Kim Ekroos

1.1 Introduction

Lipids are recognized as extremely diversified molecules, with nearly 10⁴ different structures of lipids currently being stored in the most comprehensive lipid structure database (LIPID MAPS, http://www.lipidmaps.org). The complexity is confounded by the fact that the absolute quantity of individual molecular lipids can differ among lipid species up to several million-fold depending on the matrix of origin. These features unprecedentedly complicate a precise assessment of the actual number of lipid entities and their identities and quantities making up a lipidome of a biological system. The accomplishment of this task now relies on lipidomics. Cutting edge lipidomics has already demonstrated its supremacy by revealing, for example, over 500 lipid species in human plasma [1, 2] and 250 lipid species in yeast [3]. With the currently ongoing meticulous developments in this field, it is highly anticipated that it will in the coming years facilitate delivery of a close to complete lipidomic content and precisely determined. If this increases the total number of species, for instance, in the human plasma lipidome to comprise thousand lipid species or more still remains to be seen.

The lipidome of eukaryotic cells is believed to contain thousands of lipid entities that structurally and chemically regulate cell membranes, store energy, or become precursors to bioactive metabolites [4, 5]. Lipids primarily reside in cellular membranes. The individual membranes of a cell have unique lipid compositions, required for serving their vital biological functions. For example, the free cholesterol (FC) to total phospholipid (PL) ratio in the endoplasmic reticulum (ER) membrane in mammals has been shown to be 0.15, whereas in the plasma membrane the ratio is 1 [6]. Although ER is the main site of lipid synthesis, it is the local lipid metabolism that is the prime determinant of the unique compositions of organelles. Moreover, further membrane specialization is orchestrated by lateral organization to form dynamic platforms, that is, lipid rafts, within the cellular membranes serving as functional assemblies for diverse processes such as signal transduction, membrane trafficking, and cell adhesion [7]. The physiological response and bioactive output of such lipid raft domain or cellular membrane will

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collectively be defined by the present molecular lipid structures, their local concentrations, and spatial distributions [8]. In addition, several studies have demonstrated and highlighted the importance and specificity of single-molecule lipid structures in determining the biofunctionality. Thus, based on these facts, it is highly anticipated that a defect in the underlying lipid regulation can lead to deleterious effects on the cell or organism and assist in the pathophysiology of diseases.

Precise determination of molecular lipid species becomes a prerequisite not only to gain their biological functions that might vary depending on the localization but also to gain their roles in a lipid collective. It is highly envisioned that this opens up new avenues in cell biology, biochemistry, and biophysics as it will untie the organization and function of the complex lipid metabolism machinery and its association with the construction and formation of unique cellular membranes. An essential aspect is that this information will accelerate our understanding of human diseases. This applies not only to pharmaceutical drug discovery programs but also to nutrition programs. It is known that major diseases such as atherosclerosis, infectious diseases, Alzheimer's disease, and cancer all have a lipid component in their epidemiology. Through precisely defined maps of the lipid metabolism and its regulation, more targeted delineations of the underlying dysfunctional metabolic pathways and cellular events can be obtained that are likely to result in the discovery of the culprit(s) associated with a particular disease. As it will unravel the mechanisms of action, one can envision that this will advance the discovery of new drug targets and efficacy and diagnostic biomarkers. In the field of nutrition research, the gained know-how will facilitate innovation of healthier food formulas. Finally, as the valid drug efficacy and disease diagnostic lipid biomarkers are discovered, they need to be transferred into a regulatory environment. Not only will this demand stringent analytical quality fulfilling the regulatory guidelines, but will also require the assays to be cost-effective and high-throughput oriented.

This chapter describes the types of lipid information the currently applied analytical platforms produce and how these differently assist in understanding biology. Future viewpoints of lipidomics in respect to its expected deliveries and upcoming challenges will be given. Special focus has been put on molecular lipids as these hold the answers in lipid biology.

1.2

Hierarchical Categorization of the Analytical Lipid Outputs

The applied analytical approach determines the level of details of delivered lipid information. According to the currently available techniques, the following hierarchical categorization can be made: (i) lipid class, (ii) sum compositions, (iii) molecular lipids and its related category, and (iv) structurally defined molecular lipids (Figure 1.1). Following this hierarchy, the number of entries belonging to phosphatidylcholine (PC) in human red blood cells would, for instance, be 1 lipid class, 18 sum compositions, more than 40 molecular lipids, and finally more than 100 structurally defined lipids [9].



1-(9Z-hexadecenoyl)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine

Figure 1.1 Hierarchical categorization of lipid outputs based on the analytical approach. The number of entries per category is based on PC of human red blood cells [9]. The structure of

PC 16:1n7/18:1n9 is shown. ^aExpected number of entries including all positional isomers. ^bLikelihood of number of entries, although it still remains unknown.

