spectrometry with a hybrid-quadrupole orthogonal time-of-flight mass spectrometer. *Anal Chem* **80**, 7921–7929.
105 Lehmann WD, Koester M, Erben G & Keppler D (1997) Characterization and quantification of rat bile phosphatidylcholine by electrospray-tandem mass spectrometry. *Anal Biochem* **246**, 102–110.

106 Liebisch G, Drobnik W, Lieser B & Schmitz G (2002) High-throughput quantification of lysophosphatidylcholine by electrospray ionization tandem mass spectrometry. *Clin Chem* **48**, 2217–2224.
107 Zacarias A, Bolanowski D & Bhatnagar A (2002) Comparative measurements of multicomponent phospholipid mixtures by electrospray mass spectroscopy: relating ion intensity to concentration. *Anal Biochem* **308**, 152–159.
108 DeLong CJ, Baker PR, Samuel M, Cui Z & Thomas MJ (2001) Molecular species composition of rat liver phospholipids by ESI-MS/MS: the effect of chromatography. *J Lipid Res* **42**, 1959–1968.
109 Larsen A, Uran S, Jacobsen PB & Skotland T (2001) Collision-induced dissociation of glycero phospholipids using electrospray ion-trap mass spectrometry. *Rapid Commun Mass Spectrom* **15**, 2393–2398.
110 Koivusalo M, Haimi P, Heikinheimo L, Kostiainen R & Somerharju P (2001) Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response. *J Lipid Res* **42**, 663–672.
111 Liebisch G, Lieser B, Rathenberg J, Drobnik W & Schmitz G (2004) High-throughput quantification of phosphatidylcholine and sphingomyelin by electrospray ionization tandem mass spectrometry coupled with isotope correction algorithm. *Biochim Biophys Acta* **1686**, 108–117.
112 Rouzer CA, Ivanova PT, Byrne MO, Milne SB, Marnett LJ & Brown HA (2006) Lipid profiling reveals arachidonate deficiency in RAW264.7 cells: structural and functional implications. *Biochemistry* **45**, 14795–14808.
113 Haroldsen PE, Clay KL & Murphy RC (1987) Quantitation of lyso-platelet activating factor molecular species from human neutrophils by mass spectrometry. *J Lipid Res* **28**, 42–49.
114 Ekroos K, Chernushevich IV, Simons K & Shevchenko A (2002) Quantitative profiling of phospholipids by multiple precursor ion scanning on a hybrid quadrupole time-of-flight mass spectrometer. *Anal Chem* **74**, 941–949.
115 Bleijerveld OB, Houweling M, Thomas MJ & Cui Z (2006) Metabolipidomics: profiling metabolism of glycerophospholipid species by stable isotopic precursors and tandem mass spectrometry. *Anal Biochem* **352**, 1–14.
116 DeLong CJ, Shen YJ, Thomas MJ & Cui Z (1999) Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. *J Biol Chem* **274**, 29683–29688.
117 Wenk MR, Lucast L, Di Paolo G, Romanelli AJ, Suchy SF, Nussbaum RL, Cline GW, Shulman GI, McMurray W & De Camilli P (2003) Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat Biotechnol* **21**, 813–817.
118 Ando S, Tanaka Y, Toyoda nee Ono Y, Kon K & Kawashima S (2002) Turnover of synaptic membranes: age-related changes and modulation by dietary restriction. *J Neurosci Res* **70**, 290–297.
119 Haimi P, Chaithanya K, Kainu V, Hermansson M & Somerharju P (2009) Instrument-independent software tools for the analysis of MS-MS and LC-MS lipidomics data. *Methods Mol Biol* **580**, 285–294.
120 Haimi P, Uphoff A, Hermansson M & Somerharju P (2006) Software tools for analysis of mass spectrometric lipidome data. *Anal Chem* **78**, 8324–8331.
121 Katajamaa M, Miettinen J & Oresic M (2006) MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. *Bioinformatics* **22**, 634–636.
122 Thomas MC, Mitchell TW & Blanksby SJ (2006) Ozonolysis of phospholipid double bonds during electrospray ionization: a new tool for structure determination. *J Am Chem Soc* **128**, 58–59.
123 Thomas MC, Mitchell TW, Harman DG, Deeley JM, Murphy RC & Blanksby SJ (2007) Elucidation of double bond position in unsaturated lipids by ozone electrospray ionization mass spectrometry. *Anal Chem* **79**, 5013–5022.
124 Sud M, Fahy E, Cotter D, Brown A, Dennis EA, Glass CK, Merrill AH Jr, Murphy RC, Raetz CR, Russell DW *et al.* (2007) LMSD: LIPID MAPS structure database. *Nucleic Acids Res* **35**, D527–D532.
125 Fahy E, Sud M, Cotter D & Subramaniam S (2007) LIPID MAPS online tools for lipid research. *Nucleic Acids Res* **35**, W606–W612.
126 Foster JM, Moreno P, Fabregat A, Hermjakob H, Steinbeck C, Apweiler R, Wakelam MJ & Vizcaino JA (2013) LipidHome: a database of theoretical lipids optimized for high throughput mass spectrometry lipidomics. *PLoS ONE* **8**, e61951.
127 Nimmrich V & Ebert U (2009) Is Alzheimer's disease a result of presynaptic failure? Synaptic dysfunctions induced by oligomeric β-amyloid. *Rev Neurosci* **20**, 1–12.
128 Kim D & Tsai LH (2009) Bridging physiology and pathology in AD. *Cell* **137**, 997–1000.
129 Kita Y, Ohto T, Uozumi N & Shimizu T (2006) Biochemical properties and pathophysiological roles of cytosolic phospholipase A2s. *Biochim Biophys Acta* **1761**, 1317–1322.