2-1-2022

# The foundations and development of lipidomics

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*Thematic Review Series: Lipidomics: General Introduction*

# The foundations and development of lipidomics

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**Abstract For over a century, the importance of lipid metabolism in biology was recognized but difficult to mechanistically understand due to the lack of sensitive and robust technologies for identification and quantification of lipid molecular species. The enabling technological breakthroughs emerged in the 1980s with the development of soft ionization methods (Electrospray Ionization and Matrix Assisted Laser Desorption/Ionization) that could identify and quantify intact individual lipid molecular species. These soft ionization technologies laid the foundations for what was to be later named the field of lipidomics. Further innovative advances in multistage fragmentation, dramatic improvements in resolution and mass accuracy, and multiplexed sample analysis fueled the early growth of lipidomics through the early 1990s. The field exponentially grew through the use of a variety of strategic approaches, which included direct infusion, chromatographic separation, and charge-switch derivatization, which facilitated access to the low abundance species of the lipidome. In this Thematic Review, we provide a broad perspective of the foundations, enabling advances, and predicted future directions of growth of the lipidomics field.**

**Supplementary key words** lipids • lipid metabolism • mass spectrometry • soft ionization • electrospray ionization • matrix-assisted laser desorption/ionization • shotgun lipidomics • chromatographic separation • charge-switch derivatization

## COMPLEXITY OF LIPID BIOCHEMISTRY

Cellular lipids are comprised of a plethora of unique structures that contain many hundreds of thousands (or more) distinct lipid molecular species (1). Historically, lipids have been broken down into nonpolar lipids (e.g., triglyceride (TG) and cholesterol) and polar lipids (choline glycerophospholipid (PC), ethanolamine glycerophospholipid (PE), inositol glycerophospholipid (PI), etc.) (2). In 2005, the LIPID MAPS consortium published a classification scheme that placed individual lipid molecular species into eight categories, including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, and saccharolipids (1). In each category, individual lipid molecular species are further divided into lipid classes based on their polar head groups. For example, glycerophospholipids are grouped the classes of PC, PE, PI, serine glycerophospholipid (PS), etc. according to their polar head groups containing phosphocholine, phosphoethanolamine, phosphoinositol, phosphoserine, and others, respectively, linked to a glycerol backbone (**Fig. 1**).

In addition, owing to the existence of some special structural similarity or unique characters, the molecular species in an individual class can be further classified into subclasses of the class. For example, based on the existence of different chemical bonds of the aliphatic chain to the *sn*-1 glycerol hydroxy group in glycerophospholipids, individual classes in this category are further grouped into three specific subclasses (Fig. 1). Specifically, the aliphatic chain at the *sn*-1 position of glycerol can be linked as a bond of ester, ether, or vinyl ether moiety in individual glycerophospholipid classes, which are termed as phosphatidyl-, plasmanyl-, or plasmenyl-, respectively, according to the recommended nomenclature by International Union of Pure and Applied Chemists (3). Similar subclasses of glycerolipids are also present in the classes of diglyceride (DG) and TG (4). In addition, due to differences in the backbone of sphingosine or sphinganine (i.e., with or without existence of a double bond between C4 and C5 of sphingoid base), an individual sphingolipid class can also be grouped into sphingolipid and dihydrosphingolipid subclasses.

A large number of aliphatic chains are present in lipids varying with lengths, degrees of unsaturation, locations of double bonds, *cis-trans* isomers, branched chains, etc. These variable aliphatic chains form a large number of individual molecular species. It can be readily derived that a total of $N^3$ and $N^4$ distinct molecular species (where N is the number of different

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 https://doi.org/10.1016/j.jlr.2021.100164