1.2.1 Lipid Class

The first lipid information level is lipid class. This output principally originates from the use of traditional techniques such as thin-layer chromatography (TLC) and normal-phase liquid chromatography (NPLC). These techniques together with gas chromatography (GC) have been the principal tools for assessing lipid measurements over decades. Profound descriptions of their principles and applications exist in literature. The benefit of these techniques is their capability to separate lipids into respective classes. This ability has been enormously utilized for exploring the lipid content of biological tissues and biofluids, results that strongly impacted the evolution of the lipid biology framework. However, their major drawbacks are recognized in the incapability of elucidating individual lipid entities, low detection sensitivity, and time ineffectiveness.

Lipid class measurements can also be obtained using the current lipidomic techniques. This can, for example, be achieved using shotgun lipidomics by monitoring lipid class selected fragments. For instance, precursor ion scanning (PIS) analyses of m/z 184.1 in positive ion mode selectively detects phosphorylcholine containing lipids such as phosphatidylcholine (PC) and sphingomyelin (SM) [10]. The

corresponding PC or SM lipid class levels would be obtained by adding up all signals of the identified species, for example, 50. We have previously proven this approach to be valid. Here, we monitored the molecular composition of lipid species analyzed by shotgun lipidomic analysis of total HepG2 lipid extracts before and after NPLC fractionation [11]. The molecular species composition was not affected by the NPLC separation. Moreover and most importantly, direct analysis of total lipid extracts by PIS estimated the total amount of cholesteryl ester (CE) in HepG2 cells to be 57 nmol/mg protein. In comparison, quantification using evaporative light scattering (ELS) detection together with the NPLC fractionation determined the total amount of CE to be 53 nmol/mg protein. Thus, two independent approaches and detector systems were shown to produce identical outputs. This strongly indicates that the applied lipidomic method is both qualitatively and quantitatively valid. Notably, factors such as methodological approach, selection of internal standards, and isotopic correction will influence the quantification accuracy.

Global or untargeted lipidomics, such as liquid chromatography-based full scan mass spectrometry (MS) analyses (i.e., LC-MS), could also in theory be utilized to determine total lipid class content. However, it has been recognized that ion suppression, which strongly influences quantification, might be more complex during LC-MS analysis. Since ion suppression is likely to vary during the chromatographic run due to the difference in the eluting mobile-phase composition (for gradient LC-MS methods) and sample matrix, it can lead to unequal signal responses of the different lipid species of the same lipid class even though they are present at equimolar concentrations. Data supporting this idea were recently published [1]. Optionally, the collision energy could be optimized for each analyte to correct the suppression effects. However, this becomes difficult or even unpractical as the settings are likely to be different, depending on, for example, sample matrix (different background) and LC conditions. This issue is best solved by using stable isotopelabeled lipid standards that are structurally similar to the endogenous species. Under such circumstances, targeted LC-MS approaches have shown to be superior for absolute quantification. However, since synthetic standards for each endogenous lipid species are still unavailable, lipid class quantification in absolute amounts by summing multiple various lipid species of the same class is not feasible by this method. This has been described in greater detail in Chapter 5. Thus, the quantification accuracy related to absolute lipid class content from reversephase LC-MS-based lipidomic data still awaits to be proven. Until then, it is recommended that the available published results should only be considered as estimates.

1.2.2

Sum Compositions

The level following lipid class is sum composition or brutto lipids. Common lipidomic approaches are capable of elucidating lipids with different sum compositions, for example, phosphatidylethanolamine (PE) 36:4, where 36 represent the total number of carbon and 4 the total number of double bonds in the attached fatty acids [10]. In a full mass spectrometry analysis, such type of information can already be obtained. Since no selective analysis modes are usually required, a profile of the sum lipid composition can be very rapidly acquired, either in conjunction with LC or with direct infusion approaches. For instance, in the latter, by taking advantage of the high mass-resolving power of instruments, such as an orbitrap or a Fourier transform ion cyclotron resonance mass spectrometer, a broad profile of brutto lipids can be readily identified and quantified in only minutes from unresolved samples [12]. Here, the high mass accuracy is used to separate the actual lipid peaks from the chemical noise. The simplicity, reliability, and the speed of such methods assisted by the lipid software advancements [13] have become not only attractive for standard lipidomic analyses but also very appealing for highthroughput lipidomic screenings. However, the grave weakness of this approach is that the results are still difficult to biologically interpret due to the missing details of the molecular lipids. This is more thoroughly discussed below.

1.2.3

Molecular Lipids

After sum compositions follow molecular lipids in the hierarchy (Figure 1.1). Targeted or focused lipidomic approaches such as LC-MRM and shotgun-based PIS and neutral loss scanning (NLS) are well-established techniques for the identification and quantification of molecular lipids. Their common lipid output could, for example, be PC 16:0/18:1, where the information on the type of fatty acids and their positions attached to the glycerol backbone making up the particular lipid molecule are revealed. Alternatively, this could be output as PC 16:0–18:1, where " – " describes that the positions of the fatty acids are not determined. The basis of these approaches is to monitor the lipid characteristic fragmentation ions, for example, head groups and acyl anions, to delineate the molecular species. MRM, PIS, and NLS techniques are described in greater detail in other chapters of this book and therefore only a brief overview is given here.

