REVIEW

Nutritional lipidomics: Molecular metabolism, analytics, and diagnostics

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The field of lipidomics is providing nutritional science a more comprehensive view of lipid intermediates. Lipidomics research takes advantage of the increase in accuracy and sensitivity of mass detection of MS with new bioinformatics toolsets to characterize the structures and abundances of complex lipids. Yet, translating lipidomics to practice via nutritional interventions is still in its infancy. No single instrumentation platform is able to solve the varying analytical challenges of the different molecular lipid species. Biochemical pathways of lipid metabolism remain incomplete and the tools to map lipid compositional data to pathways are still being assembled. Biology itself is dauntingly complex and simply separating biological structures remains a key challenge to lipidomics. Nonetheless, the strategy of combining tandem analytical methods to perform the sensitive, high-throughput, quantitative, and comprehensive analysis of lipid metabolites of very large numbers of molecules is poised to drive the field forward rapidly. Among the next steps for nutrition to understand the changes in structures, compositions, and function of lipid biomolecules in response to diet is to describe their distribution within discrete functional compartments lipoproteins. Additionally, lipidomics must tackle the task of assigning the functions of lipids as signaling molecules, nutrient sensors, and intermediates of metabolic pathways.

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Abbreviations: ABCA1, ATP-binding cassette transporter A1; **ARA**, arachidonic acid; **CETP**, cholesterol ester transfer protein; **CVD**, cardiovascular disease; **CYP**, cytochrome P450; **FXR**, farnesoid X receptor; **HDL-C**, HDL cholesterol; **LA**, linoleic acid; **LOX**, lipoxygenase; **MVA**, multivariate analysis; **PPARs**, peroxisome proliferator-activated receptors; **SFA**, saturated fatty acids; **TG**, triglycerides; **TRL**, triglyceride-rich lipoproteins; **UPLC**, ultrahigh-performance liquid chromatography

1 Introduction: Concepts of nutritional lipidomics

The term lipidomics is quite new, first appearing in 2001. Its definition is still being debated, from "the comprehensive analysis of all lipid components in a biological sample" to "the full characterization of lipid molecular species and their biological roles with respect to the genes that encode proteins that regulate lipid metabolism". In principle, lipidomics is a field taking advantage of the innovations in the separation sciences and MS together with bioinformatics to characterize the lipid compositions of biological samples (biofluids, cells, tissues, organisms) compositionally and quantitatively [1]. This approach of making quantitative, comprehensive molecular measurements of lipids provides access to the same research strategies of metabolomics and proteomics that are changing

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our understanding of biological processes across all of the life sciences. The first generation of research using lipidomics tools has been successful with breakthrough studies identifying lipids in novel compositions and unique functions. The biological membrane is in particular an enigmatic living structure with a highly complex and dynamic molecular composition; its properties as soft matter and its dynamic nature render it frustratingly difficult to study. In a purely descriptive sense, lipidomics studies are revealing the basic biological properties of lipids whose comparable functions have been known for polynucleotides and proteins for decades. Successes in lipidomics studies are also slow due to the fact that lipids, like polysaccharides, are the products of enzymes and thus are removed from the inherited genome of organisms. As a result of being the products of diet and metabolism, lipids in vivo can exhibit wide variation within individuals and across organisms. Because lipids are metabolites of an individual's diet and metabolic pathways, the accurate measurement of all lipids within an individual biofluid, tissue, or cell type, positions lipidomics with the potential to revolutionize nutrition research. Diet has important effects on the substrates, products, and the overall metabolism of lipids in all animals and humans. The first generation of lipidomics articles have provided proofs of principle of its ability to: develop diagnostics of disease, detect microbial contamination, establish dietary actions, identify novel signaling pathways, define cellular compartments, reveal disease mechanisms, and establish toxicological targets and mechanisms [2–8]. Building on these successes, lipidomics is poised to become a central toolset in life science research.

2 Lipids as a unique class of biomolecules

Lipids are not genetically encoded molecules but are substrates and products of enzymes constituting metabolic pathways. Lipids are derived preformed directly from the diet or by de novo synthesis from simple precursors. Variations in lifestyle and genetics influence the enzymatic activities involved in their metabolism. The wide diversity of lipid structures relate to their wide array of functions ranging from energy storage, transport, and utilization to host protection, barrier functions, and insulation. Lipids make up the main fluid structures of cellular membranes at high concentrations. At low concentrations, lipids exhibit diverse roles in cellular communication through paracrine and autocrine signaling. Cellular lipids vary only very slightly in response to wide variations in diet, providing evidence for considerable homeostatic regulation to maintain lipids at constant levels.

In spite of the fact that the core biosynthesis pathways of lipids are mainly two; fatty acid and isoprenoid metabolism, the total number of complex lipids that are assembled from these two pathways is immense [9]. The heroic scientific job of identifying and cataloguing this biological library is being organized by several consortia including the Lipid Maps project [10–13], and the European Lipidomics Initiative [14]. The immense challenge of annotating the structures functions and diverse bioactivities of complex lipids will be the basis of an increasing fraction of basic life science research as the implications of applying this knowledge to improving human health becomes ever more apparent. For lipid scientists, they now have a virtual home (http://www.lipidmaps.org/).

3 Technologies of lipid analysis

The complexity of biofluids, cells, and tissues in terms of the total numbers and structures of lipids exceeds the capability of lipidomics to identify and quantitate all of the lipid structures present. Nevertheless, it is possible to generate accurate and precise quantitative data on subsets of lipid classes based on their chemical and physical behaviors. Research today takes advantage of the increase in the accuracy, sensitivity, and speed of mass detection of MS with structural information of complex lipids [15]. The ability of mass spectrometers with higher resolution and faster chromatography to accurately determine precise masses that mathematical computations solve for the structure of lipids was a major scientific breakthrough. Only recently have the enhanced accuracy, sensitivity, and efficiency of MS made it possible to begin the exhaustive process of cataloguing the lipid composition of cells and biofluids making the field of lipidomics possible for medical diagnosis and therapeutic treatment [16]. Yet, a single complex platform is unable to solve the varying analytical challenges of the different molecular species of lipids. Analytical strategies coupling different modalities—separation science and mass spectroscopy, notably liquid and GC and MS—into complementary platforms have been most successful. The enabling capabilities of HPLC coupled to ESI-MS and novel strategies for quantitative MALDI-MS to simultaneously identify and quantitate the full range of global cellular polar lipidomes directly from crude lipid extracts of biological samples was the first proof of principle of shotgun lipidomics [17, 18].

MS is now able to identify molecules by virtue of precise molecular mass measurement, thus providing the analytical power needed to identify large numbers of complex lipids simultaneously. However, some important problems remain. The isomeric diversity of fatty acids in biology still frustrates mass analyzers because the absolute mass of some of the fatty acid positional isomers is identical. Because these fatty acid isomers are biologically different, this limitation still needs to be addressed for the field of lipidomics to continue its rapid progress. Second, mass spectrometer ion sources still remain somewhat unstable and vary dramatically with vendor and design. As a result, MS in the absence of standards is not as quantitatively accurate as metabolite measurements need to be. For many applications, measuring the subtle changes associated with early stages of disease development is necessary.