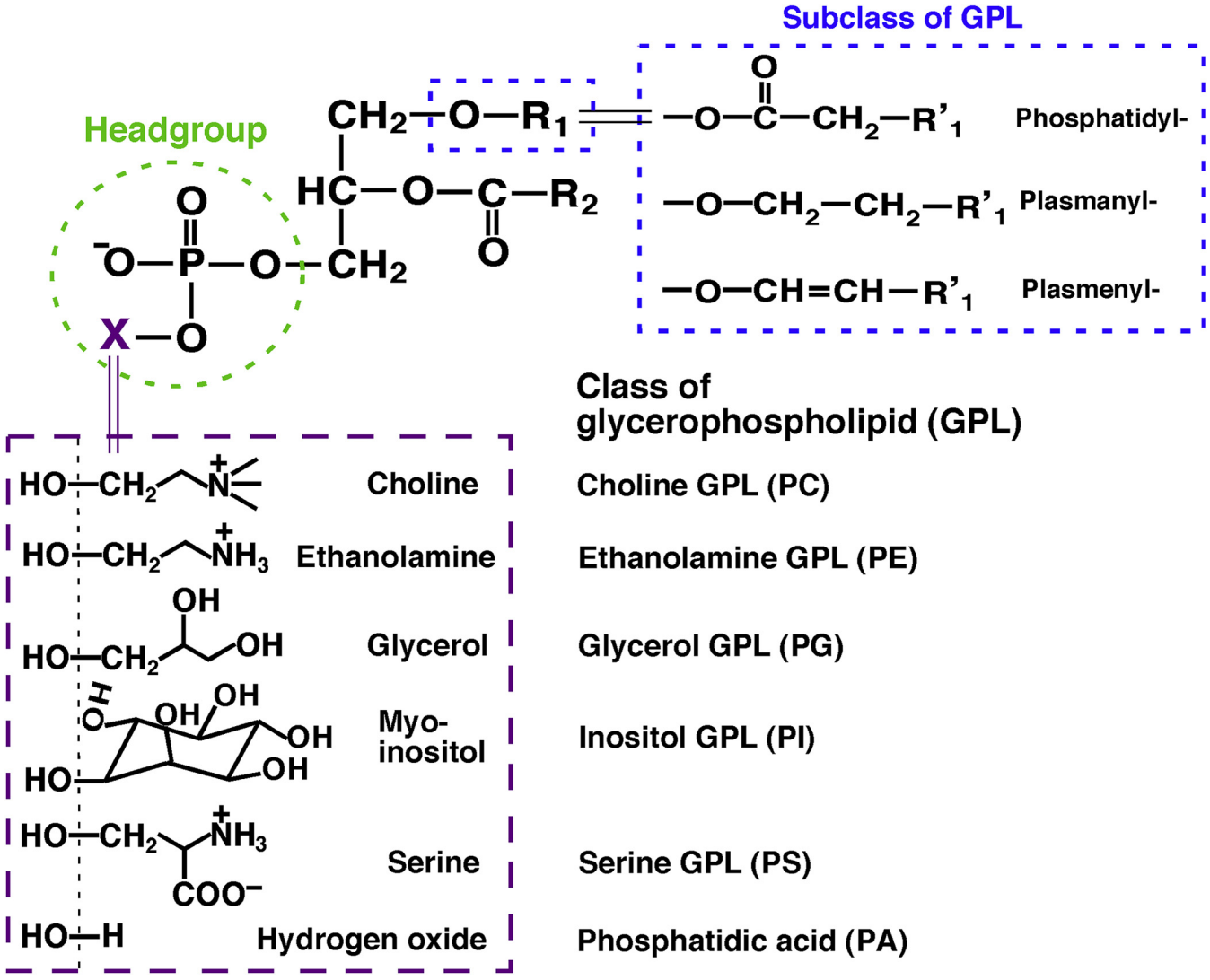


**Fig. 1.** Summary of classes, subclasses, and molecular species in glycerophospholipid. The polar moiety (X), which is connected to the phosphate group, defines the individual class of glycerophospholipid (GPL). The linkage (i.e., ester, ether, and vinyl ether) of the aliphatic chain to the hydroxy group at the *sn*-1 position of glycerol defines the structure of an individual subclass of phosphatidyl-, plasmanyl-, or plasmenyl-, respectively. The identities at $R_1$ and $R_2$, which vary with different number of carbon atoms, number of double bonds, the location of the double bonds, *etc.* define the individual molecular species of each lipid class.

aliphatic chains) can potentially be present in the classes of TG (5) and cardiolipin (6), respectively. In fact, the presence of hundreds of individual plasmenyl PE species in mammalian organs has previously been demonstrated (7).

Further excitement arose through the demonstration that many factors of biological and chemical significance could influence the variations in cellular lipids, which extended their biological effects through altering membrane charge, dynamics, and physical properties. For example, different organisms, cell types, intracellular organelles, subcellular membrane compartment, and membrane microdomains (e.g., caveola and/or rafts) contain very distinct cellular lipid molecular species and composition (8–10). These numerous molecular species change dynamically after perturbation of the environment, nutrient source, or intermolecular interactions (11–14).

The complexity of the effects of lipids on biological processes is due, in part, to their distinct physical and chemical properties that are enabling for essential cellular functions (**Fig. 2**) (16). These include, but are not limited to, the following: *i*) forming cellular membranes in biological organisms and organelles to provide hydrophobic barriers to separate cellular compartments and establish membrane potentials; *ii*) dynamically modulating cellular membrane physical properties to provide necessary matrices to regulate the activity of transmembrane proteins; *iii*) serving as intra- and extra-cellular lipid second messengers during signal transduction; *iv*) providing energy storage depots for cellular metabolism used in many biological processes; and *v*) anchoring many proteins binding to the cellular surface and membranes. Numerous studies demonstrated that, in addition to the lysosomal storage diseases (17), aberration of lipid metabolism and