In MRM, m/z of both precursor and fragment ions are specified. Precursors of interests are isolated in quadrupole Q1 and subjected to fragmentation in quadrupole Q2. Subsequently, selected fragment ions are set to pass in Q3, and the abundance of the specified fragment ions is monitored by the detector allowing quantification of targeted molecular lipids. In conjunction with LC, this approach becomes highly selective as the latter system facilitates a vast and reproducible sample cleanup prior to MRM. Reduction of sample complexity prior to MS analysis improves not only the success rate of monitoring molecular lipids but also the sensitivity of the method. However, a drawback of this approach is the limited transitions, that is, number of molecular lipids, which can be covered during an analysis run due to insufficient chromatographic peak collection caused by the limited acquisition speed of the MS.

In contrast, a shotgun lipidomics-based PIS and NLS analysis is typically not limited to acquisition time, as it has been shown that minute sample extracts can be robustly infused for an hour or even more [11]. Therefore, the lipid coverage can be

significantly greater with this approach. On a quadrupole time-of-flight (QTOF) instrumentation, we previously demonstrated the possibility to simultaneously acquire 40–50 PIS using multiple precursor ion scanning (MPIS) [14]. Using the recent QTRAP technology, we can rapidly and sensitively acquire a total of 70–80 PIS and 20–30 NLS that cover both fatty acids and lipid head group fragment ions within the quadrupole Q3, while the quadrupole Q1 is scanning lipid precursors [1]. Typically such an analysis identifies and quantifies several hundred different molecular species in approximately 30 min. These shotgun lipidomic methods have proven suitable for high-throughput lipidomic screenings.

Molecular lipid information could also be retrieved by, for instance, fragmenting (i.e., MS/MS) all eluting peaks during a chromatographic run or all precursors detected in a direct infusion full scan (i.e., MS) analysis. An example of the latter is the recently described technique sequential precursor ion fragmentation [15]. Here, precursors in a selected mass range are stepwise isolated (1 amu) in Q1 at unitbased resolution and subjected to collision-induced dissociation (CID) in Q2, while collecting more than a thousand MS/MS spectra covering every precursor in the mass range of each cycle. The power of this methodology is that it collects full MS and MS/MS of every precursor, and therefore nothing is left behind. Utilizing the high acquisition speed and mass accuracy of the recent QTOF technology, a complete lipidomic analysis covering over 400 molecular lipids in human plasma could be accomplished in less than 12 min including positive and negative polarities [15]. This is the best performance of a molecular lipidomic methodology at present. Evidently, this type of emerging instrumental technologies in combination with matching software tools will create new opportunities in molecular lipidomics as it amends the extensive acquisition times and maintains outstanding data quality and comprehensiveness. Thus, the outlooks are most promising and positively will open up new solutions for high-throughput screenings.

1.2.4

Structurally Defined Molecular Lipids

The molecular lipid information will immensely facilitate the untying of the unknown knowledge in lipid biology. Further advancements will be achieved once more structural information of the particular molecular species can be determined, such as the double bond position determination in the attached fatty acids. Analytical approaches for elucidating this type of information have recently emerged. Although the technologies are still rather immature, they deliver essential biological information. Therefore, the final level in the hierarchy is defined as structurally defined molecular lipids (Figure 1.1).

A most promising technique is OzID, which is described in greater detail in Chapter 6. The basis of this technology is that ozone vapor is introduced to the collision cell of the mass spectrometer, which will react with double bonds, for example, of fatty acids, and selectively dissociate them. This process therefore generates characteristic fragment ions that facilitate determination of the double bond position [16, 17]. This technique applies in principle to all types of double bonds. For example, Mitchell and colleagues have shown that OzID facilitates proper identification of ether lipids, that is, containing alkyl and alkylen bonds, which are typically difficult to assess by conventional MS approaches [18]. Preliminary results also suggest that this technique could distinguish *cis*- and *trans*-bonds, however this still needs to be proven. Thus, the emergence of completely structurally defined molecular lipids is awaited in the near future. Clearly, this evolution will depend on OzID and other similar nascent techniques.

1.3 The Type of Lipid Information Delivers Different Biological Knowledge

It is critical to underscore that the various detail levels represent different implications in biology. For example, lipid class information does not reveal the detailed composition of a plasma membrane, whereas molecular lipid species information is required to fulfill this task. In contrast, triacylglycerol (TAG) level in human plasma enables us to better understand the health condition. For instance, high TAG levels in human plasma (hypertriglyceridemia), has been identified as a risk factor for coronary artery disease (CAD). Thus, the different types of lipid outputs guide us to understand a biological system from diverse angles.

