Lipidomic Approaches To Dissect Dysregulated Lipid Metabolism In Kidney Disease

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Abstract

Dyslipidemia is a hallmark of chronic kidney disease (CKD). The severity of dyslipidemia not only correlates with CKD stages but is also associated with cardiovascular disease and mortality. Understanding how lipids are dysregulated, however, is challenging because of the incredible diversity in lipid structures. The dyslipidemia and its association with CKD stages and progression encompass complex interactions between genetic, environmental, and kidney specific factors that require an integrated understanding of perturbations in the network of genes, proteins, and lipids. Modern lipidomic technologies attempt to systematically identify and quantitate lipids from biological systems. The recent rapid development of a variety of analytical platforms based on mass spectrometry (MS) have enabled identification of complex lipids at great precision and depth. From current lipidomics studies of CKD patients it is apparent that overall architecture of free fatty acid partitioning between fatty acid oxidation and complex lipid fatty acid composition is an important driver of CKD progression.

Introduction

According to the Centers for Disease Control and Prevention, an estimated 37 million adults (15% of the population) in the US suffer from chronic kidney disease (CKD)[1]. Although various etiologies exist, diabetes mellitus (DM) alone is responsible for approximately 40% of cases of kidney failure [2]. Patients with CKD have a high prevalence of cardiovascular disease[3] and it is therefore of no surprise that the majority of patients with CKD present with DM and cardiovascular disease (CVD) as comorbid conditions[2].

CKD and its comorbidities lead to significant multi-organ metabolic derangements, including dyslipidemia that occurs in CKD is thought to contribute to the development of CVD[4], the leading cause of deaths in CKD[5]. Therefore, of particular interest is lipidomics, a subset of metabolomics that specifically focuses on the identification and quantification of lipids. Population-based studies over the past five decades have been limited to the measurement of traditional lipid panel which include total cholesterol, lipoproteins and triglycerides which does not account for the chemical diversity and complexity of lipids. Recent advances in analytical mass spectrometry in the past decade have enabled broader

profiling of the plasma and tissue lipidome allowing identification of lipid species by class, subclass, chain length, degree of unsaturation, chain hydroxylation, amongst other chemical characteristics[6–19]. Lipid phenotype alterations associated with CKD progression involve significant alterations in a large number of intra-class lipids due to differential elongation, desaturation, synthesis, and lipolysis that basis the alterations at class level. Hence, a system biology level analysis as opposed to individual lipid analysis is required to unravel pertinent lipid alterations. Comprehensive lipidomics studies allow for a systems-view of CKD-associated changes in lipid levels and regulation, as opposed analysis driven by a few highly altered lipid species. Although lipidomics in CKD is still a burgeoning field and therefore requires further experimental validation of its findings, lipidomic studies have established that fatty-acid oxidation and lipogenesis, thought previously to be independent processes occurring in separate cellular compartments are interrelated, perturbed concurrently in CKD, and predict future clinical progression.

In this review, we aim to introduce the reader to lipidomics studies, including a technical overview of various methods. We will discuss statistical and bioinformatics strategies that can be employed to analyze complex lipidomic data. Finally, we will then discuss the most recent lipidomics biomarker studies and mechanistic studies investigating the role of these clinically relevant lipids in CKD.

Lipidomics: an overview

Lipidomics is the comprehensive analysis of individual lipids in a biological system. Lipidomics is most often conducted with mass-spectrometers, which provide accurate mass determination with mass/charge (m/z) ratio and subsequent unequivocal structural identification by fragmentation which generates a characteristic mass spectral fingerprint. MS^1 is the unfragmented mass spectra that generates "precursor" ions and MS^2 (MS/MS) is tandem mass spectra that generates fragmented "product ions" from the precursor ions. These acquired MS^1 and MS^2 spectra can be searched against databases such as METLIN-XCMS[20,21] and LIPID MAPS[22] for compound identification. Lipids are unique in that each lipid class is associated with characteristic "diagnostic fragments", usually arising from the lipid head group, and serve as specific signatures for the lipid class. Examples of lipid head groups and associated diagnostic ions are listed in Table 1 for different lipid classes. Figure 1 demonstrates MS^1 and MS^2 spectra obtained for phosphatidylethanolamine 36:1.

