Chapter 2

Analytical strategies in lipidomics and applications in disease biomarker discovery

Abstract

Lipidomics is a lipid targeted metabolomics approach aiming at comprehensive analysis of lipids in biological systems. Recently, lipid profiling, or so-called lipidomics research, has captured increased attention due to the well-recognized roles of lipids in numerous human diseases to which lipid-associated disorders contribute, such as diabetes, obesity, atherosclerosis and Alzheimer's disease. Investigating lipid biochemistry using a lipidomics approach will not only provide insights into the specific roles of lipid molecular species in health and disease, but will also assist in identifying potential biomarkers for establishing preventive or therapeutic approaches for human health. Recent technological advancements in mass spectrometry and rapid improvements in chromatographic techniques have led to the rapid expansion of the lipidomics research field. In this review, emphasis is given to the recent advances in lipidomics technologies and their applications in disease biomarker discovery.

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Introduction

Lipids, the fundamental components of biological membranes, play multiple important roles in biological systems. Firstly, lipids make the cell a sub-system in the context of the whole and relatively independent of the exterior environment through lipid bilayer structures. Secondly, lipids can provide an appropriate hydrophobic medium for the functional implementations of membrane proteins and their interactions.[1] Thirdly, a variety of lipid molecular species can produce second messengers by enzyme reactions. Furthermore, the aberrant lipid metabolism observed in numerous human diseases such as diabetes, obesity, atherosclerosis and Alzheimer's disease[2-4] has attracted increased attention from lipid researchers. In summary, all these characteristics make lipids a focal point in cutting-edge biosciences.

Current research on lipids tends to shift from determining the individual molecular structures of single lipids in biological samples to characterizing global changes of lipid metabolites in a systems-integrated context in order to understand the crucial role of lipids in physiopathology. Lipidomics, the large-scale study of the structures and functions of a wide range of lipids,[5] is of increasing importance in this research field. In the recent decade, many significant efforts have been made to promote research activities in this new-emerging field of lipidomics. For example, the "Lipidomics Expertise Platform" supported by the EU was initiated in 2005 and offers an online resource (http://www.lipidomics-expertise.de) which gathers information on institutions involved in lipidomics and includes databases for lipidomics expertise, lipid standards and methods. Other important websites comprising a wealth of information on lipids, their structures and related technologies are the LIPID MAPS (http://www.lipidmaps.org), LIPID BANK (http://lipidbank.jp), Lipid Data Bank (http://www.caffreylabs.ul.ie), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), European Federation for the Science and Technology of Lipids (http://www.eurofedlipid.org), Cyberlipid Center (http://www.cyberlipid.org) and the Lipid Library (http://www.lipidlibrary.co.uk).

Recent advancements in mass spectrometry (MS) and innovations in chromatographic technologies have largely driven the development of lipidomics. The major biological significance of lipidomics is the advancement of the traditional lipid research related to two focal points:[6] i) how to link lipid metabolites and/or lipid metabolic pathways in complex biological systems to individual metabolic health; ii) how to interpret the changes in the lipid metabolism or in the regulation of these pathways linked to metabolic and inflammatory diseases from a physiological and/or pathological perspectives. For this reason, lipidomic investigations usually focus on the measurement of alterations of lipids at systems-level indicative of disease, environmental perturbations or response to diet, drugs and toxins as well as genetics.[7] It opens an entirely new opportunity to understand the function of lipids in biological systems.[3] Often the lipid profiles in clinical investigations related to persons that are in a disease state or have specific genetic profiles become the basis for detection of the potential biomarkers related to disease or specific gene expression when compared to those of controls [*e.g.*[8-12]]. Although the analytical methods of these studies can differ to some extent, all of them share a common feature,

that is, vast amounts of data are generated from lipid profiles. How to process and statistically analyze these large datasets in order to derive useful biological information is a key task and major challenge in lipidomics, as it is also in metabolomics. Different approaches in data analysis can be followed, but the most successful are those megavariate statistics incorporating existing biological knowledge into the statistical analysis,[6] enabling the generation of integrative knowledge for systems-level investigation of lipidomics. Discussion on this specific topic is beyond the scope of this article, but detailed descriptions about this topic have been presented in a compact review.[6]

This article will review the recent advancements in lipidomics technologies and their selected applications in disease biomarker discovery.

Diversity of lipids

Lipids consist of a tremendous number of structurally and functionally distinct molecular species that span from apolar (*e.g.* sterol esters) via neutral [*e.g.* triacylglycerides (TGs)] to polar [*e.g.* phospholipids (PLs)].[13] This large diversity in structure and function of lipids makes it a huge challenge to develop a comprehensive identification for a systems-based lipid biology approach and integrating lipid data from different analytical platforms and different laboratories. The authoritative scheme for lipid classification and nomenclature often used is the one proposed by Fahy and co-authors.[14] In this scheme, eight categories of lipids are characterized based on their chemical structure and biosynthetic perspectives with the consideration of the hydrophobic and hydrophilic elements, which include fatty acyls, glycerolipids (GLs), glycerophospholipids (GPs), sphingolipids (SPs), sterol lipids (STs), prenol lipids, saccharolipids, and polyketides. Figure 1 provides the basic structures of representative lipid molecular classes. Fatty acids (FAs), as the simplest and one of the most important lipid classes, are a basic element of all lipids. Structurally, natural FAs have saturated or unsaturated straight-hydrocarbon chains varying in length from 14 to 24 carbon atoms and having 0 to 6 double bonds. Functionally, FAs are precursors of a variety of bioactive lipid molecules. For example, arachidonic acid is the precursor of eicosanoids, which function as signalling molecules through specific receptors and play important roles in inflammatory processes.[15]

GLs, as another class of relatively simple lipids, mainly include monoacylglycerides (MGs), diacylglycerides (DGs) and TGs. These lipid molecular species are variable in the number of the FAs that are esterified to the hydroxyl groups of the glycerol backbone [see Figure 1 (b)]. It needs to be kept in mind that FAs present in MGs, DGs and TGs can permute at various positions of the glycerol backbone, and thus MGs occur as three stereochemical forms, *i.e.* 1-/2-/3- isomers. Similarly, DGs exist in nature as mixtures of *sn*-1,2-, *sn*-1,3- and *sn*-2,3- isomers. Different to MGs and DGs, TGs extensively occur in nature as the main constituent of animal fats. It is important to realize that esterification of three different FAs with glycerol to form a TG molecule leads to various different biological entities. Interestingly, natural FA moieties as found in TGs originating from plants and animals are often long hydrocarbon chains with even number of carbon atoms. This is related to the biosynthetic pathways and is crucial for the biological activity.