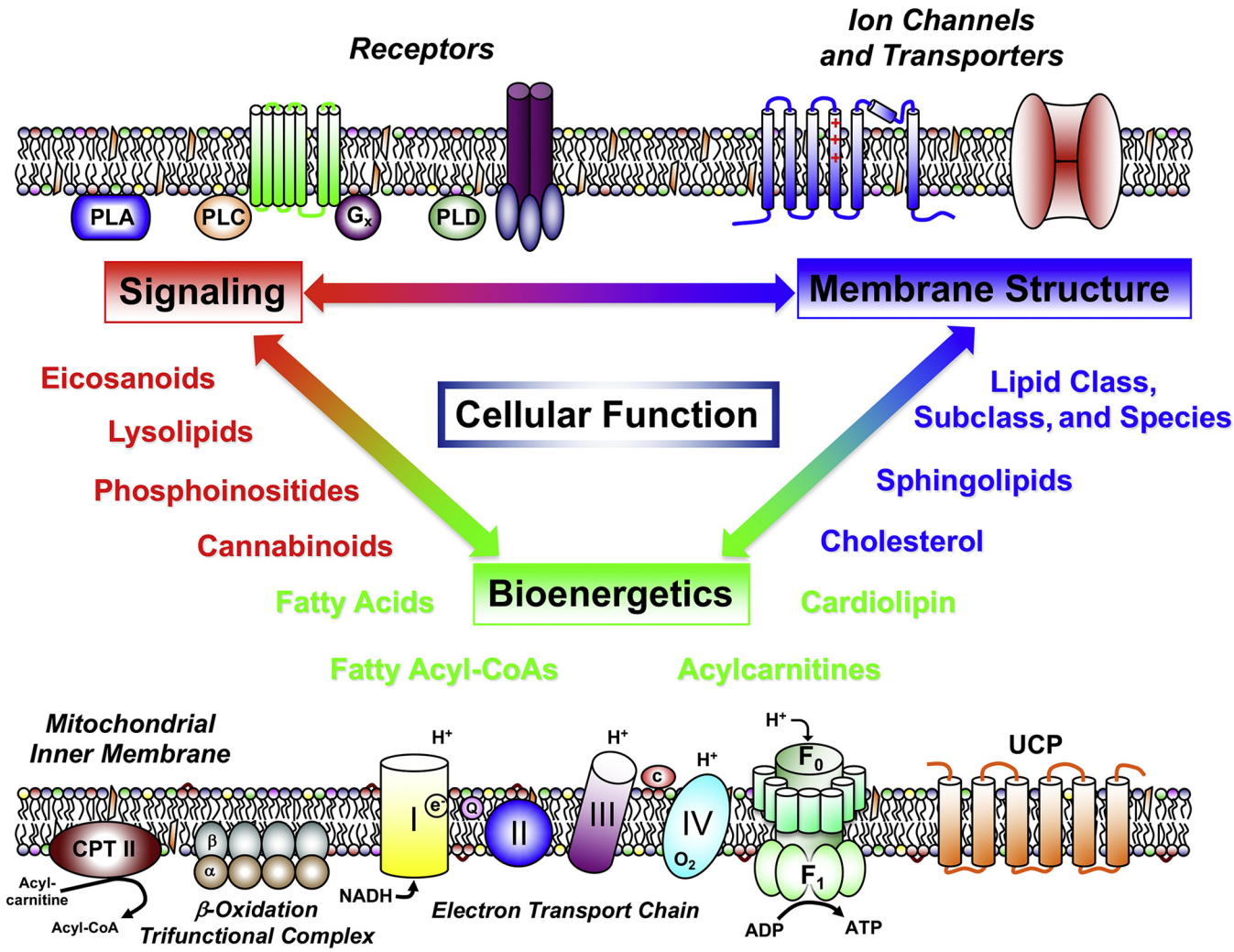


**Fig. 2.** The pleiotropic roles of lipids in cellular functions. Lipids fulfill multiple roles in cellular function including cellular signaling (top left) through the following: *i*) harboring latent second messengers of signal transduction that are released by phospholipases; *ii*) covalent transformation of membrane lipids into biologically active ligands by kinases (e.g., PI 3,4,5-triphosphate); *iii*) providing molecular scaffolds for the assembly of protein complexes mediating receptor/effector coupling (e.g., G protein-coupled receptors); and *iv*) coupling the vibrational, rotational, and translational energies and dynamics of membrane lipids to transmembrane proteins such as ion channels and transporters (top right), thereby facilitating dynamic cooperative lipid-protein interactions that collectively regulate transmembrane protein function. Moreover, lipids play essential roles in mitochondrial cellular bioenergetics (bottom) through the use of fatty acids as substrates for mitochondrial β-oxidation (bottom left) that result in the production of reducing equivalents (e.g., NADH). The chemical energy in NADH is harvested through oxidative phosphorylation whose flux is tightly regulated by mitochondrial membrane constituents including cardiolipins, which modulate electron transport chain (ETC) supercomplex formation. A second mechanism modulating mitochondrial energy production is the dissipation of the proton gradient by the transmembrane flip-flop of fatty acids in the mitochondrial inner membrane bilayer and the fatty acid-mediated regulation of uncoupling proteins (UCP). Reprinted with permission from ref. (15). Copyright 2011 Elsevier Ltd.

homeostasis is associated with many human diseases (e.g., diabetes and obesity, atherosclerosis and stroke, cancer, psychiatric disorders, neurodegenerative diseases and neurological disorders, and autoimmune disorders) (see recent reviews for references (18–25)), clearly demonstrating that lipids play many vital roles in human health and disease.

## LIPIDOMICS AND ITS RELATIONSHIP WITH METABOLOMICS

The majority of early studies on lipid biochemistry focused on one molecular species, one class, or one enzyme-mediated pathway. In those studies, researchers have clearly recognized that the metabolism of lipid species and specific molecular classes and subclasses is interwoven. To understand the biological relevance of these changes demands a comprehensive study on lipid metabolism that greatly catalyzed the emergence of lipidomics. Those pioneering studies on lipid homeostasis, signaling, and transport truly demonstrated the power of lipidomic analysis by using a variety of tools. Most importantly, those studies also provided the initial insight into the utility of identifying alterations in membrane structures and functions that mediate salutary biological

responses in health and maladaptive alterations during disease.

The term "lipidome", which refers to the entire collection of chemically distinct lipid molecular species in a cell, an organ, or a biological system, first appeared in the peer-reviewed publication in 2001 (26). The term "functional lipidomics" was described as "the study of the role played by membrane lipids" by Rildfors and Lindblom in 2002 (27). In 2003, Han and Gross outlined the scope of the research in lipidomics discipline through incorporating multiple techniques to "*i*) quantify the precise chemical constituents in a cell's lipidome, *ii*) identify their cellular organization (subcellular membrane compartments and domains), *iii*) delineate the biochemical mechanisms through which lipids interact with each other and with crucial membrane-associated proteins, *iv*) determine lipid-lipid and lipid-protein conformational space and dynamics, and *v*) quantify alterations in lipid constituents after cellular perturbations" in their thematic review (11). This definition identifies lipidomics as a new field heavily relying on the tools, technologies, and principles of analytical chemistry for the analysis of lipid structures, abundance of discrete molecular species, cell functions, and interactions that collectively identify the dynamic changes of lipids during cellular perturbations. Accordingly, lipidomics plays an essential role in defining the biochemical mechanisms underlying lipid-related disease processes through identification and quantification of alterations in cellular lipid signaling, metabolism, trafficking, and homeostasis.

Because lipids are cellular metabolic products, lipidomics falls under the larger umbrella of the general field of "metabolomics". In fact, most metabolomics studies cover the analysis of some classes of lipids or expand lipid analysis to a certain degree (28). However, special physical and chemical characteristics of lipids in contrast to most other water-soluble cellular metabolites make lipidomics a distinct discipline from general metabolomics, as discussed below.