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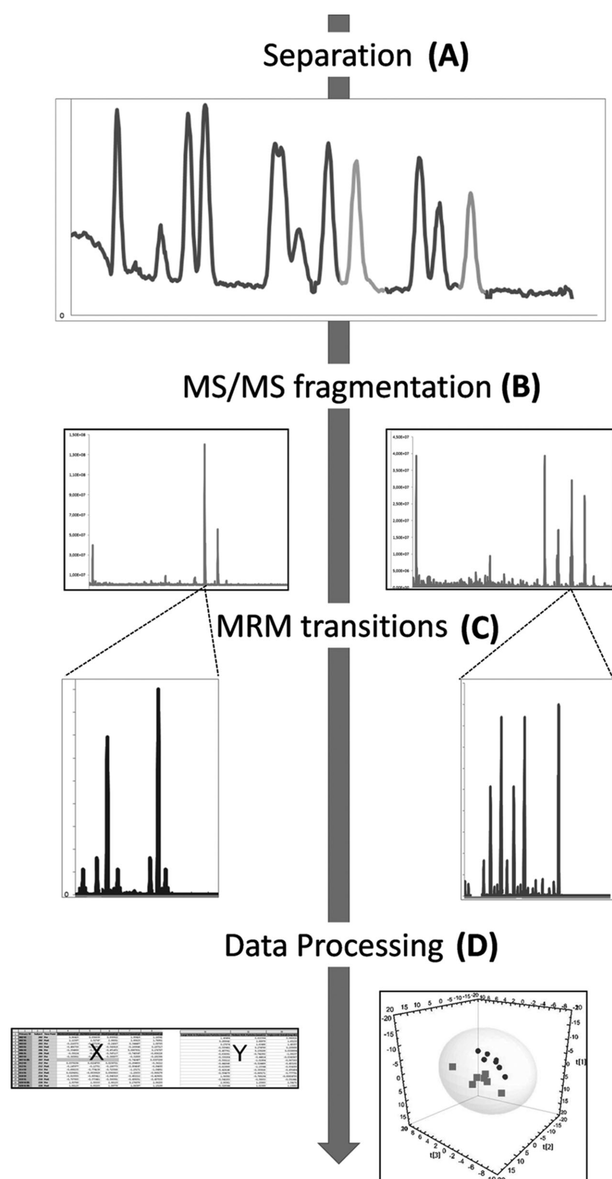

Figure 1. Targeted lipidomics using UPLC-MS/MS and MVA. Selection of the target fatty acid and oxylipin metabolites for (A) LC separation with the appropriate mobile and stationary phase. Tandem MS and multiple reaction monitoring provide molecular ions and daughter ion fragments (B) as well as specific and selective transitions (C) essential to identify and quantify the compounds, including those with co-eluting peaks. Metabolite levels are used in (D) data processing with principal component analysis and other multivariate methods (plot from Zivkovic et al. 2012) [99].

Resolution of lipid species with overlapping molecular weights of fatty acids with isomeric differences in the fatty acid structures is achievable with ultrahigh-performance liquid chromatography (UPLC) coupled to triple quadrupole LC–MS/MS detection [19] (Fig. 1). Normal phase HPLC coupled to electrospray ion-trap MS can simultaneously examine changes in multiple phospholipid classes (phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin) and the individual molecular species within each class without prior derivatization, TLC or SPE. Tandem LC coupled to MS has also been used to separate complex lipid species prior to ionization and detection by MS [20]. The strategy of combining tandem analytical methods to perform the sensitive, high-throughput, quantitative, and comprehensive analysis of lipid metabolites for high throughput of very large numbers of molecules is driving all of the fields of metabolism. This option is propelling the field of lipid biochemistry forward rapidly in describing which lipids are present in different tissues, cells, subcellular organelles, and lipoproteins. Nonetheless, the problem of quantitation is critical for understanding biological processes with the accuracy necessary to distinguish early stages of disease. For example, improved quantitation and qualitative analysis is needed to evaluate the slow, incremental deterioration of the vasculature seen in atherogenesis and the development of heart disease.

Quantification using MS requires that each analyte is compared to an internal standard and the actual amounts determined by peak area ratios [21]. These methods are clearly problematic for highly complex samples with dozens of different species of lipids because they require a large number of internal standards. Even more, the number of different linear ranges for detection required for such a highly multiplexed assay is not easily solved in a "global" analysis. Sacrificing quantification of metabolites for higher throughput is possible when only chemical identification is needed; however, using lipidomics in disease research is more challenging. Quantitative analyses are needed to detect the small differences that are the basis of varying states of metabolic health in different phases of disease. Hence, the decision to forego quantitative precision will limit the future utility of integrated databases of metabolites that are produced by these analyses. The success of the famous Framingham study in identifying variations in heart disease linked to variations in measureable biomarkers was predicated upon banking quantitative data of heart disease incidence and specific metabolites. This study protocol also highlights that it is only possible to correlate health and disease with metabolic processes if the status of those processes is accurately captured. The limitations of the Framingham and other large databases lie in the lack of quantitative and comprehensive detail for many input and outcome variables [22]. The Framingham study showed what is possible. Moving forward, it will be necessary to revisit this principle with even more ambitious studies that build quantitative databases of health variations against which individual metabolite profiles can be compared to distinguish important differences (i.e. diagnostic applications, unintended side effects of therapeutics). As lipidomics platforms become more available and more clinical populations are measured, the predictive power of the data will increase with increased number of analytes, accurate identification, and increased accuracy and precision of the quantitative data.

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4 Dietary lipid transport and signaling: From organelles to cells, from tissues to individuals, from biofluids to systems

Among the first steps toward understanding the structure and function of biomolecules has been to describe their distribution within discrete functional compartments—cells, organelles and subcellular particles. Such strategies have been a hallmark of biochemistry research for decades, and combining this approach with the tools of lipidomics has the potential to add substantially to our knowledge of lipids in areas as disparate as cellular membrane topology and lipid transport systems. Moreover, combinations of lipidomics techniques with specific sample collection, cell isolation, and subcellular fractionation methods are beginning to reveal relationships between composition and location, if not yet function.

The technologies necessary for profiling lipid metabolites have been established for many years; however, there are few reports of quantitative and comprehensive lipid metabolite profiles in the literature. Most reports provide normalized data (i.e. percentage of a particular fatty acid relative to total fatty acids within a lipid class), and these datasets consisting solely of mol% data can provide information about how composition is changing in response to a treatment or over time. A normalized analysis is valuable in assessing metabolic information that quantitative values cannot such as when the biology is at the level of intermediary or complex lipid metabolism. In contrast, quantitation is important for accessing global lipid metabolism and for reproducible relationships among metabolites over time. For example, absolute quantitation of total cholesterol in the bloodstream has provided a highly successful and actionable marker for treatment and disease diagnosis. Fortunately, the field is moving toward absolute quantitation.

Structural lipids make up the most widely studied set of lipids, particularly in the context of cardiovascular disease (CVD). Plasma total cholesterol, LDL and HDL cholesterol, triglycerides (TG), and lipoprotein particle distribution (size and number of particles) have all been widely studied, annotated, and used both as diagnostic markers and targets of intervention, with great success. Dietary changes such as decreasing saturated fat intake to lower cholesterol and increasing *n*-3 PUFA intake to decrease TG, as well as pharmaceutical lowering of cholesterol through statins have been a standard approach to modifying CVD risk for decades. New and emerging areas of dietary lipid-mediated mechanisms of disease initiation and progression as well as their prevention are being discovered through more comprehensive profiling of lipids.