Furthermore, TGs play an essential role in cellular energy storage, but they also function as mediator in the processes of metabolism and disease.[16] Some studies have shown considerable evidence that the alterations in TG synthesis and catabolism have an important contribution in disease pathology such as in diabetes,[17-18] obesity,[8,19] atherosclerosis[20] and alcohol-induced hepatic dysfunction.[21]

STs, consisting of cholesterols (Chos) and derivatives [*e.g.* cholesterol esters (ChoEs) with a four ring core structure [Figure 1 (c)], are important components of membrane lipids. One of the most eminent roles of STs lies in its regulatory function related to cell signaling and in cellular fluidity modulation.[22] Chos are highly abundant in mammals and involved in cardiovascular diseases by appearing at largely elevated level. ChoEs, derived from Chos through a long-chain FA esterifying with the only hydroxyl group of Cho, are also associated with cardiovascular diseases.[23]

As the major group of lipids, GPs cover mixtures of numerous molecular species produced by glycerol with a functional polar head group at *sn*-3 position via a phosphodiester bond esterified with various combinations of different FAs at the *sn*-1 and *sn*-2 position of the glycerol backbone. Based on the different polar head groups, GPs can be divided into glycerophosphatidic acids (PAs), glycerophosphocholines (PCs), glycerphosphoethanolamines (PEs), glycerophosphoserines (PSs), glycerophosphoglycerols (PGs) and glycerophosphoinositols (PIs) [Figure 1 (d)]. Particularly, for some classes of GPs (typically for PEs and PCs), FA chains at the *sn*-1 position can also be an ether-linkage including vinyl ether and alkyl ether. The terms "plasmenyl-" and "plasmanyl-" lipids have been recommended for them, respectively. In addition, lyso-GLs, with one of the hydroxyl groups at the *sn*-1 or *sn*-2 positions of the glycerol backbone intact and the other one esterifies to FA, should also be covered in the categories of GPs. Despite the diversity in structure, all of these GPs are main components of cell membranes and participate in various biological activities involving cell signaling, membrane anchoring and substrate transport.[24-25] In addition, some lipid molecular species such as lysoPCs, PEs, PCs and PIs have been found as potential biomarkers involved in diseases such as obesity,[8,19] pancreatic cancer[26] and ovarian cancer.[27]

SPs are a complex family of compounds derived from a sphingoid base, a basic backbone containing a 1,3-dihydroxyl, 2-amino alkane or alkene[20] as shown in Figure 1 (e). Important SPs mainly include N-acyl-derivatives of sphingosine [*i.e.* Ceramides (Cers)] and sphingomyelins (SMs), that are comprised of a FA and a phosphorylcholine head group linked to a 2-amino group and 1-hydroxyl group of the sphingoid chain, respectively [Figure 1 (e)]. Typically, the amide-linked FAs in Cers and SMs are saturated or monounsaturated long carbon chains varying in length from 14 to 26 carbon atoms.[14] Cers are precursors of SMs and are mostly located in the stratum corneum, the outermost layer of the skin, playing a crucial role in maintaining the epidermal barrier function (*e.g.* water permeability barrier) of the skin.[28] Such unique function has been demonstrated to some degree by the fact that skin diseases (*e.g.* atopic dermatitis and psoriasis) often lead to abnormalities in Cer metabolism.[20,29] SMs were found to have a role in signal transduction and can accumulate in a hereditary disease called Niemann-Pick Disease.[30]

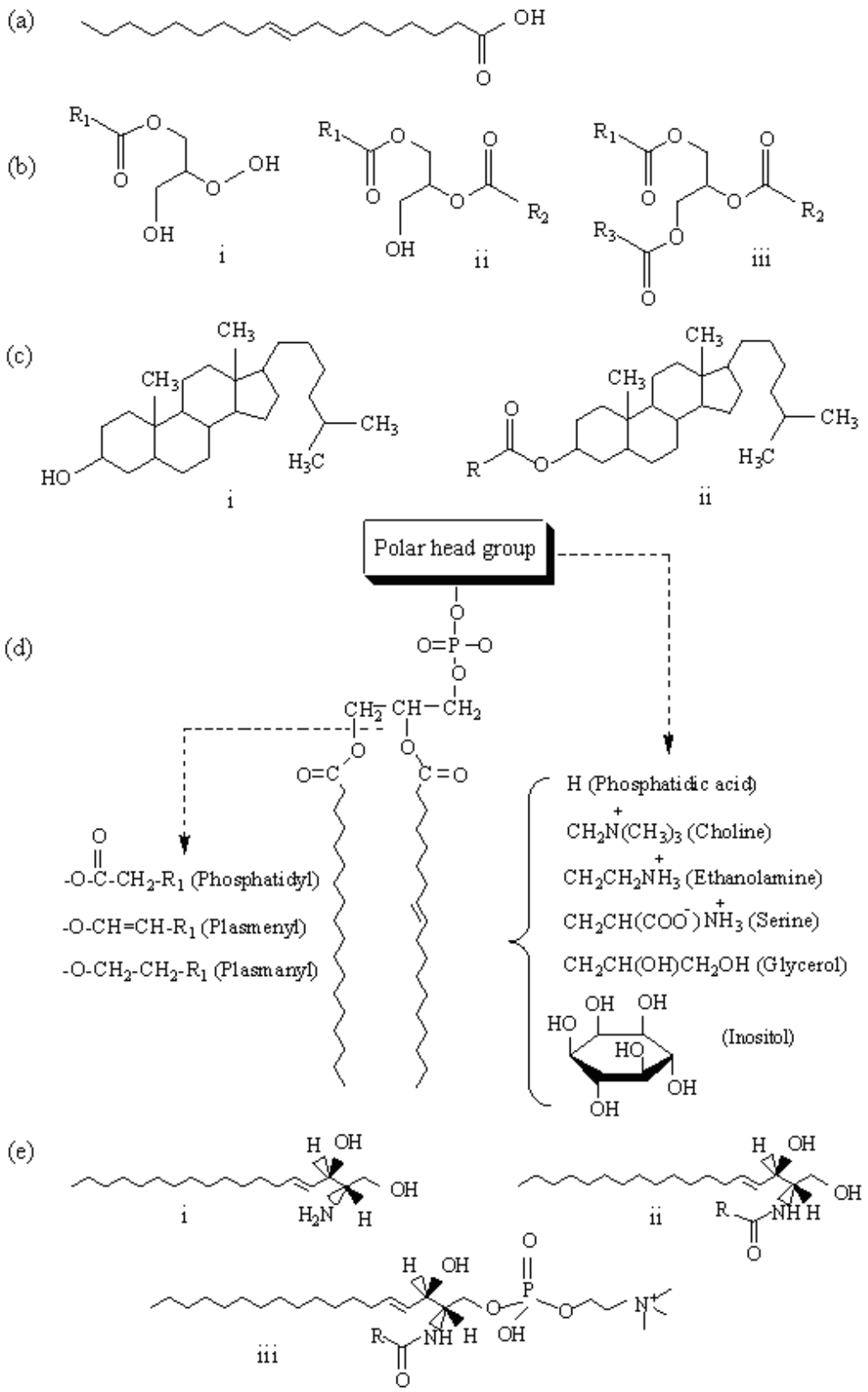

Figure 1 Basic structures of representative lipid molecular species. (a) fatty acid: oleic acid; (b) glycerolipids: i. mono-, ii. di-, and iii. tri-acylglycerides; (c) sterol lipids: i. cholesterol, ii. cholesterol ester; (d) glycerophospholipids; (e) sphingolipids: i. sphingosine; ii. ceramide; iii. sphingomyelin.