As mentioned above, our current know-how in lipid biology has been strongly impacted by the extensive measurements of lipid classes performed over decades. Lipid class information produces an essential overview of a biological system. For example, it is well known that high level of cholesterol in low-density lipoprotein (LDL) is a hallmark for increased risk of atherosclerosis. Another example would be monitoring of membrane fluidity by measuring the PC to PE ratio. Here, it has been implicated that a decrease in the ratio might induce a loss of membrane integrity [19, 20]. The repertoire of biological examples is extensive and well documented. Thus, substantial biological understanding has already been gained through studying deviations in lipid classes from their normal levels. Undoubtedly, this will remain as an essential asset for upcoming lipid research.

Information on brutto lipids has been rapidly emerging during the recent years. Sum composition information has been, for example, utilized to estimate the lipid content of cellular membranes [21], isolated viruses [22], and cells [23, 24]. The available information on total double bonds has further been used to determine the degree of saturation, that is, saturation index, which is useful for studying membrane behavior. However, sum compositions to a great extent have been assessed in studies related to diseases or dysfunctions. Here, the main objective has been to identify diagnostic or prognostic biomarkers based on observed deviations in brutto species between healthy controls and cases. Many studies of this kind have been described over the recent years. However, less focus has been put on elucidating the underlying biological mechanisms causing the observed changes. A prime reason for this is that the obtained results are normally difficult to interpret as such sum compositions do not exist in biological systems, rather it represents a collection of lipids. Moreover, there is a high risk that such a collection

$\Delta 5$	$\Delta 6$	<u>Δ9</u>	Δ11	Δ13	$\Delta 15$
n15	n12; n10	n5; n7; n9; n11	n7; n9; n11	n9	n7; n9
l6:0/20:1n15/16:0	16:0/18:0/18:1n12	14:1n5/20:0/18:0	16:0/18:0/18:1n7	14:0/22:1n9/16:0	14:0/22:1n7/16:0
l6:0/16:0/20:1n15	16:0/18:1n12/18:0	14:1n5/18:0/20:0	16:0/18:1n7/18:0	14:0/16:0/22:1n9	14:0/16:0/22:1n7
l4:0/20:1n15/18:0	18:1n12/16:0/18:0	20:0/14:1n5/18:0	18:1n7/16:0/18:0	22:1n9/14:0/16:0	22:1n7/14:0/16:0
l4:0/18:0/20:1n15	14:0/20:0/18:1n12	14:1n5/22:0/16:0	14:0/20:0/18:1n7		14:0/24:1n9/14:0
l8:0/14:0/20:1n15	14:0/18:1n12/20:0	16:0/14:1n5/22:0	14:0/18:1n7/20:0		14:0/14:0/24:1n9
	20:0/14:0/18:1n12	22:0/16:0/14:1n5	20:0/14:0/18:1n7		
	16:1n10/18:0/18:0	14:0/24:0/14:1n5	14:0/20:1n9/18:0		
	18:0/16:1n10/18:0	24:0/14:0/14:1n5	14:0/18:0/20:1n9		
	16:0/20:0/16:1n10	24:0/14:1n5/14:0	18:0/14:0/20:1n9		
	16:0/16:1n10/20:0	16:1n7/18:0/18:0	16:0/20:1n9/16:0		
	16:1n10/16:0/20:0	18:0/16:1n7/18:0	16:0/16:0/20:1n9		
	14:0/22:0/16:1n10	16:0/20:0/16:1n7	16:0/20:1n11/16:0		
	14:0/16:1n10/22:0	16:0/16:1n7/20:0	16:0/16:0/20:1n11		
	22:0/14:0/16:1n10	14:0/22:0/16:1n7	14:0/22:1n11/16:0		
		14:0/16:1n7/22:0	14:0/16:0/22:1n11		
		22:0/14:0/16:1n7	22:1n11/14:0/16:0		
		16:0/18:0/18:1n9			
		16:0/18:1n9/18:0			
		18:1n9/16:0/18:0			
		14:0/20:0/18:1n9			
		14:0/18:1n9/20:0			
		20:0/14:0/18:1n9			
		14:0/20:1n11/18:0			
		14:0/18:0/20:1n11			
		18:0/14:0/20:1n11			

Figure 1.2 Possible molecular TAG species corresponding to brutto TAG 52:1 in mammalians. Delta (Δ) and n nomenclatures describes the double bond positions. Delta nomenclature is used to assign the position of

an individual double bond or specificity of enzyme inserting it, whereas n nomenclature is used to assign individual fatty acids within a family of structurally related lipids [25].

could produce misleading results due to influence of contaminating species that are not associated with the actual study topic. For instance, as shown in Figure 1.2, in mammalians the TAG 52:1 could comprise nearly 90 different structurally defined molecular species. Thus, this aspect hinders both the biological interpretation and the success rate of the biomarker discovery. Despite this drawback, sum composition information can offer a valuable biological insight, but notably at a more general level. How to most optimally use sum composition information still remains blurred. Deconvolution strategies to outline the underlying species could be a way forward to gain insights into the biological mechanisms as this would facilitate metabolic mapping explorations.