There are numerous isomeric combinations possible for a single identified lipid feature. Figure 2 demonstrates the structural diversity of a triglyceride with a specific precursor and product m/z transition: carbon number isomers, cis/trans double bond isomer, location of double bond isomer, glycerol-chain linkage position (sn-1/sn-2/sn-3) side chain isomer, as well as stereoisomers (not depicted in fig. 2). A large drawback to many lipidomics studies published to-date is lack of such isomer separation and identification; most untargeted lipidomics studies provide information only on lipid class, total number of carbons in the side chains, degree on unsaturation, and presence or absence of carbon backbone hydroxyl groups. Lipid isomers can have distinct biological roles. For instance, phospholipase A1, phospholipase A2, and phospholipase C hydrolyze phospholipids at sn-1, sn-2, and sn-3 sites, respectively and release different lipid products that serve as different cellular

messengers[23]. Therefore, additional biological insights may be gained from lipidomics studies that can deconvolute lipid isomers.

Analytical techniques in lipidomics

Lipidomics can be conducted using a variety of techniques. Liquid chromatography-mass spectrometry is however the most used analytical method in lipidomics. LC-MS requires comparatively little processing for samples and provide high reproducibility when combined with quality-control (QC) strategies. The high sensitivity of LC-MS requires minimal amounts of sample, making it ideal for clinical studies of patient samples. All our own lipidomic studies referenced in this literature have been conducted with LC-MS. However, similar quality control and data analysis strategies can be applied to other lipidomic analytical techniques. Some of the studies discussed in the review use alternative analytical methods, each of which provide unique advantages and information that cannot be acquired with LC-MS mass spectrometry. The relative advantages and disadvantages of other analytical techniques have been briefly outlined in table 2. For further information on the details of analytical strategies, including information on general structural and biological characteristics of different lipid classes, we direct readers to these excellent reviews: [24–28].

Study design: targeted and untargeted lipidomics

Researchers first must determine whether to conduct an untargeted or targeted lipidomics study. Untargeted lipidomic studies serve as the hypothesis generating portions of the study; the aim is to discover any "features" that are differentially altered between study groups. Targeted analysis aims to detect and measure levels of pre-determined lipids of interest. Therefore, initial lipidomics study may begin with untargeted analysis, aimed at discovering as many different features between study groups, and significant findings may be validated with targeted analysis. The decision to pursue an untargeted or targeted study will inform all downstream steps: sample preparation, separation strategies, data acquisition, quality control, and data processing and analysis. The distinction between the two types of studies is perhaps especially important in lipidomics as lipids are structurally diverse molecules with varying polarities and sizes, and methods may need to be highly customized for particular species of interest.

Quality control (QC)

One of the challenges in metabolomics is to ensure as little biases have occurred during the entire experiment. Studies must be properly designed to incorporate rigorous quality controls. Errors may be introduced at any point from experimental design, sample storage, sample preparation (e.g human error in variability in pipetting) to instrumental issues (e.g batch effect, instrumental drift) to data analysis (e.g missing data, normalization). The reader is referred to previous reviews which provide in-depth discussion on these topics[29–31].

Internal standards are usually heavy-labeled isotopologue of analytes of interest that are introduced at the beginning of sample preparation to aid in QC. For lipidomics, internal standards simply do not exist for every specific lipid species of interest; instead, an internal standard representative of each class is incorporated into the study. Internal standards also serve to control for any differences in metabolite extraction, as the ratio of lipid analyte to

internal standard will remain unchanged. They also help in the identification of metabolites of interest, as they co-elute on LC and generate fragmentation patterns with a known mass-shift.

Another QC method is application of "pooled samples" made by pooling aliquots of study samples or reference samples and analyzed intermittently during an experimental queue for intra-assay variations, and between analytical experiments for inter-assay variations. Upon achieving a high coefficient of variation, the potential causes including non-random sources of bias need to be investigated. Generally, a coefficient of variation of 10–20% is considered acceptable for targeted metabolomics, although ranges up to 20–30% have been reported in previous studies[31,32].

Bioinformatics and systems integration for lipidomics:

Lipidomics has evolved tremendously in the past decade. Despite this rapid growth, significant barriers remain to incorporate this data into metabolic pathways that will allow for a precise and comprehensive evaluation of the importance of the lipidome. Lipidomic data are characterized by (i) high dimensionality in the form of more features than available samples, (ii) heterogeneity due to the collection of diverse cellular compartments, tissue types or from different experimental conditions and (iii) availability of important metadata information about the nature of the biomolecules being profiles. The goal is to use statistical methods for gaining deeper insights into lipids function, lipid-lipid interactions across different biological phenotypes *in vivo* and mapping them to functional pathways.