Analytical methods for lipidomics

Traditional strategies for lipid analysis usually pre-fractionate lipids into classes using thin-layer chromatography (TLC),[31] normal-phase liquid chromatography (NPLC)[32] or solid phase extraction (SPE)[33] and then separate particular classes of lipids into individual molecular species by high-performance liquid chromatography (HPLC) coupled with either ultraviolet or evaporative light-scattering detector.[34-35] With these traditional methods individual molecular species of many lipid classes can be analyzed. However, such "classical" techniques often either lack sensitivity or require large sample volumes and multi-step procedures for sample preparation; and the resolution is limited, *i.e.* only a limited set of individual molecular species are analyzed. Alternatively, gas chromatography (GC) has been, and is still often used for lipid analysis, but usually time-consuming procedures consisting of hydrolysis and derivatization are required as most lipids are otherwise not GC-amenable.[36] Proper GC-based methods meet the need of lipidomics with regards to the wider distribution of molecular compositions and physical properties and the wide dynamic range of concentrations of lipids covered; often mass spectrometric detection is used.

With the advent of soft ionization technologies such as matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) for MS, possibly coupled to LC, made the rapid and sensitive analysis of the majority or a substantial fraction of lipids possible in one analysis. Consequently, new soft-ionization MS-based analytical strategies have been, and are still, emerging in lipidomic research.[37-40] Strategies currently used in lipidomics include direct-infusion ESI-MS and ESI-MS/MS, LC coupled with ESI-MS or MS/MS, and MALDI combined with Fourier transform ion cyclotron resonance MS (MALDI-FTICR-MS) or time-of-flight-MS (MALDI-TOF-MS), In addition, for some classes of lipids LC coupled with APCI-MS is used.[41] Another approach used is nuclear magnetic resonance (NMR) spectroscopy, which will also be covered to some extent in this review.

Despite all advances recently made, the diversity of structures, properties and the wide range of concentrations of lipids provide a huge and almost impossible challenge for the analytical methodology when aiming at a single technological platform capable of measuring and identifying all lipids in a single sample simultaneously. As a consequence, multiple, often complementary, analytical approaches are currently used in the field of lipidomics, and will be discussed below. It should be mentioned that the different approaches can be applied to analyse a limited set of lipids, a specific class of lipids or pathway, or in a global approach a wide range of lipids (Figure 4).

Direct-infusion ESI-based MS technologies

In a direct infusion ESI-based MS approach, the analytes are directly infused into the MS without prior chromatographic separation (Figure 2). A crude lipid extract can be directly infused, or a fraction obtained after further sample preparation. The sensitivity of current ESI-MS methods has been dramatically enhanced over the years and is about 2-3 orders of

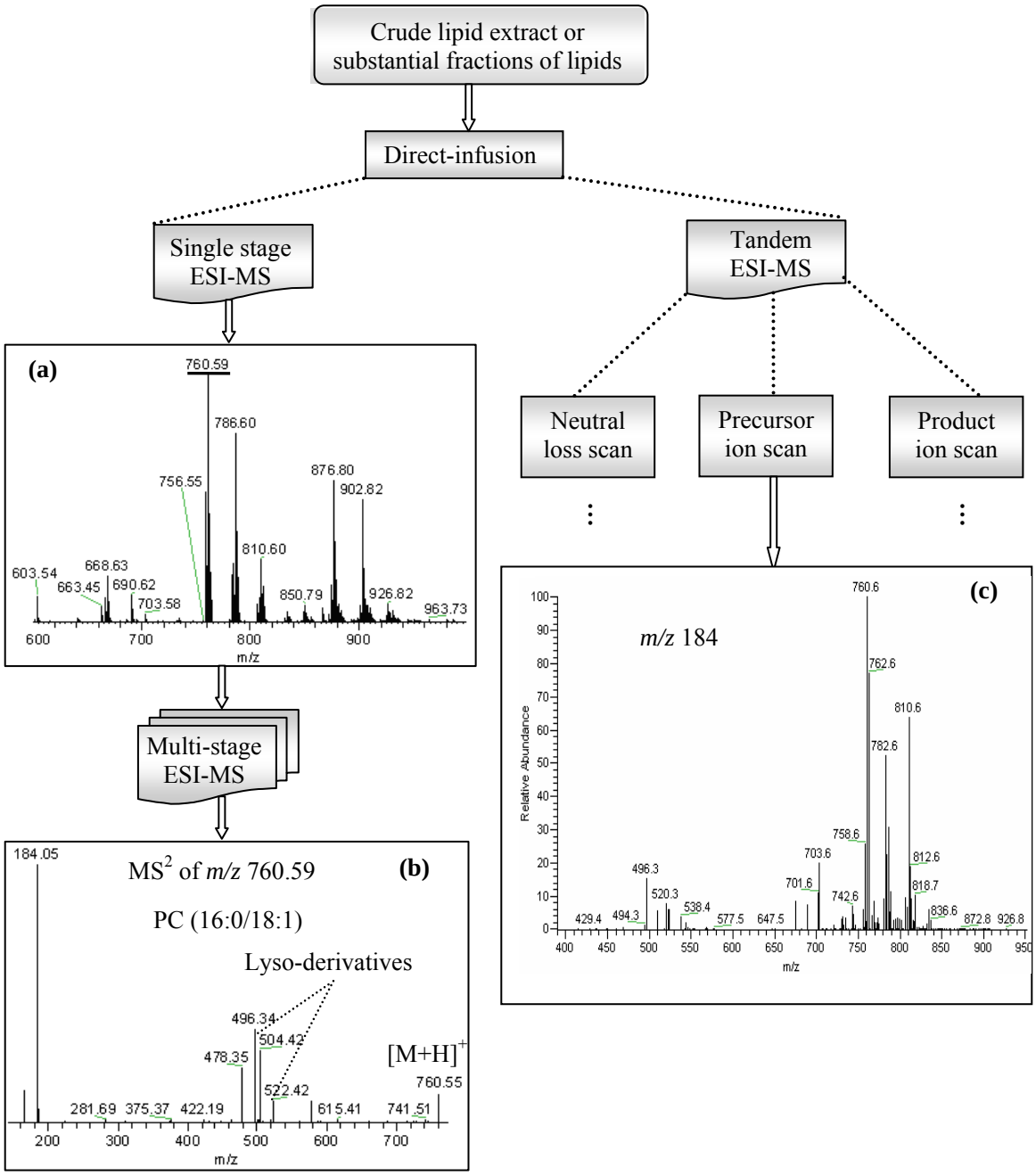

Figure 2 A typical flowchart of direct-infusion ESI-based MS or tandem MS strategies. a) An ESI-MS spectrum of lipids from mouse plasma lipid extract in the mass range of m/z 600-1000; in this single-stage ESI-MS experiment a large number of ions were detected. b) ESI-MS2 spectrum of m/z 760.59 in positive ion mode is shown as an example; many characteristic product ions are observed (for example, m/z 184 is the choline-phosphoryl ion, m/z 496.34 and 522.42 are lyso-PC (16:0) and lyso-PC (18:1) protonated molecules). Therefore, m/z 760.59 is assigned as PC (16:0/18:1). c) ESI-MS spectrum of lipid species in the mixture which have the propensity to produce a fragment with m/z 184; such a precursor ion scan is very powerful to profile classes of lipids in complex mixtures.