Most promise in lipid research rests on the molecular lipids and structurally defined lipids as these are highly expected to open up new paths in biomedical research. As described above, biology is not regulated at the lipid class or sum composition level, but rather at the level of actual molecular lipid species. As already mentioned, the likelihood of misidentification significantly increases in a sum composition analysis, due to which molecular lipid species information is masked in these data. This becomes evident from Figure 1.2, recognizing the potential of vast arsenal of underlying species of a single brutto entity. Undoubtedly, reliable analysis of the molecular lipid species is of utmost importance as they may have very well-defined functional roles. Therefore, the molecular lipid species

information (together with structurally defined molecular species) should give us the highest success rate in identifying the culprits in the causal lipid metabolic networks leading to metabolic dysfunction states. This is discussed in more detail in the following section.

1.4 Untying New Biological Evidences through Molecular Lipidomic Applications

Chapter 2 intriguingly reviews the multifaceted lipid architecture in cells with emphasis on the capability of lipids to form morphologically different membrane structures for maintaining the cell function. Fascinatingly, a functional human red blood cell requires over 100 distinct PC molecules at individual concentrations and distribution in its plasma membrane (Figure 1.1). Why such a high number of individual molecules is required still remains unknown. However, it demonstrates the biological complexity of lipids. Moreover, the authors describe that platforms, that is, lipid rafts, existing within membranes have highly specific functions. However, the knowledge of which lipids make up the lipid rafts is still limited. In addition, the platforms are likely to undergo reformations in their compositions, both in lipids and proteins, to alter their function. Obviously, the molecular lipid composition of this platform plays a vital role and therefore needs to be delineated in great detail to understand its function. Only molecular lipidomics accompanied with structurally defined molecular species can address this type of questions. This fulfills precise identification and quantification of each present lipid species, although issues such as insufficient sensitivity will remain as the sample amounts will be extremely small. Undoubtedly, this is an enormous challenge that lipidomics will be facing in the coming years; hence, lipidomics should be orchestrated with biophysical and biochemistry experiments to answer the cell biological questions.

Several studies have demonstrated the importance and specificity of single-molecule lipid structures rather than a lipid (and protein) collective in determining the biofunctionality. An impressive work has been shown by Shinzawa-Itoh et al.. They showed by sophisticated experiments that the oxygen transfer mechanism in cytochrome c oxidase requires a specific phosphatidylglycerol molecular lipid with palmitate (C16:0) and vaccenate (C18:1n7) at the sn-1 and sn-2 positions, respectively, on the glycerol backbone [26]. Altering the double position from n7 to n9 (from vaccenate to oleate) inactivated the function. Thus, a small conformational change in the attached fatty acid is sufficient to inactivate the oxygen transfer mechanism. Moreover, Menuz et al. showed that C24-C26 carbon ceramides mediated the death of a Caenorhabditis elegans mutant that failed to resist asphyxia, whereas ceramides with shorter chains had the opposite effect [27]. We have recently been able to obtain similar findings in mammalians. Here, we showed that the C24 carbon ceramide induced ER stress, whereas the shorter chain (C20-C22 and C16) ceramides had no effect on HL-1 cardiomyocyte cells [28]. Finally, Ewers et al. recently showed that the structure of the ganglioside GM1 determines the simian virus 40 (SV40)-induced membrane invagination and infection [29]. They

demonstrated that GM1 molecules with long acyl chains facilitated entering of SV40 through the host cell plasma membrane, while GM1 molecular species with short hydrocarbon chains failed to support the invagination and endocytosis and infection. Evidently, these examples already underscore the essence of molecular lipids and technologies for their discoveries.

Exhaustive research lies behind our current knowledge of lipid metabolic pathways. Although the lipid metabolism is well characterized, information about the molecular lipid metabolism still remains unknown. The main reason for this is that the applied technologies were capable of determining only those lipids that are at the lipid class level. Even though gas chromatography-based analyses have directly allowed tracking of the fatty acid and oxidized fatty acid metabolisms, there still remain a significant number of unknowns in their metabolisms, especially of the latter. This is mainly due to lack of sensitivity and specificity of the applied approaches.