The composition of circulating fatty acids reflects habitual diet [23, 24], genetics [25–27], metabolic phenotype [28–30], lifestyle [31], hormonal regulation [32] and their interactions. Quantitative lipid metabolome data were used to determine the differential effects of dietary α–linolenic acid and *n*-3 PUFA (>20 carbon chain-length) on heart and liver phospholipid metabolism [33]. A recent study used UPLC coupled with ESI-quadrupole TOF MS to analyze the plasma lipidome (including 260 identified lipid species) of healthy subjects in response to fish oil [34]. Circulating fatty acids are key regulators and surrogates of lipid anabolic and catabolic pathways. For example, in a 12-wk dietary intervention in overweight subjects, 500 kcal reduction per day plus calcium supplementation or 3–4 servings of dairy products could not be specifically related to observed changes in anthropometric measurements. Yet, baseline circulating levels of free fatty acids 18:1n9 and phosphatidylcholine 18:0 explained up to 33% of the variance of changes in waist circumference, percent body fat, and lean mass [35]. These data suggest proof of concept that lipid surrogates of metabolism could predict responsiveness to preserve lean mass at the expense of catabolizing adipose tissue during energy restriction. Future studies are needed to validate how metabolic phenotypes described by circulating lipidomic measurements predict responsiveness to any dietary intervention.

Several factors influence fatty acid composition including the fatty acid composition of the background diet (both short term and long term) but also macronutrients, micronutrients as well as food structure. Saturated fatty acids (SFA), MUFA, trans-fatty acids, and PUFA all modulate plasma lipids and lipoproteins, as does the relative intake of *n*-3 and *n*-6 PUFA. For example, consumption of diets higher in MUFA and PUFA, particularly *n*-3 PUFA, exert beneficial effects on plasma lipids whereas diets higher in SFA and trans-fatty acids are associated with deleterious effects on plasma lipid profiles and CVD risk [36, 37]. As a percent of energy, MUFA intake during caloric restriction in overweight individuals was inversely associated with changes in lean mass but positively associated with changes in percent body fat [38]. These data suggest that during energy restriction, dietary MUFA shift energy partitioning toward muscle catabolism and adipose anabolism. These results highlight the necessity to elucidate how dietary fat composition influences not only plasma lipids but also metabolic indices of lipid metabolism such as body composition.

The signaling functions of lipids are more difficult to study than their structures. The cellular and nuclear membranes are the sources of signaling molecules, or oxylipins, including the eicosanoid family. There are three major families of oxylipins—the cyclooxygenase metabolites that include prostaglandins and thromboxanes, the lipoxygenase (LOX) metabolites that include leukotrienes and some hydroxy fatty acids, and the cytochrome P450 (CYP) metabolites that include other hydroxy fatty acid epoxides and their downstream metabolite hydroxy fatty diols. Of course, all of these lipids can be metabolized to other, often biologically active lipid amides. *n*-3PUFAs eicosapentaenoic acid (EPA, 20:5*n*3) and docosahexaenoic acid (DHA, 22:6*n*3), and *n*-6 PUFAs dihommo-gammalinolenic acid (20:3*n*6), and arachidonic acid (ARA, 20:4n6) serve as the substrates for these enzymes. Each fatty acid can be either derived directly from the diet or converted in the liver from *n*-3 and *n*-6 PUFA precursors alpha-linolenic acid (18:3*n*3) and linoleic acid (LA,

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18:2*n*6), respectively. Alpha-linolenic acid and LA themselves can also act as substrates for the LOX and CYP pathways. Over 85 distinct oxylipin metabolites can be measured simultaneously on a single platform and comprise a complex network of communication for both the initiation and termination of inflammation-related events [39, 40].

The lipids that form diverse signaling molecules are the basis of much of biology's alarm communication system, triggered by various forms of "stress" and mediating appropriate cellular responses. Lipid structures themselves are in many respects chemically fragile. Phospholipids in particular and the PUFA they contain can readily break down via simple, thermodynamically favorable chemical reactions (hydrolysis, oxidation) into products with solubility and structural features distinctly different from their intact precursors. This means that with a biomolecular layer of fragile phospholipids surrounding every cell, virtually every type of stress to a cell is likely to liberate lipid fragments, the presence of which contributes to a signaling system appropriate to the recognition and management of stress. The biological and chemical properties of lipids that make them such a valuable signaling system for cellular stress makes them difficult to study. Only with extensive experience and familiarity with mass spectra from a variety of samples with a range of levels of auto-oxidation and controlled experiments with little auto-oxidation have researchers been able to distinguish between biological samples that likely contain predominantly auto-oxidation products versus biologically relevant enzymatically derived signaling molecules. Not surprisingly, the importance of oxidation of lipids to biological processes still remains a poorly understood field of biology, and similarly its inhibition through antioxidants is equally poorly understood and controversial. Lipidomics can provide considerable input to solve the complexities of lipid oxidation in vivo and the value of antioxidant molecules in nutrition.

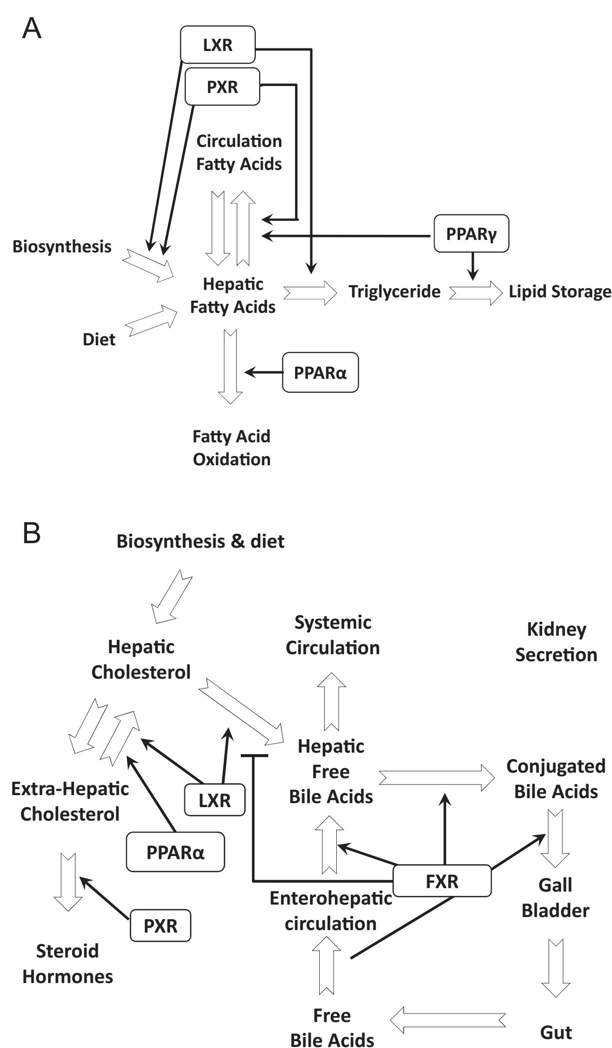

Figure 2. Lipid homeostasis regulation by nuclear receptors. (A) Fatty acid and triglyceride homeostasis. (B) Cholesterol and bile acid homeostasis. The arrows represent that the lipid biosynthesis, metabolism, or transport pathways are positively regulated by the indicated nuclear receptors. The "T" lines represent inhibition. Pregnane X receptor (PXR), farnesoid x receptor (FXR), liver X receptor, and PPARs.