magnitude greater compared with that of the earlier fast-atom bombardment MS,[42] and can be even further increased when using nanospray-MS with injection flows in the nL/min

range.[43] When using ESI-MS/MS, further improvement in sensitivity can be achieved; limits of detection of lipid molecular species were reported within the nanomolar concentration range in early studies to femtomolar amounts in recent applications.[44] Moreover, soft ionization by ESI has the advantage that the structural identification of lipids is more straightforward using different MS/MS experiments such as precursor ion scan, product ion scan and neutral loss scan. Various applications of lipid profiling of biological samples using ESI-MS and ESI-MS/MS have been reported.[45-48]

In general, direct-infusion ESI-MS lipidomics studies can be divided into two categories: shotgun lipidomics[5] and targeted or focused lipidomics.[37] Shotgun lipidomics was developed by Gross and Han; they used an intrasource separation in ESI-MS for the high-throughput analysis of lipids directly from lipid extracts of biological samples without chromatographic separation prior to mass detection.[44] After multiplexed lipid extractions under certain conditions, lipid classes were separated in the ion source according to their inherent charge, or their charge after interaction with a cation or anion in the matrix using positive or negative mode, and then detected using full-scan MS or MS/MS.[44] Based on these strategies, individual molecular lipid species of most major and many minor lipid classes could be directly identified and quantified. This methodology aims at determining comprehensive profiling or mapping of a substantial fraction of lipid mixtures. For example, the use of shotgun lipidomics with tandem MS was successful in characterization and direct quantitation of cerebroside species from lipid extracts of rat brain tissues.[49] Hydroxy- and nonhydroxy-cerebroside species were easily identified by employing differential fragmentation patterns of chlorine adducts using a tandem MS scan mode. Quantitation of cerebroside molecular species was directly carried out after correction for different 13C-isotopomer intensities relative to the internal standard in either positive or negative ion mode. The authors stated that the method had a linear range of above 1000 in the low concentration region, which suggests that it is a promising approach for studying the cellular spingolipid lipidome.[49]

Different from shotgun lipidomics, targeted lipidomics focuses on selected categories of lipid molecules by using precursor ion scan and neutral loss scan of their characteristically functional groups, so that minor but important lipids can be detected with an enhanced detection limit under optimal conditions for the lipid class of interest because of the more sensitive identification obtained by the focused scan modes above-mentioned.[37] For example, this method is highly efficient in detecting for instance PLs when using precursor ion scan and neutral loss scan of their polar head groups and fatty acyl moieties. A prominent example of the role of targeted lipidomics comes from a study on specific detection of lysoPA in serum extracts by tandem MS.[50] Precursor ion scan and neutral loss scan of specific m/z values (*e.g.*, 79,153) were used in profiling of LysoPAs. Quantitative analysis of lysoPAs from calf serum was performed by taking ion suppression effects of the matrix into account. This method is highly selective, rapid and convenient without prior chromatographic separation. It could be useful in discovering potential biomarkers for a specific disease involved lysoPA metabolism.

Some other ESI-MS-based studies on lipid profiling have been reviewed in ref.[51-52] In these studies, either positive or negative ion mode was performed for lipid profiling taking the ionization preference of different classes of lipid molecular species into account. These studies aimed at a full characterization of membrane lipids in a total lipid extract from cells, body fluids and tissues by sequentially carrying out characteristic precursor, product ion or neutral-loss scans for each lipid class while the sample is continuously infused into the MS detector. These methods were useful for comparative analysis where the result of an experiment is compared to a control. In summary, direct-infusion ESI-MS based technologies allow a powerful approach for rapid analysis of lipid metabolites that may reflect metabolic changes resulting from diseases. However, such approaches for lipid analysis by direct infusion have potential limitations in resolving isobaric compounds and also encounter a risk of ion suppression which may lead to decreased sensitivity in the analysis of very low abundant lipids when tandem MS technologies are not available[53] and problems in quantitation if no proper internal standards are used. Actually, a combination of a global profiling including appropriate MS/MS experiments enables both, sensitive analysis and detection of a wide range of lipids.

LC-MS based technologies

Both, shotgun and targeted MS-based lipidomics approaches for a global or targeted analysis of lipids, respectively, have the advantages of high sensitivity, speed and ease of automation. However, for both approaches, the resolution of isobaric lipids is limited if no dedicated MS/MS experiments are used, and the potential risk of ion suppression of very low abundant lipids cannot be eliminated. This limitation can be overcome by using high efficient online chromatographic separation prior to MS detection so that complex lipid mixtures are to some extent resolved prior to MS analysis. Depending on the lipids of interest, usually NPLC or reverse-phase (RP) LC method are coupled with various MS detectors. Occasionally, GC coupled with MS is used as an alternative in studies of some specific lipid molecular species such as fatty acids (FAs) using hydrolysis and derivatization procedures, [36] but this topic is not included in this review. The hyphenation of LC and MS or tandem MS have proven to be powerful in the comprehensive analysis of complex lipid mixtures or in the analysis of specific categories of lipid species. On the one hand, the use of LC prior to the MS reduces ion suppression effects by decreasing the number of competing analytes entering the MS ion source at the same time. On the other hand, chromatography greatly guarantees the consistency of quantitative and qualitative results because each chromatographic peak is located in a 2-D space with a characteristic retention time versus a specific *m/z*. In addition, the separation of the isobaric and isomeric lipid species is possible in the LC part of an LC-MS or LC-MS/MS method.

As a typical example, a lipid platform based on RPLC-linear ion trap-FTICR-MS was recently developed in the authors' groups for the profiling of lipids in human and mouse plasma by using a fused-core C8 column.[12] Strategies for comprehensive method validation and relative quantification including selection of internal standards and normalization of the sample peaks with those of the appropriate internal standards were

discussed in the work. More than 160 lipids belonging to eight different lipid classes were detected and identified in a single LC-MS run. The analysis time was less than 25 min and the column equilibration took only 5 min, which is far less than that of traditional LC methods for lipid analysis. Figure 3 displays a typical LC-ESI-linear ion trap-FTMS chromatogram of mouse plasma. This method has been successfully applied in combination with multivariate statistical analysis to classify plasma lipid profiles of male and female as well as of heterozygous and homozygous p53 mutant mice. The results showed that the method can be used as part of systems biology approaches to measure changes in levels of endogenous lipid metabolites in human and animal body fluids after exposure to drugs.