As previously stated, each of most lipid classes may contain hundreds to thousands or many tens of thousands of individual lipid molecular species. This large number of individual molecular species in a class are formed by de novo synthesis, phospholipase-acyltransferase remodeling (e.g., glycerophospholipids) or represent the consequence of different isoforms of enzymatic activity (e.g., sphingolipids). From a chemical perspective, an individual node of a metabolic network indicates the transformation of a molecule connected by upstream or downstream metabolites. Unfortunately, neither the content of individual lipid molecular species nor the mass of an entire lipid class can provide information on the flux into and out of a metabolic node. Typically, analysis of metabolic flux requires measurement of stable isotope labeling from pulse-chase experiments. Thus, an understanding of metabolism in lipidomics is much more complex than that in metabolomics.

In contrast to other cellular metabolites, lipid molecular species which contain at least one hydrophobic region are extractable with some type of organic solvents to a certain degree, and their solubility is dependent on the polar water-soluble group. This physical property makes the majority of lipids readily recovered and largely separated from other cellular metabolites. However, this amphiphilic structure also readily forms aggregates even in organic solvents as the concentration of lipids increases and exceeds the ability of a given solvent to solubilize them as monomers (16). This physical characteristic makes quantification of intact individual lipid molecular species difficult and inaccurate by MS under certain conditions since different aggregates show very different ionization response factors (29). Furthermore, the existence of a large number of isomeric/isobaric lipid species in cellular lipidomes makes very challenging and special in comparison to metabolomics.

## THE HISTORY OF LIPIDOMICS DEVELOPMENT

### Classical lipid analysis with a variety of analytical techniques

In the history of lipid analysis, many tools including gas chromatography (GC), HPLC, TLC, MS with different types of ion sources (e.g., electron ionization, chemical ionization, field desorption, thermal desorption, etc.), nuclear magnetic resonance, spectroscopic techniques, etc. have all been used for lipid analysis alone or coupled each other (2, 30, 31). Each of these techniques played important roles historically in elucidating lipid structures, identifying new lipids, and quantifying lipid abundance with appropriate internal standard(s) and with noted limitations (32, 33).

With the development of fast atom bombardment-MS and later atmospheric pressure chemical ionization-MS coupled with HPLC (online or offline), analysis of intact lipid molecular species became possible (34–41) without interfering amounts of in-source fragmentation. Using these techniques, class-specific analysis of individual molecular species was also practically performed with neutral loss or precursor ion monitoring of polar head groups (36). Remarkably, a prototype of functional lipidomics was demonstrated through profiling intact phospholipid molecular species. In brief, Gross exploited the power of HPLC and fast atom bombardment-MS and identified plasmalogens as the major phospholipid constituents of sarcolemma (34), sarcoplasmic reticulum, and their presence in mitochondrial membranes (35) of canine ventricular myocardium. Those studies demonstrated that arachidonic acid was highly enriched in plasmalogen molecular species in sarcolemma and sarcoplasmic reticulum. Because the release of

arachidonic acid from their storage depots is the rate-limiting step in signal transduction in eicosanoid cascade, the aforementioned findings presaged the identification of plasmalogen-selective phospholipases in the release of arachidonic acid from plasmalogen species (42–44). Furthermore, through the use of specifically labeled diacyl and plasmalogen molecular species markedly differential molecular dynamics and averaged conformational states were identified for plasmalogens in comparison to their diacyl counterparts (45–50).

### Prelipidomics era (before 2003) by ESI-MS and MALDI-MS analysis

Analysis of intact cellular lipids was greatly accelerated with the advances in ionization technologies, which was in large part fueled by the development of ESI and MALDI by Nobel Laureates, John Fenn, and Koichi Tanaka (e.g., (51, 52)), respectively, in late 1980's. This is largely because of their much softer ionization and easier practice than any other previously developed ionization techniques. Applications of these techniques for the analysis of lipid species sequentially occurred in literature in early 1990 as exemplified as follows:

In 1991, Henion's team, in collaboration with Shieh, analyzed the standard mixtures of monoglyceride, DG, and TG in chloroform/methanol (7:3, v/v) after direct infusion with a Sciex TAGA 6000E triple-quadrupole mass spectrometer (53). This represents one of the earliest studies on neutral lipids by ESI-MS. They made four novel findings, including *i*) there was minimal in-source fragmentation; *ii*) the response factors of molecular ions depended on the polarity of the classes and on the number of double bonds; *iii*) magnitude of ionization was inversely correlated to the acyl-chain length (54); and *iv*) molecular ions with sodium or ammonium adducts could be readily formed. At a similar time, Weintraub's group characterized the fragmentation pattern of sodiated platelet activation factor (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) by ion source-induced fragmentation with a single quadrupole ESI mass spectrometer, which was further validated with experiments using a triple-quadrupole mass spectrometer (55).

Not long afterward, Han and Gross (56) reported quantitative analysis of phospholipids using ESI in both positive- and negative-ion modes and demonstrated the profiles of phospholipids in lipid extracts from human erythrocyte plasma membrane as sodium adducts in the positive-ion mode and deprotonated ions in the negative-ion mode with a Finnigan (now ThermoFisher) mass spectrometer using direct infusion. In their studies, they recognized the impact of the dipole in phospholipid head groups and electric field-induced charge separation on ionization of many cellular polar lipids. By appropriate matrix adjustments of lipid solutions (i.e., changing solution pH or ionic strength), the selective ionization of different lipid classes and/or categories was accomplished. This selective ionization of lipid classes represented a de facto separation method executed within the instrument's ion source and has been referred to as intrasource separation (57–59). This concept was developed into an important component of a shotgun lipidomics technology called "multi-dimensional mass spectrometry-based shotgun lipidomics (MDMS-SL)" (60, 61). This technology enabled the accurate identification and quantification of hundreds to thousands of individual lipid molecular species directly from the extracts of biological samples. Examples from the early studies include changes of cellular lipidomes after activation of platelets (62) and in diabetic myocardium (63).