5 Lipid regulation and cellular homeostasis

Systemic regulation of lipid anabolism and catabolism is critically dependent on cellular energy homeostasis, membrane structure and dynamics, and signaling. Dysregulation of lipid metabolism results in disease. Cellular regulation of dietary and endogenous lipids occurs through the action of nuclear receptors that act as sensors for cellular lipids. One such nuclear receptor superfamily–peroxisome proliferator-activated receptors (PPARs)—functions as transcription factors to regulate the expression of lipid metabolic genes [41]. PPARs play essential roles in the regulation of cellular differentiation, development, and carbohydrate, lipid, and protein metabolism [42–44]. Additionally, microarray and gene ontology analyses provided new functional clusters of genes that were not previously known to be directly regulated by PPARs such as chromatin remodeling, DNA damage response, Wnt signaling proteins, and mitogen-activated protein kinase signaling [45]. Transcriptional regulation by PPARs requires hetero-dimerization with the retinoid x receptor and when activated by a ligand, the dimer modulates transcription via binding to a specific DNA sequence element called a peroxisome proliferator response element in the promoter region of target genes (Fig. 2A).

PPAR-α, predominantly expressed in tissues that oxidize fatty acids that include liver, muscle, and brown adipose tissue, regulates lipid metabolism and transport, fatty acid oxidation, and glucose homeostasis [46]. PPAR-α regulated genes are those encoding fatty acid transporters, proteins involved in export (apolipoprotein B), the microsomal triglyceride transfer protein, fatty acid binding proteins, and acyl CoA

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dehydrogenase [46]. They also include P450 enzymes and epoxide hydrolases that regulate levels of potent lipid chemical mediators. Activation of these genes results in increased uptake and oxidation of free fatty acids, increased triglyceride hydrolysis, and upregulation of apolipoprotein AI and II (apoA-I and apoA-II) gene expression. The net effect is increased fatty acid oxidation, decreased serum TG, a rise in HDL, and an increase in cholesterol efflux. The ligands for PPAR-α are fatty acids, leukotrienes, pesticides, and environmental contaminants and drugs including nonsteroidal anti-inflammatory drugs and fibrates [47–49]. PPAR-α knockout mice exhibit steatosis, myocardial lipid accumulation, and hypoglycemia during short-term starvation or after high-fat diet administration [50, 51]. In mice fed fenofibrate or fish oil, both PPAR-α agonists, plasma TG were significantly decreased. Yet, fenofibrate was found to specifically downregulate genes involved in the complement cascade and inflammatory response and fish oil specifically downregulated genes involved in cholesterol and fatty acid biosynthesis and upregulated genes involved in amino acid and ARA metabolism [52]. These data suggest that despite being similarly potent PPAR-α agonists, activation by fish oil that contains two anti-inflammatory fatty acids (DHA and EPA) may influence multiple pathways either by their additive effects or multiplicative effects through de novo metabolism into oxidized products that are also PPAR agonists [53, 54]. These data reflect one difference between food-based and pharmacological interventions in that the former contains mixtures of ligands at low doses that target multiple pathways compared with the former that often contains one or two highly concentrated synthetic compounds with fewer targets. The complexity of food to simultaneously target multiple pathways is attractive for nutrition research and highlights the value of comprehensively and quantitatively measuring lipids to identify their molecular targets and their mechanisms.

5.1 Bile acid and cholesterol metabolism

Bile acids role in the mammalian system is much broader than simply to aid in the digestion and absorption of dietary lipids. They have been recognized as important signaling molecules with systemic endocrine functions [55]. Bile acids are natural ligands for the nuclear receptor, farnesoid X receptor (FXR), which co-dimerizes with retinoid x receptor to regulate lipid [56], glucose [57], and energy homeostasis [58] in addition to regulating bile acid synthesis [59], conjugation [60], transport [61], and detoxification [62]. Using ChIP sequencing, our group found that FXR [63] and RXR-α (unpublished) bind to the same locations in about 2000 hepatic genes suggesting their roles are coupled in the liver. FXR functions as a receptor for a wide range of bile acids, including cholic and deoxycholic acids as well as their glycine and taurine conjugates. FXR is primarily expressed in the liver, kidney, and intestines, and overall inhibits hepatic de novo bile acid production [64] via feedback regulation through the repression of cholesterol 7 alpha-hydroxylase [64, 65]. In the liver, FXR reduces bile acid toxicity by upregulating bile acid modifying enzymes including sulphotransferase 2A1 [66], UDP-glucuronosyltransferase 2B4 [62], and CYP 450 3A4 [67, 68] and increases bile acid conjugation to taurine or glycine [69]. Increasing the amphipathic nature of bile acids is necessary for their transport to the gallbladder and intestine. In the intestine, FXR reduces bile acid absorption by inhibiting the expression of apical sodium-dependent bile acid transporter, increasing bile acid transport through the enterocyte via ileal bile acid binding protein [70], and promoting recycling of bile acids to the liver via organic solute transporter α and β [71]. FXR knockout [72] and hepatic RXR-α knockout mice have elevated total bile acids, plasma triglyceride, and cholesterol levels, and they are susceptible to developing steatohepatitis [73] and colon cancer [74]. These data clearly indicate that FXR regulates bile acid turnover, suppressing the synthesis of new bile acids and stimulating biliary excretion to prevent excessive bile acid induced toxicity.

The global signaling capacity of bile acids is supported by the expression of bile acid receptors FXR in tissues outside of the enterohepatic circulation, including the kidney, vasculature, heart tissues, and immune cells [75–77] and suggests their systemic role. Using a shotgun approach with RP UPLC coupled to ESI quadrupole TOF MS, plasma bile acids glycocholic acid, glycochenodeoxycholic acid, and glycodeoxycholic acid were identified as highly responsive to an oral glucose tolerance test in healthy humans [78]. These data found that bile acids displayed biphasic kinetics with a maximum 4.5- to 6-fold increase at 30 min after glucose ingestion and a significant decrease over the next 60 min followed by an increase after 120 min [78]. The bile acid response to the oral glucose tolerance test could reflect the body's adaptation to promote glucose sensitivity and oxidation [79]. Additionally, plasma bile acids exert a high circadian rhythmicity such that bile acids failed to oscillate in the livers of clock-deficient mice [80]. The role of bile acids in relation to diet, metabolic phenotype, lifestyle, intestinal microbiota, genetics, and effects on whole body metabolism is a new and exciting area of lipidomics research.