In the case of LC-based MS/MS technology, a comprehensive strategy for analysis of PLs utilizing a NPLC-QTRAP MS/MS system has been reported by the authors' group.[54] A combination of highly selective triple-quadrupole MS/MS and high-sensitivity ion trap product scans were used in the same analytical platform for human plasma PL profiling. The "enhanced MS'' single-quadrupole mode (for detection) and the "enhanced" product ion scan (for identification) were sequentially performed under both negative and positive scan modes considering the structural characteristics of lipids. Information including molecular mass, the polar head group and the fatty acyl substituents were obtained. This method was successfully applied to classify plasma PL metabolic profiles of type 2 diabetes mellitus (DM2) and controls.[9]

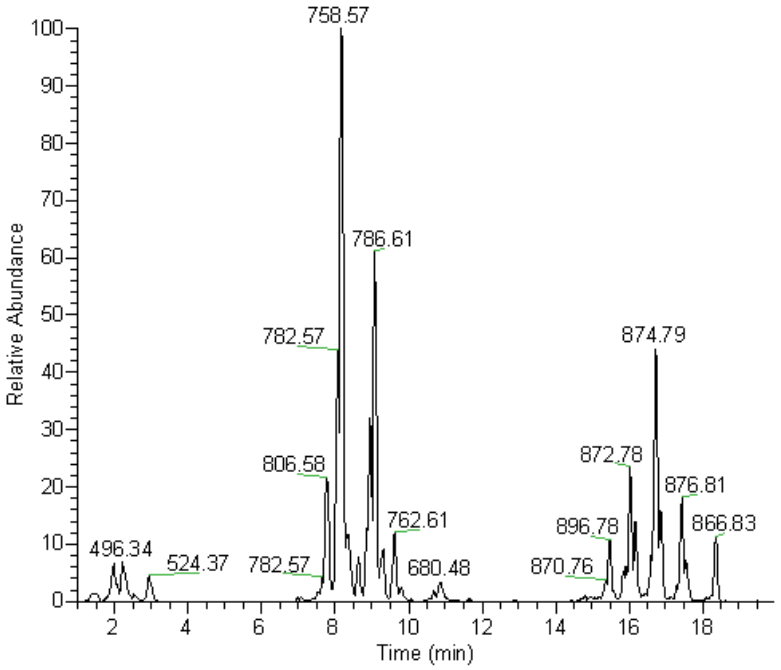

Figure 3 A typical base peak chromatogram of mouse plasma after LLE sample preparation obtained by RPLC-ESI-linear ion trap-FTMS [ref. [12]].

In another interesting example, a shotgun tandem ESI-MS with NPLC and/or RPLC was used for analysis of PLs.[48] Data-dependent MS/MS scanning (*e.g.* precursor ion and

neutral loss scanning) mode was used throughout the method. This method clearly revealed the advantage and disadvantage of both NPLC and RPLC in the analysis of complex lipid mixtures. Improved separation results of PLs are obtained by RPLC-MS compared to those by NPLC-MS. In total, about 60 PC and 50 PE species were identified using RPLC-ESI-MS/MS while about 50 molecular species including PC, PE, PI and PS were identified using NPLC-ESI-MS/MS in a PL mixture of rat liver; more applications are summarized in [ref.[55]].

Although LC-based MS or tandem MS analysis of lipids of particularly PLs have been extensively reported, quantitative validation data are rarely provided. Sommer et al.[56] developed a novel method by using NPLC and RPLC coupled with ESI-MS and ESI-MS/MS for qualitative and quantitative analysis of complex lipid mixtures. They stated that their approach enabled determination of low levels of lipids for each molecular species. Based on the method developed, semi-quantitation of several lipid classes was obtained via external standards. However, the authors stressed that the quantitation currently presented needs to be further optimized. Actually, the difficulty in exact quantitation of lipids is quite understandable, because lipids cover such large concentration range of compounds with a wide diversity and there is still a lack of well characterized standards over the whole range of molecular structures and functionalities.

MALDI-based MS technologies

MALDI-MS is used as a high-throughput technology in peptidomics and proteomics, but has also been used for the analysis of lipids,[57] *e.g.*, low molecular weight PLs.[58] Important advantages of MALDI-MS are the speed of analysis and simplicity of operation: the analytes are ionized under relative soft conditions by laser desorption using an ultraviolet-absorbing matrix; the choice of the matrix ,often 3,5-dimethoxy-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid, depends on the properties of the analytes. One important disadvantage of MALDI is the presence of a lot of background in the lower mass range due to the matrix molecules. In addition, MALDI-MS is generally less quantitative compared to ESI-MS. MALDI-MS has therefore been less used for the analysis of lipids, and small organic molecules in general. However, coupling MALDI with FTICR-MS overcomes most problems with background due to the matrix, as the matrix peaks and lipids are resolved due to the high resolution of FTICR. FTICR outperforms MALDI-TOF-MS also on mass accuracy, sensitivity as well as tandem MS capabilities,[59] but not on acquisition speed. MALDI-MS has been successfully applied to characterize PLs in mammalian tissue sections of mouse brain, heart and liver[60] with simple and robust sample preparation and high reproducibility and accuracy. It allowed the rapid and precise monitoring of PLs within mammalian tissues. MALDI-TOF-MS, although is not as powerful as MALDI-FTICR-MS in terms of mass accuracy, sensitivity and resolution, is widespread mainly due to its lower price. It has been used to (semi)-quantify lipid species either by using internal standards[61] or by comparing the intensity of the lipid peak with a known matrix peak].[62] MALDI-TOF-MS combined with TLC has also been applied to analyze the PL composition of bronchoalveolar lavage (BAL) fluid from humans and minipigs,[63] in which

detailed quantitative analysis of all PL species was performed; the results demonstrated that MALDI-TOF-MS can be used to differentiate PLs of different species and accurately quantify the highly abundant lipid species (*e.g.* PG and PC in BAL).[63] Other related MALDI-TOF-MS methods can be examined in an excellent review.[64] Although being faster MALDI-MS-based methods have many limitations with regards to quantitating complex mixtures (*e.g.* whole or a substantial fraction of lipid extracts). On the other hand, MALDI-MS offers the possibility of imaging of lipids in, *e.g.*, tissues, which recently gets more attention.[57]

NMR-based technologies

NMR spectroscopy has demonstrated to be a powerful technology in metabolite profiling of biological samples (*e.g.* body fluids, cells and intact tissues). It can be performed with often limited sample preparation and is a rapid and straightforward analytical approach without extensive optimization of instrumental parameters that are often required in LC-MS.[65] NMR-based metabolomics has been applied to body fluids or pathological tissues, enabling to obtain diagnostic biomarker profiles for various diseases and the response to pharmaceutical treatment.[66] To more accurately define the biological processes related to abnormal metabolism in tissue directly, high-resolution magic angle spinning ^{1}H-NMR-derived fingerprints are acquired to study metabolic changes in tissues and/or perturbations in biological systems.[67] Natarajan et al. used ^{1}H NMR to establish PL profiles of invasive human breast cancer cells to investigate the regulation of PLs before and after being treated with the anti-inflammatory agent indomethacin.[68] In this study, lipid extracts of human mammary epithelial cell were analyzed to understand the effect of malignant transformation and progression on membrane PL metabolism. The authors reported that choline PLs play an important role in progression of the malignant phenotype.[68-69]