As a large proportion of lipids are not mapped to a particular pathway, most lipids in lipidomics datasets fall outside of our current capabilities of bioinformatics tools and databases, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG)[33] and Human Metabolome Database (HMDB)[34]. One reason for this discrepancy is that pathway databases provide a genome-centric view, that is pathways defined by genes/enzymes that regulate the lipidome. In contrast, experimental lipidomics provides a chemistry-centric sampling defined by a chosen analytical method, on molecules whose diversities to a great extent are driven by modifications of simpler fatty acids as their building blocks. The overlap of these two views typically includes primary metabolic pathways (e.g phospholipase A2 clevage of sn-2 fatty acid in phospholipids to yield free fatty acid and lysophosphatidic acid), while coverage of secondary pathways and lipid metabolism (e.g sn-1 versus sn-2 fatty acid distribution amongst complex lipids such as triacylglycerols) is scarce. Alternatively, the LIPID MAPS Gene/Proteome Database[22] provides a tabular list of reported interactions between genes, proteins, and individual lipids. However, this does not place these interactions in a pathway map and relies on precise lipid identification, whereas most lipidomics experiments do not provide precise identification (for example, lipids are mostly identified by lipid class and the number of carbons and double bonds present, not by the precise position of double bonds). Therefore, the current pathway databases either describe lipid metabolism at the class level or at the individual, uniquely identified level, providing coverage that is too broad or too precise for current lipidomic detection methods. The user is then left to decide how to extrapolate this information. Here, we will outline statistical, and systems-biology approaches we have utilized to analyze lipidomics data.

Structure and composition based lipidomic analysis

Selecting lipids or lipid classes of interest from an untargeted study for further investigation can be accomplished through many different strategies. The endogenous elongation and desaturation processes of fatty acids induce generation of longer and more polyunsaturated products from simpler shorter fatty acids, so that the quantity of endogenous lipids within each lipid class becomes the net effect of the efficacy of these processes and a function of synthesis versus lipolysis, which in turn determines the total concentration of the corresponding lipid class at any given time. As a result, the intra-class members of each lipid sub-class are highly correlated, and hence no single lipid may adequately represent the intra-class disease specific alterations. On the other hand, lipid phenotype alterations associated with CKD progression, involves significant alterations in a large number of intra-class lipids due to differential elongation, desaturation, synthesis, and lipolysis that basis the alterations at class level. Hence, a system biology level analysis as opposed to individual lipid analysis is required to unravel pertinent lipid alterations.

Due to the numerous potential acyl-carbon backbones combinations that can occur in lipids, grouping the identified lipid species within a class by secondary characteristics of carbon chain length and/or degree of unsaturation in order to reduce the analytical complexity could be a powerful step in data analysis. The groupings can reflect the inherent biological differences associated with degree of unsaturation and carbon bond number. For example, acylcarnitine metabolism differs for short-chain (C2-C6), medium-chain (C8-C14), and long-chain acylcarnitines (>C14); short-chain acylcarnitines can be derived from glucose, amino acids, and fatty acids whereas medium and long-chain acylcarntines are derived usually exclusively from fatty acids. Fatty acid degradation is carried out by acyl-CoA dehydratases of different degree of acyl-carbon length preference[35]. However, researchers can also determine how many groups per lipid class and which lipid species can be aggregated to a specific group empirically. In our studies, we have used principal component analysis to generate lipid class subgroups[36–38]. In the study of the Clinical Phenotyping and Resource (CPROBE) cohort[37], we compared the interaction term between carbon number and number of double bonds for each lipid class (Figure 3), and found significant enrichment for long-unsaturated plasma free fatty acids (FFAs) in stage 2 CKD patients and in shorter (C16-C18)-unsaturated FFAs in stage 5 CKD patients, and the opposite in complex lipids such as TAGs, diacylglycerols (DAGs), and phosphatidylethanolamines (PEs). Overall, these findings show incremental shift toward higher abundance of circulating unsaturated C16-C18 free fatty acids and longer polyunsaturated complex lipids by worsening CKD from stage 2 to 5.