Lipid-regulating genes are reflective as measurable indices in circulation, the best studied of which is cholesterol, and its many metabolites. Cholesterol plays an essential role in an array of biochemical, structural, and signaling functions in biology. It is a major component of cellular membranes and lipid rafts, is involved in intracellular trafficking and signal transduction, is a precursor for the synthesis of bile salts, steroid hormones, vitamin D, ubiquinone, dolichol, and coenzyme Q10, and acts as an anchor for membrane proteins such as farnesyl isoprenoid groups [81–83]. Cholesterol and its oxidation into oxysterols regulates HDL metabolism through transcriptional regulation in intestinal epithelia. In hamsters, dietary cholesterol upregulated the expression of apical intestinal ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 [84] which function to efflux cellular cholesterol [85–87]. The mechanism of action

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is through the activation of LXR-α [88]. LXR-α senses and responds to levels of cellular cholesterol by modulating the expression of genes that regulate cellular levels of cholesterol by targeting genes involved in the synthesis of bile acids [89], cholesterol efflux [90], and intestinal cholesterol absorption [90]. Specifically, when activated, LXR-α stimulates the expression of ABCA1 in the intestine [91, 92]. Although ABCA1 is required for transporting cholesterol from peripheral tissues to the liver, during reverse cholesterol transport, this transporter may play an important role in regulating intestinal cholesterol absorption [93,94]. When caco-2 cells were incubated with labeled cholesterol, ABCA1 and ATP-binding cassette transporter G1 gene expression was increased with concomitant basolateral efflux of cholesterol to HDL and apoA1 [95]. These data suggest that ABCA1 plays a role in intestinal production of HDL and cholesterol absorption. If so, inducing ABCA1 expression by dietary cholesterol would be a molecular strategy to regulate cellular cholesterol turnover. Overall, FXR and liver X receptor coordinately regulate bile acid, oxysterol, and lipid homeostasis (Fig. 2B).

6 Diversity as groups of individuals whose phenotype defines a particular risk

Although people are different and not everyone responds equally to a given diet or intervention, studies that reveal subgroups of individuals with a common metabolic phenotype or response have begun to emerge. For example, researchers identified three distinct phenotypes in healthy subjects in response to high versus low-glycemic index meals [96]. The group with the highest number of subjects had prototypical responses to the different meals, but two smaller groups showed unexpected deviations, with one group showing signs of early insulin resistance and the other showing an exaggerated leptin response. Response to *n*-3 PUFA has also been shown to be divergent. For example, in African American subjects grouped according to their 5-lipoxygenase genotype ("dd", "d5", or "55"), fish oil supplementation had differential effects [97]. In the high coronary heart disease risk group (the "dd" subjects), consumption of 5 grams per day of fish oil failed to even increase plasma and red blood cell EPA and DHA and lower the *n*-6:*n*-3 PUFA ratio. Only the subjects with the "d5" genotype had the expected decrease in plasma TG in response to the *n*-3 PUFA supplementation. These data highlight the variation in response to dietary fatty acids based on genotype.

Phenotype is likely to become even more revealing and ultimately more actionable as a way to assess and monitor response to diets. Again, *n*-3 PUFA are a powerful example, highlighting the variation in response among individuals. In healthy subjects, the predominant changes in plasma oxylipins in response to *n*-3 PUFA were increases in the *n*-3 PUFA derived CYP pathway epoxides and diols, along with decreases in *n*-6-derived metabolites, particularly those produced by the LOX pathway (Fig. 3) [98]. In IgA nephropathy patients, similar increases in *n*-3 PUFA derived epoxides and diols were observed; however, there was a divergent response in patients for whom kidney function improved versus those for whom it did not improve or worsened [99]. The same potently pro-inflammatory and cytotoxic LA and ARA-derived LOX metabolites that decreased in response to *n*-3 PUFA supplementation in the healthy subjects described by Shearer et al [98], decreased in IgA nephropathy patients whose kidney function improved but not those whose kidney function did not improve. These data suggest that there are different lipid metabolic phenotypes that determine response to different dietary fatty acids. These findings highlight the importance of measuring lipidomic profiles for detecting different metabolic phenotypes and assessing their response to dietary interventions and treatments. In the case of IgA nephropathy, these differences in response may be part of the reason for the inconsistent findings of beneficial effect of *n*-3 PUFA in the treatment of the disease. The measurement of lipidomic profiles could potentially identify those patients for whom *n*-3 PUFA treatment is beneficial.

7 Diagnostics and health assessment

7.1 Measuring lipids as biomarkers of disease

Lipidomics is better able to identify and characterize the complexities of lipid regulation than measurements of single biomarkers using traditional biochemical methods [100]. The lipidome is not definable in the same sense as the genome. Unlike the genome, which remains static, lipids change in every cell and body fluid, notably in response to food intake [101]. All of our cells and biofluids contain a finite number of key lipid metabolites, and lipid homeostasis is generally maintained so that the actual variations in any given lipid pool are typically minor relative to the abundance of the lipids. These basic molecules and their fluxes through human metabolism, i.e. those that all humans have in relatively constant amounts [102], include substrates, intermediates, and products of endogenous metabolism [103]. Hence, the lipidome will remain a discussable biological construction in which pragmatic clinical utility will require that assumptions, protocols, and reference conditions are standardized. Nonetheless, lipidomics is already proving to be informative in revealing the complex metabolic effects of diet [104], in predicting responders to drugs [105] and changes in body composition during energy restriction [35], and in identifying metabolic aberrations associated with disease [106].

Lipidomics has demonstrated its investigative power by its key role in the discovery of a novel class of signaling molecules termed lipokines [107]. Mice deficient in adipose tissue lipid chaperones (aP2 and mal1) are protected from the complications of metabolic syndrome when fed a high-fat diet. Researchers discovered that the mechanism underlying this effect was increased lipogenesis in the adipose

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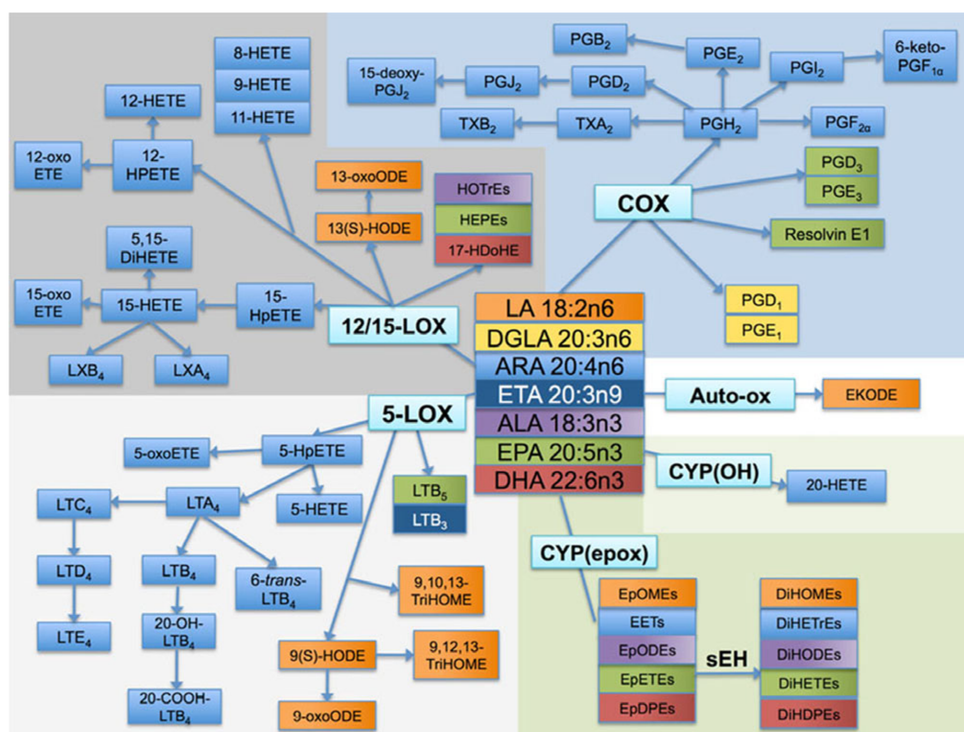