NMR has strong quantitative capabilities but cannot always directly identify metabolites in complex mixtures; an additional challenge is the similarity of the spectra with respect to the limited structural carbon chain information. However, from another point of view, NMR is favorable for identifying PLs via resolving the structure of the polar head group. Unfortunately, the sensitivity of NMR is limited compared to MS-based approaches. For many high abundant lipid species this is not an issue but the lipids at trace levels in biological systems are not easily measured. Because of this, and the somewhat less resolving power compared to MS for very complex mixtures, NMR is therefore usually less often preferred in lipidomics compared to MS at this point in time.[70] Actually, in lipidomics a shift from NMR to MS-based technologies is clearly observed in recent publications. However, the value of NMR in lipidomics should not be underestimated, especially in the tissue analysis. Recent new NMR instrumental developments might influence this situation in the future.[71]

Applications of lipidomics in disease biomarker discovery

Since lipids possess a variety of biological functions in the processes of life such as formation of cellular membranes, energy storage and cell signaling, they can be expected

to reflect much of the metabolic status in health and disease.[44] In addition, many studies have proven that lipid metabolic disorders or abnormalities can lead to various human diseases including diabetes,[2] obesity,[8] arteriosclerosis,[20,72-73] coronary heart disease[74] and brain injuries.[75] Taking obesity as an example, it is a well-recognized problem in industrialized countries caused by unbalanced uptake and expenditure of calories.[44] Obesity is one of the most vital risk factors of cardiovascular disease and diabetes.[76] High serum lipid levels, especially the largely elevated level of low-density lipoprotein (LDL) and TG and decreased level of high density lipoprotein (HDL) ChoE are commonly found in individuals with abdominal obesity.[76-77] Such distribution of lipids in serum is mainly caused by overproduction of lipid peroxidation and changes of FA compositions of certain lipid molecular species that may possess particular biological functions.[2,77] Therefore, monitoring the alterations of lipid metabolites of certain molecular species in biological samples as influenced by external stimuli or disturbance by disease processes will be helpful for the discovery of lipid metabolites indicative of metabolic disorders or diseases. This is an active research field in lipid metabolomics (lipidomics).

Figure 4 summarizes the different approaches in lipid research, which can be described as targeted lipid analysis, lipid profiling and global lipid profiling. In a targeted lipid analysis approach, the focus is on a few lipids which are expected to be important. In a lipid profiling approach, the focus is on a specific group of lipid metabolites, a certain class or pathway. In a global lipid profiling approach, a very wide range of lipids is analyzed, as wide as possible. However, the workflow of the different approaches resembles each other to a large extent. Biological samples including body fluids, cells and tissues spiked with appropriate internal standards are first extracted, crude lipid extracts are then either pre-fractionated into lipid fractions or kept intact for further separation. Next, MS detection is employed either using direct-infusion of a sample or using chromatographic separation (*e.g.* GC or LC) of certain or substantial lipid fractions or whole lipid extracts to generate lipid data. In this way, lists of lipid metabolites with absolute or relative concentrations are generated from control and diseased subjects. Next, the data are normalized and subjected to statistical data analysis for identifying those lipid metabolites which are discriminatory for diseases. The final step is the biological interpretation of obtained results into systems biology knowledge such as systems pathology, systems pharmacology/toxicology by linking the potential biomarkers with metabolic pathways or network. In this step often a huge challenge is encountered as new findings cannot always directly be linked or fitted in existing biological network knowledge. In lipidomics, knowledge at the biological level is often related to classes of lipids and not to the bioactivity of single lipid species within classes. This opens up a still unexplored area of biological sciences.

Up to now, many studies have shown that lipidomics appears to be essential in determining novel lipid molecular species that serve as potential biomarkers in many lipid-related diseases. Related reviews or summaries can be found in literatures.[78-81] Detailed applications of lipidomics in discovery of potential lipid biomarkers have been carried out for certain metabolic diseases such as obesity,[8,19] diabetes,[9,23] cardiovascular disease[82] and cancers.[83]

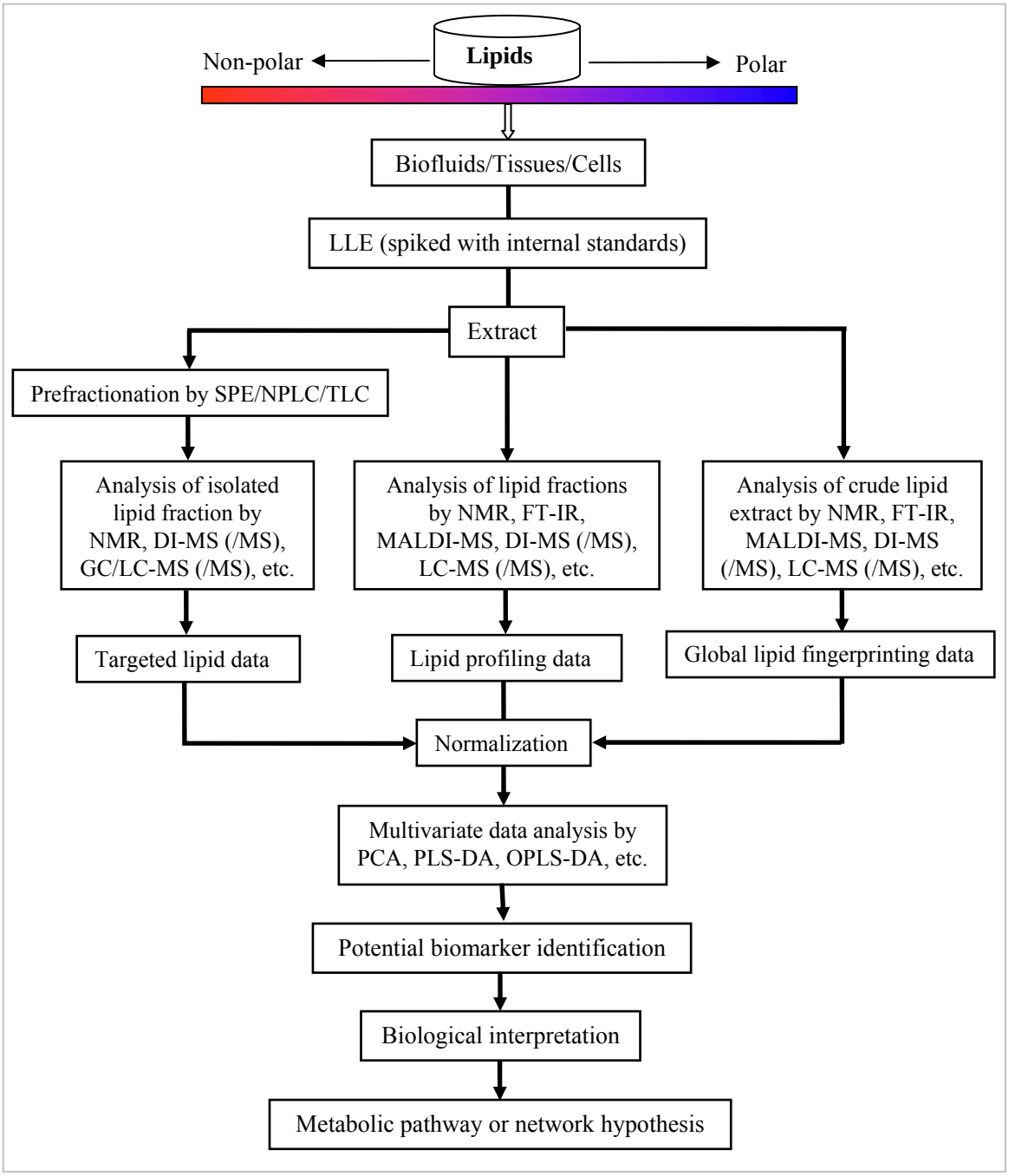

Figure 4 Different approaches in lipid research for biomarker discovery. DI, direct infusion; other abbreviations, see text.