At a similar time, LC separation coupled with ESI-MS analysis was first reported by Kim *et al.* (64) after their successful application of LC-thermospray MS analysis of phospholipids (65, 66). In this first study, the researchers exploited a C18 column to separate individual phospholipid molecular species and monitored protonated and/or sodiated molecular ions. In the study, they characterized the fragments from molecular ions after collision-induced dissociation (CID) in the positive-ion mode. Furthermore, they examined the differences of dynamic range and response factors between lipid classes and between molecular species in a class after optimization of experimental conditions. The developed LC-ESI/MS method was then applied for determining phospholipid molecular species in C-6 glioma cells after incubation with 22:6 fatty acid for different time intervals. LC-ESI-MS/MS was soon applied for the analysis of long chain fatty acids and their esters (67). Characterization and quantitative comparison of PE, lysocardiolipin, and cardiolipin molecular species from lipid extracts from cytoplasmic membrane of bacteria offline after HPLC separation were also conducted by ESI-MS at the similar time (68).

At this early period, characterization of commercially available lipid species was reported in many publications. Kerwin *et al.* (69) characterized major phospholipid classes including their subclass constituents, as well as SM and ceramide molecular species in both positive- and negative-ion modes with different adducts of molecular ions after CID. Han and Gross (70) reported the fragmentation patterns of PC, PE, and SM from sodiated and chlorinated molecular ions, and deprotonated phosphatidic acid, PS, and cardiolipin after CID. The group determined the ratios of fatty acyl carboxylates yielded from *sn*-1 and -2 positions for discrimination of phospholipid regioisomers and proposed potential fragmentation mechanisms. These studies led to develop a method for discriminating the regioisomers of lysoPC (71). Different from these studies on characterizing broad lipid classes, deep characterization of lipids class by class was begun in this period. For example, Hsu and Turk started this type of research on

sulfatide (72) and TG molecular species (73) and then virtually on all the classes of phospholipids, sphingolipids, and glycero- and glyco-lipids, and unraveled the fragmentation mechanisms of these classes (see the review (74) for an example). Murphy *et al.* characterized the location of double bond(s) in fatty acyl-chains of glycerophospholipids after reaction with ozone (75) and the fragmentation patterns of glycerophospholipids esterified with eicosanoids (76). It should be mentioned that MALDI-MS after postsource decay was also applied for profiling and/or characterization of glycerophospholipid and sphingolipid fragmentation (77–79), which was clearly served as a foundation for the applications of MALDI-MS for biological samples (80, 81).

In addition to the aforementioned intrasource separation for large scale analysis of lipids, other technologic developments for large scale lipid analysis were successfully pursued. This includes prototypes of shotgun lipidomics, which includes class-specific, MDMS-SL, and a new method based on high mass accuracy/resolution spectrometry as briefed below:

In 1997, Brügger *et al.* (82) described the method for profiling of different classes of glycerophospholipids via neutral loss or precursor ion scanning of a head group fragment specific to a class of lipids to a certain degree. With addition of two internal standards to correct for different ionization response factors of individual lipid species in the class, the team achieved the quantitation of these species present in a biological sample. This method becomes one of the shotgun lipidomics approaches (83) and has been applied for many lipidomic studies (84–87).

In 2001, Han and Gross (54) described an approach for quantitative analysis of individual TG analysis using 2D MS constituted with all neutral loss scans of fatty acyls in the TG pool. In the method, the researchers mathematically presented the different types of correction factors for $^{13}C$ isotopolog distribution at the first time and also demonstrated the correlation of response factor of lithiated TG molecular ions with the total numbers of double bonds and carbon atoms in the fatty acyl-chains of individual TG species. Unfortunately, it should be pointed out that the molecular species-dependent ionization response factors have not been well recognized in the lipidomics community. This 2D MS method for analysis of TG species combined with a simulation strategy enabled scientists to identify and quantify hundreds to thousands of individual TG species (88). Moreover, the principles described in the development of MDMS-SL were broadly applied in ratiometric measurements with appropriate internal standards (29, 57, 60, 89).

In 2002, Ekroos *et al.* (90) described a method for quantitative profiling of total extracts of endogenous phospholipids, in which simultaneous acquisition of precursor ion spectra of multiple fragment ions using a quadrupole time-of-flight mass spectrometer to detect major classes of phospholipids in a single experiment. A mixture of isotopically labeled endogenous lipids was used as comprehensive internal standards. This method was expanded to use other strategies such as the inclusion of neutral loss scanning, data-dependent driving, all ion fragmentation, etc. using high mass accuracy/resolution instruments such as quadrupole-orbitrap type instruments (91–94).

There were two articles published in the early period of lipidomics development worthy to be mentioned. The study by Koivusalo *et al.* (95) extensively determined the effects of fatty acyl-chain lengths and lipid concentration on ionization response factors using a mixture of 14 PC molecular species. They found that response factors decreased as chain length increased, and the increased concentrations led to marked reduction of response factors of PC molecular species containing longer fatty acyl-chains. Unfortunately, two important factors regarding the processing of data failure to correct for $^{13}C$ isotopolog distribution and lipid aggregation were not suitably considered. In contrast, a study by Delong *et al.* (96) demonstrated that LC separations resulted in variable losses of different classes of lipids. Further, they demonstrated the concentration-dependent linearity in the low concentration regime and the effects of CID energy on response factors. The knowledge obtained from these early studies greatly impacted the development of lipidomics and the broad utility of ESI-MS.