Figure 3. Reprinted from *Metabolomics*, Volume 8, Issue 6, 2012, 1102–1113, Angela M. Zivkovic, Jun Yang, Katrin Georgi, Christine Hegedus, Malin L. Nording, Aifric O'Sullivan, J. Bruce German, Ronald J. Hogg, Robert H. Weiss, Curt Bay, Bruce D. Hammock, Fig. 1, with kind permission from Springer Science and Business Media. Fatty acid precursors and their oxylipin products.The fatty acids linoleic acid (LA; 18:2*n*6), α-linolenic acid (ALA, 18:3*n*3), ARA, 20:4*n*6, dihommo-γ-linolenic acid (DGLA; 20:3*n*6), eicosatrienoic acid (ETA; 20:3*n*9), eicosapentaenoic acid (EPA; 20:5*n*3), and docosahexaenoic acid (DHA; 22:6*n*3) are precursors to a number of oxylipin products produced via the cyclooxygenase, lipoxygenase, and cytochrome P 450 (CYP) enzymes. The oxylipin products of the cyclooxygenase pathway include prostaglandins (PGE1, PGD1, PGH2, PGF2α. PGE2, PGB2, PGD2, PGJ2, 15-deoxy-PGJ2, PGI2, 6-keto-PGF1α, PGE3, PGH3, and resolvin E1) and thromboxanes (TXA2, TXB2). The oxylipin products of the lipoxygenase pathway include hydroperoxyeicosatetraenoic acids (HpETEs) and dihydroxyeicosatetraenoic acid (DiHETE), (further converted to hydroxyeicosatetraenoic acids (HETEs)), hydroxyoctadecadienoic acids (HOTrEs), hydroxyeicosaptenaenoic acids (HEPEs), hydroxydocosahexaenoic acid (17-HDoHE), and leukotrienes (LTA4, LTB4, 20-OH-LTB4, 20-COOH-LTB4, 6-trans-LTB4, LTC4, LTD4, LTE4, LTB3, LTB5) as well as the hydroxyoctadienoic acids (HODEs), and trihydroxyoctamonoenoic acids (TriHOMEs). The products of the CYP hydroxy (OH) pathway include 20-HETE, and the products of the CYP epoxy pathway include the epoxyeicosatrienoic acids (EETs), epoxyoctadecadienoic acids (EpODEs), epoxyoctamonoenoic acids (EpOMEs), epoxyeicosatetreaenoic acids (EpETEs), and epoxydocosapentaenoic acids (EpDPEs), as well as the downstream soluble epoxide hydrolase (sEH) metabolites dihydroxyoctamonoenoic acids (DiHOMEs), dihydroxyeicosatrienoic acids (DiHETrEs), dihydroxyoctadecadienoic acids (DiHODEs), dihydroxyeicosatetraenoic acids (DiHETEs), and dihydroxydocosapentaenoic acids (DiHDPEs). Each fatty acid precursor and its oxylipin products are colored the same: LA = orange; DGLA = yellow; ETA = dark blue; ALA = purple; EPA = green; DHA = red; and ARA = light blue.

tissue, which led to suppressed hepatic steatosis and increased muscle insulin sensitivity. Importantly, the de novo lipogenesis marker, palmitoleate (16:1n7), was shown to be the signal that mediated the protective effects, and was discovered through a lipidomic approach measuring lipids quantitatively in plasma, adipose, liver, and muscle tissue. Another recent study simultaneously measured fatty acids, oxylipins, and endocannabinoids in overweight and obese subjects with type II diabetes mellitus and discovered specific pathways affected by this disease including increased stearoyl CoA desaturase activity and increases in specific oxylipin classes, particularly epoxides and ketones of 18-carbon fatty acids [108]. Lipidomic profiling has also been used to detect diseases not directly associated with lipid metabolism. Phospholipids and ether-linked phospholipids were accurate and sensitive markers of prostate cancer, for example [109].

7.2 Dynamics of lipid metabolism

Lipids are the functional signatures of the interactions between genotype and environment, offering clinical relevance for the individual. Using the response-to-challenge model adds several dimensions to existing methods for revealing lipid metabolism. The fasted condition is a metabolic state when diet is absent. During fasting in an insulin-sensitive model, the insulin counter-regulatory hormones, glucagon, and epinephrine, peak while circulating insulin levels are

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low. As a result, free fatty acids are liberated from adipose tissue and are oxidized by extrahepatic tissues; the liver and kidneys are gluconeogenic and hepatic glycogenolysis is active. During this period, energy partitioning shifts to a catabolic and oxidative state. In the fed state, insulin peaks, glucagon, and epinephrine are suppressed and energy partitions to an anabolic state. Circulating lipids change in response to both of these states, yet the fasted condition receives the most attention. To achieve a complete picture of lipid metabolism, both fasted and fed states need to be rigorously studied.

The concentrations of circulating lipids are dynamic, and actively respond to environmental stimuli and diet. One time point only reveals a snapshot, whereas metabolites measured over a time-course provide a short film of the dynamic interactions among lipid pathways. Measuring changes in lipid metabolism provide a valuable assessment of metabolic regulation. The postprandial state, or the state following a meal, has recently come to be known as an important transient period during which significant vascular damage can occur. Given that most of the population in the United States is in a postprandial state for most of the day and given the rise in obesity and its associated complications (i.e. metabolic syndrome, type 2 diabetes mellitus, and heart disease), the postprandial state has become an increasingly important area of investigation. In fact, postprandial responses have recently been implicated in the causal processes of CVD [110] and metabolic syndrome [111]. For example, postprandial circulating TG are proving to be an independent predictor of CVD risk when compared with measurements in the fasted condition [112] that was recently reviewed in Jackson et al. [113].

Postprandial lipemia is characterized by a rise in triglyceride or triglyceride-rich lipoproteins (TRL) and their remnants in the immediate hours following food intake, and is an independent risk factor for CVD [114]. The amount and type of fat given in a meal has been shown to influence the rates of metabolism of TLR, their residence time in circulation, and in turn their potential impact on LDL and HDL particle size distribution and HDL cholesterol (HDL-C) concentrations. Data from animal experiments have shown that apoB48 and apoB100 TRL remnants bind and infiltrate the arterial wall leading to concomitant retention of cholesterol within the intimal sites of carotid arteries [115]. Another mechanism explaining the increased CVD risk with postprandial lipemia is through modulation of HDL and LDL size distribution and HDL-C concentrations through the action of circulating cholesterol ester transfer protein (CETP). CETP mediates the transfer of TG from TRLs to HDL and LDL particles in exchange of cholesterol ester. Both the absolute number of apoB-containing particles and concentration of TG in these particles favors CE enrichment of TRL and TG enrichment of HDL particles [116, 117]. The action of hepatic lipase on TG-rich HDL particles results in the production of small HDL particles, which are targeted for catabolism by the kidneys. Additionally, renal catabolism of circulating apoA1 associated with TG-rich HDL particles is enhanced [118, 119]. Low-circulating apoA1 and HDL-C are both independent risk factors of CVD [120, 121]. TG enrichment of LDL particles by CETP gives rise to the formation of small dense LDL particles, which were found to be associated with established CVD [122]. Small dense LDL particles have enhanced binding capacity to LDL receptor independent binding sites in extrahepatic tissues and reduced binding capacity to the LDL receptor [123, 124] which would extend their residence time in circulation and potentially increase their infiltration rate into the arterial wall. Small dense LDL particles are pro-atherogenic by accumulating in the vascular endothelium, and depositing cholesterol in the subendothelial space [125]. Postprandial endothelial dysfunction is positively associated with the concentration of TRL in healthy individuals and in patients with elevated fasting TG [126]. Thus, postprandial lipemia influences lipoprotein size, distribution, and metabolism.