As mentioned previously, the use of LC separation prior to MS scanning enables detection and identification of minor-abundant lipid species but may possess some specific functions or serve as potential biomarkers indicative of disease. As a typical example, the authors' group developed a NPLC-based tandem MS lipid profiling for plasma PL metabolic profiling in discovery and identification of potential biomarkers implicated in DM-2.[9] A total of 69 adults (34 DM-2 patients and 35 controls) with the age range of 30-80 years were selected for the examination of their blood plasma using LC-MS/MS followed by multivariate statistical analysis to study the alterations in PL metabolic

profiles of DM-2 patients and controls. Four lipid molecular species including two PE and two lysoPC molecular species were identified as potential biomarkers for classifying the DM2 patients from the control populations. This study holds promises for screening and identifying potential biomarkers involved in diabetes through construction of lipidomic analytical platforms using NPLC-MS technologies.

Apart from applications in human diseases, the strategy of lipidomics-driven biomarker discovery has also been used in fields of nutrition and health necessary for health promotion and disease prevention. In an interesting application, Draisma *et al.* used LC-MS lipid profile of fasting blood samples from healthy twin pairs with the average age of 18-year-old in conjunction with statistical analysis to study the contribution of genetic background and/or environmental exposure to the blood plasma lipid profile.[84] A total of 54 participants from 23 families (21 pairs were monozygotic and 8 of their siblings; 30 males and 24 females) were selected for this study. The results show that a significant number of co-twins forms close clusters based on hierarchical clustering of lipid profiles from healthy monozygotic twins. This demonstrates that similarities in genetic background and/or environmental history among individuals indeed yield similar lipid profiles.[84] Moreover, the important role of environmental influences on monozygotic twins is demonstrated by the fact that several monozygotic co-twins whose recent experiences might have decreased the within-pair similarity did not cluster closely. In this study, the authors stated that lysoPCs and SMs differentiate samples from different families more than other lipid classes. This study suggests that useful information on the health status can be obtained by monitoring global lipid profiles.

In addition, lipidomics is also applied to biological samples from transgenic animal models such as p53 mutant mice[12,85] and the apolipoprotein E (APOE) transgenic mice[86] which may develop metabolic disorders, diseases or cancers. An excellent example comes from the study of lipid profiling of APOE*3-Leiden mice.[10] In this study, LC-MS lipid profiles of liver tissues were obtained for wild type and APOE*3-Leiden transgenic mice that were suffering from diet-induced hyperlipidemia and an early state of atherosclerosis. Two populations of mice were found (wild type and disease group) and lipid molecular species contributing to the difference between the two populations were identified by using principle component analysis. Two lysoPC species lysoPC (16:0/0:0) and LysoPC (18:0/0:0) were found at higher levels in the APOE*3-Leiden mice, which was consistent with a report elsewhere.[77] In addition, a number of TG species were found significantly higher in the transgenic mice. Interestingly, similar results were found in p53 transgenic mice.[12]

Other applications of lipidomics in biomarker discovery of human diseases or animal models of mammalian diseases are summarized in Table 1. It is found that lipidomics has been extensively used in epidemic diseases (*e.g.* obesity,[8,19] diabetes[23] and cardiovascular disease[82]), cancers (*e.g.* pancreatic cancer,[26] ovarian cancer[27] and breast cancer[68]), inflammations (*e.g.* rheumatoid arthritis[87] and chronic glomerulonephritis[88]), dysfunctions (mitochondrial dysfunction[40] and epithelial barrier dysfunction[89-90]), genetic diseases (*e.g.* Barth syndrome,[91-92] Fabry disease[93] and Gaucher's disease[94]), neuropathic disease (*e.g.*

Alzheimer's disease[95]) and response to drug treatment (*e.g.* statin-induced myopathy[96] and differential effects of rosuvastatin and atorvastatin on cardiovascular risk therapies[97]). Applications of lipidomics used in four representative rat or mouse disease models (*e.g.* Kyoto and Wistar rats of fatty liver,[98] Zucker diabetic fatty rat of type II diabetes mellitus,[99] APOE*3 Leiden transgenic mice of atherosclerosis and live inflammation[100] and purebred male wild-type C57bl6 mice of starvation-induced hepatic steatosis [101]) are also included in Table 1. Although different methods were used in these studies, a variety of lipid biomarkers that are involved in the above mentioned lipid metabolic studies were discovered by comparing the lipid profiling of control populations with that of diseased objects. For example, Cer lipid molecular species were found to be elevated significantly in neuropathic diseases[95] and long-chain TG lipid species are highly accumulated in obesity[8,19] and cardiovascular diseases.[82] Although many explorative biomarkers are displayed in Table 1, it is difficult to assign or classify them in detail to specific diseases. There are at least four reasons accounting for this originating from a biological and/or analytical nature: i) lipid metabolism in biological system is highly complex and is working at various different biological levels throughout the whole organism, which is influenced by many external factors; ii) endogenous lipid metabolites are very complex mixtures covering structurally distinct compounds which have generally not been studied in more detail than at the class level typically; iii) lipid extraction efficiency can be largely dependent on the selected organic solvent which may give different analytical outcome; iv) different analytical platforms or even the same platform but operated in different laboratories may produce different results. Collectively, biomarkers discovered for one disease reflect to a great extent the status of that established condition, but it can still be used as a reference for target discovery of a similar disease. It should also be noted that the discovery of a potential biomarker is only the beginning of the lengthy process of validating the biomarkers for a clinical setting or even more for a diagnostic purpose.

Although various analytical ways were presented to detect changes in lipid metabolism related to disorders or diseases, multivariate statistical analysis was almost invariably performed to assist in identifying novel lipid molecular species which might server as potential biomarkers. Recently, the disease pathology and intervention effects in obese Zucker diabetic rats and hypertriglyceridemic humans were studied by lipidomics and analyzed using a new data analysis approach called Clustering Objects on Subsets of Attributes (COSA).[102] Figure 5 visualizes the similarities between regulation of selected plasma lipids between human and rat, as selected from the rat study using COSA. This study proved that the COSA method is very powerful in selecting different subsets of variables, different clusters taking into account that different physiological conditions or phenotypes can be regulated by different biomolecules.[102] Extensive discussions on the strategy or performance of data analysis is outside the scope of the current review, but has been discussed for example in a review on biomarker discovery using high-dimensional lipid analysis.[6]

Table 1 Applications of lipidomics in disease biomarker discovery.