Much promise now relies on lipidomics to untie the lipid metabolism at molecular level (and structural defined). Lipidomics exhibits the analytical preferences for this quest. We have recently demonstrated the power of lipidomics in elucidating the lipid metabolism in yeast [3]. Although the pathways are outlined in the form of lipid classes, the experiments identified that the individual classes comprise of unique lipid species, thus indicating that the metabolism is regulated at the molecular level. However, there is still no solution in respect to how to connect the molecular lipids in the metabolism. An utmost challenge lies in the measurements of single metabolic events. It can be expected that a whole-cell measurement represents the total sum of events that concurrently occur in the cell. Thus, how to separate, for instance, a local metabolism of PC in the plasma membrane from a coexisting PC synthesis in ER or metabolism in other organelles remains unclear. Fortunately, enlightening approaches tackling this are emerging. One approach is to perform metabolic flux experiments using stable isotopes that incorporate selectively and efficiently into lipids of interest. The approach will then be to specifically measure the labeled lipids during a time course to produce a kinetic readout of the lipids of interest. In this way, the synthesis or catabolism rates of lipids could be established. Haynes et al. recently described the use of a stable isotope-labeled precursor ([U-¹³C]palmitate) to analyze *de novo* sphingolipid biosynthesis by tandem mass spectrometry [30]. Moreover, Pynn et al. incorporated a deuterated methyl-p9labeled choline chloride to quantify biosynthesis fluxes through both the PC synthetic pathways in vivo in human volunteers and compared these fluxes with those in mice [31]. In conjunction with sophisticated lipidomics, they were able for the first time to show that phosphatidylethanolamine-N-methyltransferase (PEMT) pathway in human liver is selective for polyunsaturated PC species, especially those containing docosahexaenoic acid. Kuerschner et al. utilized a highly unique isotopic label facilitating detailed lipidomics-assisted tracking of labeled molecular lipids and in concert with their cellular localization by fluorescent microscopy [32]. The label does not necessary need to be detectable by advanced microcopy techniques. For instance, a general (nonfluorescent) labeled lipid could be precisely determined by imaging lipidomic techniques (described in Chapter 7). Very recently, this

labeling approach in conjunction with lipidomics lent to the discovery of exclusively one sphingomyelin species, namely, d18:1/18:0, interacting directly and being highly specific with the transmembrane domain (TMD) of the COPI machinery protein p24 [33]. The results demonstrate that the exclusive molecular sphingomyelin acts as cofactor to regulate the function of a transmembrane protein and thus again point out why biological membranes are assembled from such a large variety of different lipids and the essence of single entities. Taken together, labeling experiments in concert with molecular and imaging lipidomics compose the right ingredients for tackling the delineation of the molecular lipid metabolism. Together with subcellular dissection of the cell, enzymatic silencing or inhibitory experiments, and supported bioinformatics tools, these show the most promise for the discovery of the biological roles of molecular lipids and mapping of the molecular lipid pathways.

1.5 Molecular Lipidomics Approaches Clinical Diagnostics

The foreseeable biological specificity residing in molecular lipids make them prime candidates for drug and biomarker discovery. However, the success rate will substantially depend on the selected experimental design and its accomplishment. The use of isotopic tracers, lipid imaging, and subcellular dissections are likely to be key assets. These types of experiments are rather trivial to perform in vitro, however, for instance, isotopic labeling in vivo is still a very challenging task. Another asset is the integration of genomic and proteomic data with the molecular lipidomic data set. However, it still remains blurry in how to mine such large data sets. How biological representative the currently existing results are remain very unclear, bearing in mind that the applied lipidomics results are mainly based on lipid sum compositions. No clear evidences exist demonstrating that the "omic" mining results correspond to the results from basic biochemistry and biology. Thus, the risk for misinterpretation can still be rather high in such hypothesis-driven experiments. A final strength is the combination of biochemistry, analytical chemistry, biophysics, biology, bioinformatics, and medicine know-how and expertise. Taken together, the receipt for discovery of new drugs and lipid biomarkers rests on how to retrieve most out of the above-described assets (and others) alone and/or collectively, and in most accurate way. Convincingly, an optimal component setup will take us beyond our current understanding in lipid biochemistry and biology. It is anticipated that the localization and function of the lipid metabolic machinery, including its active components and interactive companions, to be inclusively illuminated. The resulting novel lipid maps will foster the discovery of novel mechanisms of action (MoA), drug targets and drug efficacy and disease diagnostic biomarkers.

As clearly illuminated in other chapters, lipids are highly awaited serving as drug efficacy and disease diagnostic and prognostic biomarkers. A main reason for this is that lipids are physiological readouts of the complex gene-driven system that is affected by environmental factors. Molecular lipidomics in combination with the

appropriate clinical samples and biobank material can therefore be highly considered for escalating the improvement of disease diagnostics and prediction. This applies not only to a certain disease but also to many therapeutic areas, including cardiovascular diseases, neurological states, cancers, metabolic diseases such as diabetes, and inflammatory processes. It is anticipated that a single or up to a handful of molecular lipids rather than 20-50 different biological molecules fulfill the diagnostic purposes. Moreover, as lipids are considered as intermediate phenotypes that are actually much closer to the disease state in question than for instance genetic information, they could also serve as candidates for companion diagnostics in the pharmaceutical arena, which is moving increasingly toward the specialized therapeutics model. Similarly, as lipids have been highly preserved throughout the evolution of life, that is, highly similar lipid contents throughout the mammalian species, they can be highly considered for the assessment of translational medicine and thereby help in identifying the optimal experimental animal model most closely mimicking the human disease. A promising example has recently been described by Chan et al. [34]. They could identify a correlating behavior of GM3 and CE in certain Alzheimer's transgenic mouse models and in Alzheimer's disease patients. Although these results are most encouraging, they need to be further proved considering that the transgenic mice displayed highly dramatic lipid changes that were not seen in humans.