Challenging lipid metabolism in individuals with a strategically designed meal coupled to lipidomics is an experimental instrument for measuring the dynamic and interactive changes of lipid pathways in the postprandial state. In the near term, designing dietary challenges will depend on the targets of biochemical pathways and the responsiveness of physiological and clinical outcomes in question such as blood pressure and insulin sensitivity. The complexity of lipid pathways is mediated by diet in several ways: (i) as substrates and products of these pathways, (ii) as modulators of enzymatic activities, (iii) as stimulators of hormonal regulation of enzymatic activities, and (iv) as effectors of gene expression regulation. For example, shotgun lipidomics identified fluctuations in MUFA and SFA FFA, and acyl carnitines that reflected the switch from β-oxidation to glycolysis and fat storage during an oral glucose tolerance test in healthy subjects [78]. Developing a standardized challenge to scrutinize lipid metabolism combines lipid biochemistry and effects from food, points of regulation at various transcriptional and posttranslational levels, cellular lipids and abundance, and interactions between organ systems and the plasma compartment.

There are several advantages to measuring lipids in the fed condition to discriminate individual variation as metabolic phenotypes. In a study investigating the response of healthy individuals measured three times in response to the same challenge meal, as many as 50% of the total measured lipids were “individual discriminators”. Individual discriminators are metabolites for which the variation in metabolic response to the defined dietary challenge within each subject was significantly lower than the variation among the three subjects. These findings highlight the fact that certain metabolites are indicators of response to a specific meal rather than simply reflections of recent and habitual dietary fatty acid composition [127]. When designed appropriately, the challenge can be a tool to perturb specific pathways of interest. A challenge in which all lipid pathways are interrogated and measured simultaneously would be ideal, but this feat would require formulation of dietary components that are substrates to all pathways in question, and not abundant in the food supply

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and circulation. Assessment of pathway activities can be done for isolated pathways with ease.

8 Designing interventions: Cohort selection criteria

In studies designed to discover the relationships between diet and health among different cohorts within the general population, selection criteria can be critical. Study criteria must deliver statistical power to accommodate multiple posthoc statistical tests possible. Some outcome variations stem from biological variability within groups. Sex and BMI influence peak/nadir and time courses of postprandial hormonal responses [128]. Sex also influences lipid metabolism in a variety of ways, including greater very low density lipoprotein secretion rates and lower postprandial TG concentrations in women compared with men [129]. Metabolic status such as obesity determines circulating postprandial leptin dependent on the macronutrient composition of a dietary challenge [130]. Differences in postprandial lipid metabolism are also caused by normal dynamic fluctuations in metabolism as part of diurnal rhythms and other cyclical events. For example, in women, lipid metabolism is affected by the phase of the menstrual cycle [131]. Other determinants include health status (e.g. diabetic versus healthy) [132], age [133], and even place of residence, which reflects cultural differences as well as differences in food availability and composition [134]. Not surprisingly, genetic factors interact with diet to contribute strongly to interindividual variation in lipid metabolism, and contribute significantly to phenotype [135]. For example, the combination of a variant lipoxygenase genotype and increased dietary intake of the *n*-6 PUFA resulted in increased intima-media thickness, a measurement of atherosclerosis progression whereas, the intake of *n*-3 PUFA was negatively associated with intima-media thickness [136]. Metabolic outcomes of interest will determine the appropriate time intervals and frequency of blood draws [137]. The controllable confounding variables that could potentially interact with dependent variables of interest need to be identified a priori.

Because small variations in the exact composition of meals result in variable responses and because the influence of diet is variable depending on the individual, studies must be designed to capture both variables. The traditional approach of using a randomized, placebo-controlled design, in which half of the participants are randomized to treatment and the other half are randomized to the control arm, fails when the aim is to understand the phenotypic differences among individuals. The crossover design is powerful when each participant acts as his or her own control, undergoing both the treatment and control arms repeatedly to obtain estimates of intraindividual variation. In the future, as tools develop to control the composition and structure of food coupled to more comprehensive measurements of repeated time samples using lipidomics, investigators and clinicians will gain a powerful diagnostic tool to understand an individuals' postprandial lipid metabolism.

9 Use of chemometrics to reveal complexities of lipid metabolism

The concept of chemometrics has its origin in chemistry. The overall aim to mathematically extract relevant information from high-dimensional empirical data makes chemometrics ideally suited for omics research [138]. The high dimensionality of datasets in lipidomic studies makes it often difficult to visualize the results, and correlations between different variables may compromise the utility of traditional statistical methods. To address the issue of multidimensional and correlated data, a research field of bioinformatics and computational methods in lipidomics research is emerging, extensively reviewed by Niemelä et al. [139]. We have limited the scope of our review to the use of chemometrics for statistical experimental design and multivariate analysis (MVA) in nutritional lipidomics.

9.1 Statistical experimental design

A systematic approach of maximizing meaningful data while limiting the contribution of noise already in the stage of setting up the experiment is crucial when dealing with the vast amount of data produced in a lipidomics study. Statistical experimental design has been used extensively for this purpose, for instance in predictive metabolomics and has great potential in improving the quality of nutritional lipidomics studies as well [140–142]. It can advantageously be used throughout the workflow, from defining the aim to the final extraction of information. As a consequence, it limits the noise and makes the study contain data with the information relevant to the specific study aim/hypothesis addressed. The idea is to obtain well-balanced data by spanning the variation in a systematic way, which can be done through for instance factorial and D-optimal designs [143]. Furthermore, multivariate design can be used to optimize the selection of objects that span the biological variation [144, 145].