There were many studies during this early era of lipidomics that increased the power of this technology including large scale analyses of lipids, applications of ESI-MS for understanding biological phenomena, and the use of MALDI-MS with appropriate matrices for lipid analysis. Readers interested in these studies should find them in the literature in early review articles published in early 2000 (58, 80, 97, 98).

## The emerging of lipidomics and names of various "types" of lipidomics

Despite the appearance of the terms "lipidome" (26), "functional lipidomics" (27), and "lipidomic analysis" (99) in literature before 2003, the emergence collection of thoughts in the field was facilitated through the use of definitive definitions in 2003 (11, 100), demonstration of the power of synergistic technologies (11, 101, 102), and the deeply penetrating power of lipidomics in biological applications (11, 103–105). Blair *et al.* (101) first demonstrated a novel technique to study eicosanoid enantiomers and regioisomers through use of electron capture atmospheric pressure chemical ionization-MS after chiral chromatography and quantified molecular species using stable isotope dilution.

At a similar time, the LIPID MAPS consortium which was led by Dr Edward A Dennis and funded by the National Institute of Health was formed to facilitate the development of lipidomics in multiple areas. These included systematic classification of lipids (1), preparation of lipid standards for quantification, and

development of databases (106, 107). The aim of this consortium was to characterize lipid metabolites and to quantify changes in their levels and subcellular localization within a cell. This movement along with the publication of many informative review articles around 2005 (29, 58, 108–111) greatly accelerated the growth and development of lipidomics.

The emerging of lipidomics was well indicated with the bloom of the books, and special issues described the methods and applications in lipidomics in this period. In the late years, lipidomics studies on a special category of lipids have been frequently given a subtitle of lipidomics, such as sphingolipidomics (112–114), phospholipidomics (115, 116), mediator lipidomics (110), oxidative lipidomics (117, 118), etc. Similarly, lipidomics applications to an area of research have also been commonly named a subtype of lipidomics, such as neurolipidomics (119–121), plant lipidomics (122–124), yeast lipidomics (125, 126), dynamic lipidomics (127–129), etc. It would like to be particularly emphasized that lipidomics is not a sole analytical science; the determined/annotated lipid molecular species have to follow the biological principles (130).

## CURRENT STATUS OF LIPIDOMICS

Lipidomics has undergone a fundamental distinction in the types of experiments and strategies that are performed. One strategy, now known as targeted lipidomics seeks to identify specific metabolites that are known to exist and alterations in their abundance. This type of research is often hypothesis driven. A second method, untargeted lipidomics identifies global alterations in lipid molecular species and their abundance for both known and unknown lipids. Untargeted lipidomics is typically used for hypothesis generation through identification of unanticipated changes in both known and unknown lipid molecular species and changes in their abundance. Typically, the latter is used to develop hypotheses and new perspectives that were not previously known. There exist some platforms which follows in-between. For example, MDMS-SL targets to individual lipid classes but untargets to individual molecular species of a lipid class of interest.

For targeted lipidomics, derivatization has been widely applied taking advantage of the physical properties of the analyte and functional groups in the targets that can easily be derivatized. For example, charge-switch derivatization can lead up to 100-fold increases in signal to noise ratios and be used to confirm the initial presence of the functional group and make a mass shift to remove target analytes from crowded regions of the mass spectrum. Of course, with increased sensitivity and high resolution/accurate mass measurement, the combination of technology development and derivatization can vastly improve definitive identification and quantification of extremely low abundance analytes for both shotgun lipidomics and LC-MS approaches (131). In global analyses, increase in the coverage of lipid classes and molecular species is a key factor. The broader the coverage is, the more likely the approach will allow dissection of entire metabolic pathways of lipid classes/subclasses and individual species in a system. Understanding the interrelationship between classes and species within a metabolic pathway or connecting metabolic networks is essential for chemical characterization. Fortunately, multiple synergistic approaches have vastly improved the power of identification and quantitation in the understanding of the changes of hundreds to thousands of individual species (60, 93, 132, 133). It should be emphasized that LIPID MAPS, now supported by the Wellcome Trust and providing expanding databases, educational materials, and numerous tools and software, should be the rich resources for identification and quantification of cellular lipidomes.

In the last 20 years, development of lipidomics technologies has vastly improved by the following: *i*) extensive characterization of the structures of known lipid classes and subclasses and uncovering both new classes and new molecular species of lipids (e.g., (4, 74, 134–138)); *ii*) sensitive quantification of lipid species at attomole to femtomole levels from a variety of biological samples (e.g., (132, 139–146)); *iii*) applications for biomedical and biological studies through pathway/network analysis; *iv*) biomarker development that facilitates prediction, diagnosis, and prognosis of disease states (see recent reviews for references (83, 147–156)); *v*) determining the alterations of lipids in spatial distribution via mapping complex organs by MALDI imaging (see recent reviews for references (157–162)); and *vi*) advances in bioinformatics to facilitate real time data processing (e.g., (88, 163–174)).

Currently, in our view, the following areas become the focal points in lipidomics, which largely reflects the development of the field:

### Deeply penetrating analysis of individual lipid molecular species

As the development of lipidomics proceeded, strategies for chemical analysis of lipid metabolites have greatly extended to identify regioisomers, stereoisomers, and diasterotopic molecular species. These include both the use of multistage fragmentation to identify *sn*-1 versus *sn*-2 chains, location of double bond(s) in fatty acyl-chains, and *cis-trans* isomerism. To this end, newly developed technology/instrumentation including ultraviolet photodissociation MS (175) and a variety of chemical reactions/derivatization approaches have been broadly used for deep analysis of lipid molecular structures, including Paternò–Büchi reaction (176–178) and click chemistry (179–181). Readers interested in this area of work can find the comprehensive review of deep phenotyping with chemical reaction/derivatization approaches by Xia in this Thematic Review series.