Once novel drug efficacy and prognostic and diagnostic molecular lipid biomarkers are discovered, the next step will be to move their monitoring into clinical laboratories. However, before this can take place it is required that the biomarkers are thoroughly validated. For instance, the identity and quantity of the discovered lipid biomarkers should be verified simultaneously as the analysis of other independent cohorts should validate the findings. Optimally, the biomarker validation should be performed in different clinical or diagnostic laboratories. In this cascade of the biomarker development, it will be required to adapt the lipidomic assay according to the regulatory requirements following the US Food and Drug Administration (FDA) guidelines. Consequently, the lipidomic assay has to undergo a thorough validation procedure, which includes, for instance, the determination of the method accuracy, precision, lower limit of quantification (LLOQ), long- and short-term stability, freeze-thaw cycles, and robustness. An example of a calibration curve of ceramide d18:1/17:0 including corresponding quality controls (QC) is shown in Figure 1.3. Here, the performance of the used LC-MRM method can be verified by the observed linear instrument response and performance of minimum three different QC samples in accordance with the FDA guidelines. Thus, this type of results confidently indicates that validated methods for measuring molecular lipids such as ceramides can be established. Indeed, Scherer et al. recently demonstrated a rapid and validated LC-MS assay for the measurement of plasma sphingosine 1-phosphate, sphinganine-1-phosphate, and lysophosphatidic acid [35]. Bearing in mind the labiality and the analysis difficulty of these lipids, these results prosperously demonstrate the feasibility of using lipids as validated diagnostic endpoints. The upcoming challenges, however, include the availabilities of qualified



Figure 1.3 Calibration curve with corresponding QCs. Samples prespiked with various amounts of the analyte ceramide 18:1/ 17:0 and extracted followed by a postspiked external standard served for constructing the calibration curve (n = 7). Extracted individual samples of equivalent biological matrix containing known concentrations of the analyte (low QC, QC 3; middle QC, QC 2; and high QC, QC 1) served as quality controls (n = 6 per QC). All samples were measured by LC-MRM. A linear regression coefficient R^2 of 0.9988

demonstrates a linear instrument response inside the selected concentration range. From this, the lower limit of quantification (LLOQ) is determined. The mean values of the measured QCs are within \pm 15%, thus fulfilling the accuracy and precision requirements set in the FDA guidelines. The obtained results justify the FDA acceptance criteria and are essential parts in the full method validation process. x-axis represents the calculated concentration of the analyte (μ M) and y-axis the measured analyte to external standard ratio.

internal standards, qualified sample handling, high-throughput adaptation, and FDA-approved MS-based setups.

Finally, but not the least, molecular lipidomics is recognized as an essential toolkit in nutrition research. Here, the scope will be to assist in optimizing food recipes aiming for improving human health. For instance, an interest is to reduce the omega-6/omega-3 ratio as a high level, such as 15: 1, in the diet of today's Western world, which promotes the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases syndrome. It has been shown that a ratio of 2.5: 1 reduces rectal cell proliferation in patients with colorectal cancer and a ratio of 2-3: 1 suppresses inflammation in patients with rheumatoid arthritis [36]. As the current studies are typically based on total fatty acid analyses, scarce or even no insights are produced on the underlying lipid metabolisms. Such delineation by molecular lipidomics and especially the emerging OzID lipidomics would produce a detailed overview of the fatty acid and lipid metabolic machinery and how this is regulated. An example of such delineation is given by Ståhlman et al. where they pinpointed selected metabolic shifts in the TAG metabolism of dyslipidemic subjects [37]. They showed that dyslipidemia was associated with elevation in TAG molecular species containing vaccenic acid, especially

TAG 16:0/18:1n7/16:0, indicating an involvement of delta-9 desaturase and elovl-5. It needs to be noted that this is the first time complex endogenous lipid species have been identified and quantified at this detailed level. In the case of omega-6/omega-3 ratio, a target of a similar outlining could be to identify and utilize the metabolic switches that favor the omega-3 production or other beneficial metabolites.