9.2 Multivariate analysis (MVA)

Traditional univariate statistical methods commonly used, such as Student's *t*-test, are sensitive toward missing data and assume variable independence, which makes them less suitable than MVA for lipidomics and other large-scale omics data. Since omics data often contain a large number of variables tested simultaneously, large datasets are generated with potentially intradependent variables, which represent a risk for cumulative errors and biased results. To that end, MVA, through different mathematical tools, creates robust models for analyzing the complexity of the lipidomic data. This is done by reducing the dataset to a few latent variables that represents the majority of the variance among the measured variables. MVA methods can be divided into unsupervised projection methods and supervised methods where the user

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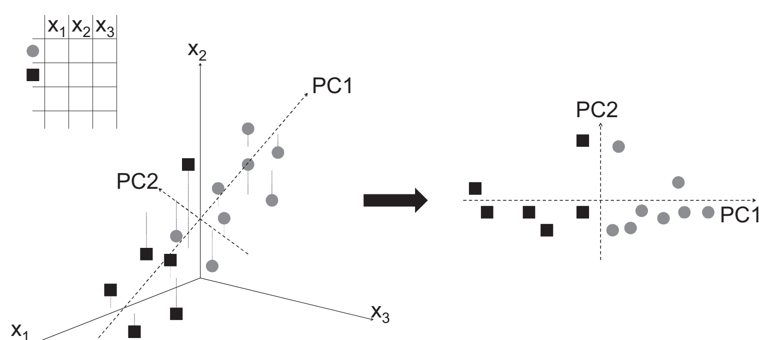

Figure 4. Principal component analysis is an unsupervised multivariate projection method for pattern recognition in multidimensional data tables. To illustrate the concept of principal component analysis, the variables (x_1, x_2, x_3) describing the objects (black squares and gray circles) are reduced from a three-dimensional space to a 2D plane by modeling the principal components in the direction of the largest variability. The resulting score plot (to the right) facilitates the interpretation of clusters and outliers among the objects. This concept might be extended to reducing unlimited number of variables to typically one to three principal components in order to summarize the major portion of the variability.

defines which variables belong to the X dataset (e.g. lipidomic profile) and which belong to Y dataset (e.g. intervention group).

Principal component analysis is a popular unsupervised projection method for pattern recognition often used to obtain an overview of all the observations/subjects included in the study, both visually and mathematically [144]. This overview plotted in a 2D (or 3D) coordinate system is achieved by projecting the multidimensional data to a few orthogonal principal components, also called latent variables (axes in the coordinate system). The first component (PC1) summarizes the largest possible portion of the systematic variation in the data set and the following principal components contain decreasing portions of the variation in the dataset (Fig. 4). Regression analysis between large data sets can be achieved through the supervised methods of projections to latent structures (PLS), and its extension orthogonal (O) PLS [146]. In OPLS, systematic variability in the X data set is separated into two parts, related and unrelated to Y. In doing so, interpretability is greatly enhanced and the resulting coordinate system will contain variability in X related to Y on the x-axis and unrelated to Y on the y-axis.

For clinical applications, principal component analysis and OPLS methods have been used in a number of metabolomics studies, reviewed by Madsen et al. [147]. However, in nutritional lipidomics, such methods are still not used to its full potential. A few examples of successful applications exist, exemplified by studies of responsiveness to dietary cholesterol [148–150], and to a dietary lipid challenge [127], as well as studies of altered lipid metabolism in relation to osteoarthritis [151]. In the future, we anticipate an increasing number of studies using a combination of the strengths in both univariate analysis and MVA to reveal complexities of lipid metabolism.

10 Translation: What is not known, what needs to be developed

The ultimate test of any new scientific strategy is its ability to bring knowledge to practice. The field of translational science is establishing standardized protocols and metrics to accelerate the processes of bringing scientific breakthroughs to human health. Lipidomics is ideally suited to participate. The translation of nutrition research is both forward and reverse. Translation is forward when it brings scientific breakthroughs in the laboratory to public policy and marketplace action. Translation is reverse when it alerts the scientific community of the gaps in knowledge that underlie diversity in diet-dependent health outcomes in the population [152]. Lipidomics will enhance the power of lipid knowledge in applications in diagnostics; the early recognition of health disparities, in interventions; and the recommendations for inclusion or removal of lipid components in diets and in theranostics. The application of theranostics, a key component of personalized medicine in which treatments are tailored based on the test results [153,154], will lead to the demonstration of efficacy and safety of various dietary interventions in individuals in practice.

Lipids have a long history in diagnostics of health, (serum cholesterol, TG, LDL, HDL, free fatty acids) precisely because they are highly responsive to pathologic, metabolic, dietary, and genetic diversity. This diagnostic power of lipids will increase as lipidomics studies reveal patterns of lipids that are reflective of normal and aberrant states and in parallel, technologies to measure these lipids comprehensively and more accurately improve to bring lipid measures to clinical practice [155].

Lipids can be highly active as dietary ingredients whether that SFA that alter metabolism or the *n*-3 PUFA that alter signaling pathways. Lipidomics technologies are revealing novel mechanisms of the actions of dietary lipids and the effects of diet on lipid metabolism. The comprehensive nature of lipidomics is particularly effective in evaluating the breadth of nutritional interventions, both efficacy and unanticipated side effects [156–159]. The future importance of lipidomics cannot be overstated in part because we still know so little about lipids. The cataloguing of lipids will continue a pace with the ever more sensitive and high-throughput analytical platforms emerging [18]. The great excitement will come with a more detailed and predictive understanding of the functions of lipids in vivo and the directed manipulation of those functions through explicit dietary manipulation. However, to understand lipid functions in vivo, new toolsets will

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need to be developed that extend the reach of science into the complex structures of lipids as they function.

Lipids are metabolites and soft structures. We need to understand both. Lipidomics analytics exceeds our ability to separate subcellular compartments of lipids in which the soft structures function. Cell biology can assist greatly by developing the means to capture/sample functional units of subcellular compartments as they exist in vivo. A portend of this direction is the lipidomic study of mitochondria [160]. Yet we need to go farther. Novel biologically based separation systems are likely to be critical to complement the more traditional physically based separation methods. The recognition that retroviruses escape their cellular hosts by co-opting their lipid membrane microdomains led to novel separation methods for lipid rafts, greater insights into viral replication and novel drug and dietary targets for membrane functions [161].

As metabolites, lipids are part of complex pathways and while the absolute concentration of each intermediate is valuable information, the actual flux through pathways is a key to understanding function. Analytic platforms capable of measuring varying concentrations of metabolic intermediates as a function of time (fluxomics) are a holy grail of lipidomics and kinetics. The first generation of these studies is already providing a glimpse of this approach [162]. Combining flux with spatial discrimination is a dream that is tantalizingly close with MS imaging [163]. Perhaps the most perplexing aspect of biological lipids is that in spite of the diversity of complex lipids, the absolute and relative concentrations of the lipids themselves and even the fatty acids that are esterified to them remain remarkably constant. How cells sense and regulate the relative abundance of lipids remains unknown, except for one, cholesterol. Breathtakingly elegant studies led by Brown and Goldstein, laid out the basic cholesterol-sensing mechanisms, the signals transducing the effective cholesterol concentrations within the ER and the resulting coordinate regulation of cholesterol, synthesis, uptake, and mobilization to normalize intracellular concentrations [164]. Discovering the mechanisms by which the concentrations of docosahexaenoic acid, ARA, etc., are regulated in cells is a tantalizing challenge for the field of lipidomics whose solution would lead immediately to strategies to control these molecules for therapeutic benefits.

11 Conclusions

The field of lipidomics is bringing lipids into the mainstream of research and translation. Lipids themselves, the long underappreciated biomolecule class, are beginning to reveal their functions through lipidomics in sufficient detail to guide nutritional interventions. It is now the role of the lipid specialists to recruit scientist collaborators in fields from nanoscience to clinical medicine. The resulting collaborations will set a new standard for multidisciplinary and integrative science.

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