### Accurate quantification and standardization

As discussed in the "Introduction" section and recognized by our group a decade ago (182), accurate quantification of lipid species is particularly important for lipidomics especially in confounding situations where special attention is needed to exclude structures with the same elemental composition. To this end, the use of internal standards can aid accurate quantification and diagnostic derivatization in this process, as previously discussed (183).

However, an inter-laboratory ring trial with quantification of lipids in human plasma has demonstrated the huge variations of the quantified levels of lipid molecular species (184), indicating the potential issues with accurate quantification of individual lipid species. Therefore, standardization and harmonization in an accurate, quantitative, high throughput manner has recently caught the attention of lipidomics community (185–187), leading to the formation of Lipidomics Standards Initiative Consortium and then the establishment of International Lipidomics Society. It becomes more and more clear that guidelines for the entire workflow of lipidomic analysis, from preanalytics, lipid extractions, MS, data processing and reporting, need to be developed and standardized. To this end, a guideline for reporting MS-based lipidomics has recently been proposed (187). Readers interested in this area of work could also find further discussion by Kofeler *et al.* (188) in this Thematic Review series.

### Clinical lipidomics

Clinical lipidomics is a new extension of lipidomics, which aims to investigate metabolic pathways and networks through quantifying the complete spectrum of lipidomes in cells, biopsies, and/or body fluids of patients, and to link the lipidomic components to clinical proteomics, genomics, and phenomics to accurately diagnose human diseases (189, 190). This type of study expands the original biomarker discovery/development using the lipidomics approach into a much broader, clinic-related research area. This area of research includes *i*) identification of molecular mechanism(s) mediating diseases for potential therapeutic development; *ii*) connection of altered lipid profiles with gene variants of patients via a genome-wide association study on selected particular genes through which the metabolic pathways are mediated and the network is interconnected; and *iii*) all types of the information from lipidomes such as the content and composition of individual species, as well as the mass ratios of these species for subtyping disease phenotypes and/or subgroups of patients to serve the purpose of precision medicine for accurate treatment; etc. (e.g., (191–195)). Readers interested in this area of work could also find the comprehensive review by Meikle in this Thematic Review series (196).

### Functional lipidomics

Defining the function of a class, a subclass, and individual molecular species of lipids in a cell, organ, or organism is a long-standing task of lipid biochemistry, which represents the definition of functional lipidomics (27). However, functional lipidomics not only includes this type of long-standing studies but has evolved further in studying altered lipids in specific disease states and stages of an organism's life cycle. As illustrated in **Fig. 3**, functional lipidomics expands *i*) uncovering the altered lipids between states by lipidomic analysis; *ii*) identifying the alterations in metabolic pathways and networks leading to the changed lipidomes at proteome, transcriptome, and genome levels; *iii*) investigating the biological/pathological sequala of the altered lipids; and *iv*) developing potential therapeutics for the treatment of diseases and/or aging based on the identified signaling, regulators, and sequala.

Functional lipidomics was proposed at the earliest stage of lipidomics development (27, 197, 198) and has been caught particular attention in the field and conducted in many studies (e.g., (150, 199–203)). It is regrettable that the majority of lipidomic studies are still at their basic levels, i.e., profiling lipidomes, and unable to penetrate into the interpretation of changes in lipidomes and identification of the underlying molecular mechanism(s) leading to lipid changes. This is largely because of the disconnect between analytical chemists who are capable of developing lipidomic methodology to conduct comprehensive lipidomic analysis and those possessing broad knowledge in lipid metabolism and having resource including animal models to test their theories of associated biology and the effects of drugs on the observed process.

### Lipidomics of subcellular organelles

The cellular function is tightly associated with the internal organization of cells, in which multiple subcellular compartments exist possessing specialized roles. These organelles largely hold the secrets for normal cellular function, progression of disease, and a plethora of interactions among cellular molecules that define life. Lipids play a key role in organelle functions. Different organelles contain very different lipidomes, which are known to vary with a circadian rhythm (204) and patho(physio)logical conditions (10, 205). For example, ether lipids and polyphosphoinositides play important roles in exosomal biology (206). Therefore, lipidomics provides detailed information of these organelles, which has the potential for insights into the possible origin, structure, and functional alterations of individual organelles. Fingerprinting the changes of lipid molecular species of an organelle after a perturbation also has the power to connect changes in organelle function with the perturbation, thereby providing insights into molecular mechanism(s) of lipid