1.6

Current Roadblocks in Lipidomics

The success of a MoA, drug, or biomarker study decidedly depends on the bioanalytical quality. Since the stability information of molecular lipids in various matrices or milieus remains scarce or unknown, the sample handling should be performed with precaution. Here, not only storage of the samples but also the sample preparation practices and processes throughout the workflow should be considered. Careful sample collection is required where samples are preferably quickly frozen and stored at the appropriate storage conditions if they cannot directly be subjected to lipidomic analysis. This has recently been reviewed by Jung et al. [1]. It has been shown that certain biological matrices can be safely stored for years at -80 °C [38]. However, the stability can dramatically vary depending on the time, type of lipid, type of sample, and type of storage material and solvent. For instance, Hammad et al. recently showed that ethylenediaminetetraacetic acid (EDTA) is the preferred anticoagulant for sphingolipids [39]. This indicates that the material should carefully be selected to avoid unspecific reactions and interferences with the lipids of interest. Furthermore, the materials should also be resistant to the harsh solvent treatment, as typically a lipid extraction is performed in hazardous organic solvents such as chloroform, which itself can unfavorably react with lipids [40]. An example of the stability of lactosyl ceramide d18:1/24:0 from human serum is shown in Figure 1.4. This sphingolipid is shown to be rather stable when stored (10 days) in chloroform:methanol (1: 2, v/v) at -20 °C in standard eppendorf tubes (Eppendorf AG). Unexpectedly, its concentration is more than threefold elevated when stored in 96-well plates from the same vendor. The reason for this remains unknown. However, this shows that a small change in the storage condition can have fatal consequences and thus points out the essence in sample handling. Moreover, it is recognized that the number of freeze and thaw cycles can influence the end result [41]; however, two freeze-thaw cycles did not affect the levels of this sphingolipid.

Another challenge is that the end results are strongly influenced by the chosen extraction methodology and lipidomic instrumentations, leading to deviations in the final lipidomic outputs. Although much effort is currently put in making of synthetic standards, a substantial lack of proper nonendogenous standards is still a facto. Therefore, users have been stranded using the available synthetic standards, which regrettably in most cases have been insufficient for absolute quantification of monitored lipids. Thus, most of the currently available lipidomic reports are based on relative or semiabsolute lipid outputs, which can be difficult to compare due to



Figure 1.4 Effect of storage material on the stability of lactosyl ceramide d18:1/24:0. The sphingolipid was extracted as described in Ref. [1] from serum of two healthy donors. The total chloroform:methanol (1: 2, v/v) extract was analyzed by LC-MRM [28] fresh (solid bars),

after 10 days of storage in standard 2 ml eppendorf tubes (small squared bars), and after 13 days of storage in eppendorf 96-well microtiter plates (large squared bars) at -20 °C. Independent samples were analyzed and error bars indicate standard deviation (n = 6).

deviations in both applied methodology and applied internal standards. A relative or semiabsolute lipidomic measurement can be considered valid for retrieving the first glims of lipidomic differences. However, once the focus is turned to determine, for example, the precise size of cellular membranes or lipoproteins and the lipid biomarker in clinical diagnostics, absolute measurements becomes a prerequisite.

Indisputably, lipidomics suffers from the lack of standardization. Naturally users apply their own preferred protocols and methodologies to assess their lipidomic quests. Regrettably, deviations in, for example, sample handling, extractions, synthetic standards, instrumentations, and data processing tools between laboratories lead to inconsistencies in the lipidomic results, which in turn complicate further data comparison and combination efforts. Transparent data collections will be a fundamental foundation triggering the direction of future lipidoimcs. Rigorous standardization and validation of the lipidomic processes are therefore urgently needed. Once established, new generation of lipidomic assays can set off and widely be adapted to clinical practices. This on the other hand leads to new challenges since such an adaptation typically demands high-throughput performance assays. We have recently demonstrated the feasibility of such attempts in discovery work [1]. The virtue of this high-throughput molecular lipidomic workflow relies on its high reproducibility and controllability, gained through robot-assisted sample preparation and lipid extraction and multiple lipidomic platforms integrated with a sophisticated bioinformatics system. Currently, it offers the highest throughput in

delivering simultaneously the most comprehensive and quantitative lipidomic outputs at the molecular lipid level. For instance, approximately 5 days are required to determine the concentration of over 500 molecular lipids in 20 different lipid classes of 96 human plasma samples. Thus, this setup illuminates and demonstrates the first attempts toward high-throughput quantitative molecular lipidomics and, although not confirmed, prosperously supports its suitability in a regulatory setting.

1.7

Conclusions

The lipidomics era is currently occurring. Applications in basic and applied research have clearly pointed out and demonstrated its indispensable value. It will open up new avenues in the biomedical research community, with high expectations that this discovery toolkit will enhance biomarker discovery and provide novel information to target discovery programs as it will prospectively shed new light onto the affected metabolic and signaling pathways. Undoubtedly, it is the delineation of molecular lipids and their precise determination that will lead the way forward and accelerate our understanding of molecular lipids, the integrated lipidomic networks, and decoding the coordinately regulated pathways.

New attempts will be taken to overcome the challenges lipidomics currently faces. The standardization of sample preparation and analytical and bioinformatic procedures has to be properly addressed. Once solved, this launches the transition of lipidomics into clinical laboratories. In parallel, the demand for high-throughput technologies that do not compromise on the data quality is required. Emerging new MS technologies and methodologies already show promises by offering quantitative information on over 400 molecular lipid species obtained in less than 12 min [15]. If these platforms fulfill the regulatory requirements still needs to be seen.

It is now the time for researchers in biochemistry, analytical chemistry, biophysics, biology, bioinformatics, and medicine to gather and utilize lipidomics in the most productive way. It is the collective wisdom that will take us beyond our current know-how in lipid biology.

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