Sample	Disease / animal model of mammalian disease	Analytical method	Biomarker	Reference
Liver tissue, serum	Obesity	UPLC-MS	Long chain TG, Cer, lysoPC, ether PLs	[8, 19]
Mouse heart	Diabetes	ESI-MS	CL	[9, 23]
Human serum	Cardiovascular disease	^{1}H-NMR	ChoE, long chain TG	[82]
Human plasma	Pancreatic cancer	^{1}H-NMR	PI	[26]
Ascites fluids	Ovarian cancer	ESI-MS	Acyl-, alkyl- and alkenyl-LPA, lysoPI, sphingosylPC	[27]
Breast cell lines	Human breast cancer	^{1}H-NMR	Choline PLs	[68]
Human plasma, synovial fluid	Rheumatoid arthritis	^{31}P-NMR, MALDI-TOF-MS	PC, lysoPC	[87]
Human plasma	Chronic glomerulonephritis	NPLC-MS/MS	PI, PS	[88]
Mouse heart	Mitochondrial dysfunction	ESI-MS	CL	[40]
Epithelial cell	Epithelial barrier dysfunction	GC-MS, LC-MS/MS	n-3 PUFAs	[89-90]
Dried bloodpots	Barth syndrome	HPLC-MS/MS	MonolysoCL, CL	[91-92]
Human urine	Fabry disease	ESI-MS/MS	SPM (22:0), glucosylCer (22:0), Cer trihexoside (24:1), lactosylCer (24:1)	[93]
Human serum, pericardial fluid and peritoneal fluid	Gaucher disease	MALDI-TOF-MS	Cer monohexoside	[94]
Human brain	Alzheimer's disease	ESI-MS/MS	Cer	[95]
Human plasma	Statin-induced myopathy	UPLC-MS	PE (36:1), PE (40:4), TG (54:2), TG (54:3), TG(56:5), SPM (d:18/22:0), PC (O-38:5), PC (O-34:3), ChoE (18:0), ChoE (18:2)	[96]
Human plasma	Differential effects of two statins on cardiovascular risk therapies	HPLC-MS	SPMs, PCs	[97]
Rat liver	Kyoto and Wistar strains of rats[a]	NMR	PC	[98]
Rat tissues	Zucker diabetic fatty (ZDF) rat[b]	ESI-MS	PC and PE	[99]
Mouse liver and plasma	APOE*3 Leiden transgenic mice[c]	HPLC-MS	Cho	[100]
Mouse liver and blood	Purebred male wild-type C57bl6 mice[d]	HPTLC, RPLC-MS	free Cho, TG, ChoE	[101]

[a] rat model of fatty liver disease
[b] genetic rat model of type II diabetes mellitus
[c] transgenic mice of atherosclerosis and live inflammation
[d] wild-type mice of starvation-induced hepatic steatosis

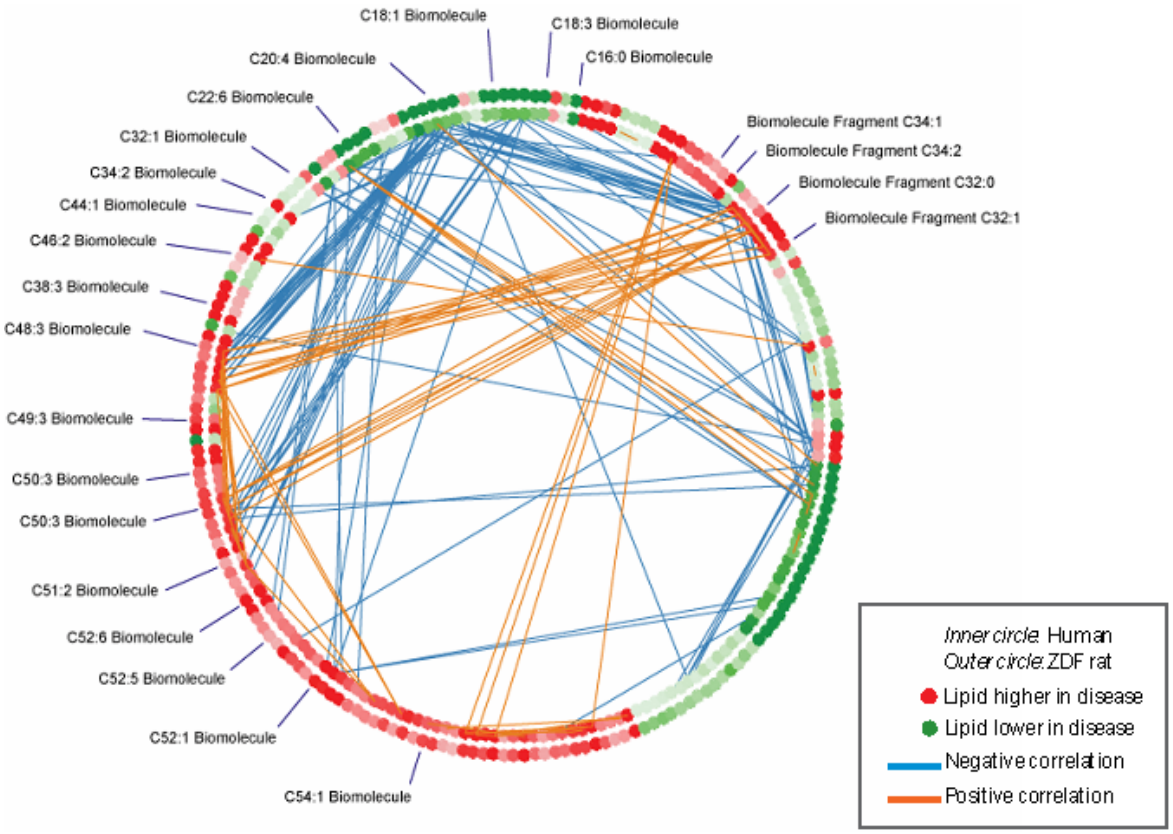

Figure 5 Visualizing similarities between regulation of selected plasma lipids between human and rat for study of hypertriglyceridemic individuals and obese Zucker diabetic rats using COSA [ref.[102]].

Perspectives

Lipidomics is an emerging methodology holding promise for a systems-based study of a wide range of lipids. Recent advances in MS technologies and improvements in chromatography have greatly enhanced the developments and applications of lipidomics. Currently, novel lipidomics technology enables an unprecedented power in lipid research in which alterations of lipid metabolite profiles can be associated with diseases, and changes in lipid metabolism or pathway modulation by lipids due to diseases can be detected in complex biological systems. This provides new insights in *e.g.* metabolic and inflammatory diseases, and the role of lipids in biological systems in general. The symbiosis of lipid profiling and multivariate statistics in a lipidomics approach can help us in discovering potential biomarkers, understanding disease pathology, drug response monitoring in pharmacology and toxicology, translational medicine and in depth deciphering mechanisms of lipid-mediated disease. The integrative collaboration of analytical, statistical and lipid biologist are a requirement in this field to improve this field further and achieve more breakthroughs in lipid research. Better and more data on very low abundant lipids and better predicting data models will reveal more of abnormalities in lipid metabolism associated with diseases.

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