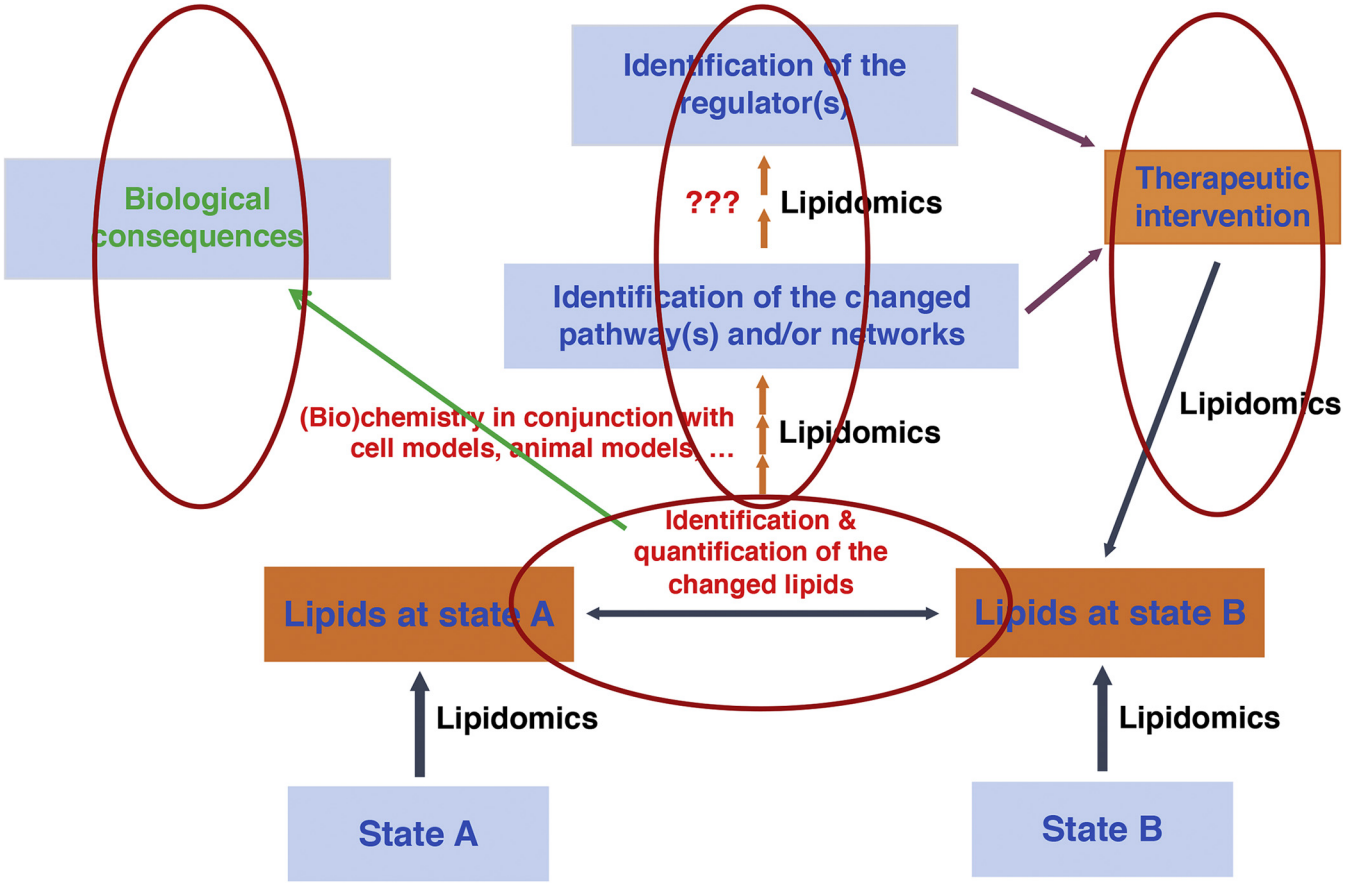


**Fig. 3.** Schematic illustration of functional lipidomics. Functional lipidomics represents a new research direction in lipidomics, in which the altered lipids between different states are uncovered through lipidomic analysis; then the molecular mechanism(s) underpinning the changed lipids and the biological/pathological sequala of the changed lipids are identified; and finally, potential therapeutics for treatment of diseases and/or aging based on the identified signaling, regulators, and sequala are developed.

changes and altered functions to a certain degree. Dynamic labeling of lipids in pulse chase experiments can determine the turnover rate and flux of individual lipid molecular species under specific perturbations, thereby providing a real time dynamic picture of lipid flux in individual organelles.

However, organelle lipidomics still remains as a challenge for many scientists largely because of the difficulty to isolate a large quantity of relatively pure organelles. Fortunately, this issue will likely be resolved with the development of sensitive and spatially focused methodologies including secondary ion MS, as previously demonstrated (207). Moreover, new technologies, robotic fluid handling systems, and spatial reconstruction methods should greatly facilitate lipidomics of subcellular organelles. Accordingly, it can be foreseen that lipidomics at the organelle levels will become a part of the armamentarium of lipidomics in the near future.

## PERSPECTIVE

In the near future, the focal points at the current lipidomics (see above) will inevitably evolve and be solved. Moreover, the following areas of research in lipidomics appear in demand and should be well developed in the future. These include, but are not limited to, *i*) further increases in the coverage of lipid classes and individual species (particularly for those very low abundance species) using an automated, quantitative, high throughput lipidomics in combination with instrumental advances and chemical breakthroughs; *ii*) single cell lipidomics, as stimulated with the fruitful development and powerful applications of single cell genomics and demonstrated with limited studies (208–210), will likely become a rising star; *iii*) MS imaging should evolve into more quantitative and multi-dimensional; *iv*) more complex dynamic studies in metabolic flux to reveal the reaction rates in lipid metabolism to comprehensively determine lipid metabolism in the molecular levels and provide true understanding of the roles of lipids in biomedical sciences; and *v*) integration of lipidomics with other omics considering the relationship with genes, transcripts, and enzyme data to perform metabolic pathway reconstruction and flux analyses is in high demand, which is still at the very early stage of development (211). Collectively, as an interdisciplinary field, lipidomics will

continue its exponential growth and become fully integrated with the other omics technologies and phenotypic alterations.

## Data availability

All data are contained within the article.

*Acknowledgments*

This work was partially supported by National Institute on Aging Grants RF1 AG061729, RF1 AG061872, and U19 AG069701, and National Institute of Neurological Disorders and Stroke Grant U54 NS110435, as well as from the UT Health SA intramural institutional research funds, the Mass Spectrometry Core Facility, and the Methodist Hospital Foundation.

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*Conflict of interest*

The authors declare that they have no conflicts of interest with the contents of this article.

*Abbreviations*

CID, collision-induced dissociation; MDMS-SL, multidimensional mass spectrometry-based shotgun lipidomics; PC, choline glycerophospholipid; PI, inositol glycerophospholipid; PS, serine glycerophospholipid.

Manuscript received August 4, 2021, and in revised from December 13, 2021. Published, JLR Papers in Press, December 22, 2021, https://doi.org/10.1016/j.jlr.2021.100164

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