Network analysis

Lipids, in particular, are capable of extensive inter-class and intra-class conversion, and network analysis may identify targets of dysfunctional lipid metabolism in CKD. However, such networks tend to be very strongly connected, since correlations do not delineate direct from indirect associations. To that end, the focus has shifted to partial correlation networks that identify direct associations, but require more sophisticated algorithms to construct especially when the number of metabolites/lipids exceeds that of available samples[39,40]. Once a data driven network is obtained, one can identify strongly

interconnected subnetworks of metabolites/lipids by network clustering algorithms (e.g. see [41]) that can act as input to a topology-based pathway enrichment algorithm.

An example of the latter strategy is in a study of lipid interactions in the Chronic Renal Insufficiency Cohort (CRIC)[42] and CPROBE cohorts[37], wherein we constructed a partial correlation interaction network based on plasma lipidomics data and then identified through network clustering algorithms strongly interconnected subnetworks. The latter were tested for enrichment based on the topology-based pathway enrichment algorithm Network-based Gene Set Analysis (NetGSA), which assesses differential abundance and differential correlations simultaneously amongst various different lipids within a dataset (figure 4). Networks were built separately for non-progressors or progressors for CRIC patients and stage 2–3 (early-stage) or stage 4–5 (late-stage) for CPROBE patients. Comparisons of these lipid networks between the CRIC non-progressors and progressors and CPROBE early-stage and late-stage patients found decreased interconnectedness between lipid species in the progressor and late-stage CKD patients compared to the non-progressor and early-stage CKD patients. The decrease in number of these connections or "edges" were especially evident in the clusters of long-chain TAGs and clusters of cardiolipins (CL) and PE. We interpreted the loss of edges between these lipid species in the worse CKD groups to represent reduction in desaturation-elongation mechanisms between TAGs sub-network, and mitochondrial CL-PE subnetwork. These findings highlight that mitochondrial lipidome changes are associated with neutral lipid changes and this process may play a causal role in mitochondrial dysfunction.

Lipidomics and other -omics integration

Lipidomics integration with other datasets, particularly tissue transcriptomics and proteomics data can lend to more meaningful interpretations of how changes in plasma or serum lipidome in CKD reflect and/or impact kidney disease pathogenesis. Integration of data may occur without any preselection for genes or metabolites of interest using network analysis tools, or analysis can be conducted on relevant genes/proteins associated with metabolites of interest. There are many strategies available in the literature (see review [43]). One simple strategy is to examine Pearson correlation coefficients, corrected for multiple testing to select significant associations, and network visualization tools such as MetScape, which provides a framework for visual integration of metabolomic and transcriptomic profiles enabling visualizing connections between datasets[44].

The initial experimental design is perhaps the most salient factor in multi-omic data integration. In the study of Pima American Indian DKD cohorts[36], we were able to take advantage of transcriptomics data acquired from kidney biopsies taken close in time to the serum collection. Of note, kidney biopsies were micro-dissected into glomerular and tubulointerstitial compartments before gene-expression analysis. We sought to identify lipid-regulating transcripts that correlated significantly with the levels of lipid classes that were found to discriminate between progressors and non-progressors amongst the cohort. In particular, we discovered that the transcript levels for Acetyl-CoA Carboxylase Alpha (ACACA), the rate limiting enzyme of fatty-acid biosynthesis, correlated positively with serum long-chained saturated diacylglycerol levels in the glomerular compartment (nominal

P = 0.0062 and FDR = 0.060) and with serum intermediate chain low-double-bond TAGs (nominal P = 0.0042, FDR = 0.009) in the tubulointerstitial compartment. While it is still unclear whether kidney ACACA contributes to the observed serum lipid changes associated with DKD progression, renal upregulation of ACACA may poses its detrimental effect on kidneys by upregulation of local *de novo* lipogenesis and impairing mitochondrial β-oxidation. These findings underscore how integrating lipidomic findings with other available -omics datasets could identify tissue-specific genes relevant to disease pathogenesis in CKD.

Lipid metabolism in chronic kidney disease

Kidney energy metabolism in CKD

The kidney is an energy intensive organ. The substrate and energy generating pathways are cell-specific in the kidney, and depend on nutritional challenges or availabilities. In the glomeruli, podocytes harbor a strong preference for anaerobic glycolysis, and rely little on mitochondrial oxidative phosphorylation for energy generation, although they are capable of fatty acid oxidation (FAO)[45,46]. Significant heterogeneity of substrate preferences exists amongst the different kidney tubules, depending on their location along the nephron and the kidney[47]. On the other hand proximal tubules, which have a large mitochondrial content, prefer to use free fatty-acids, along with glutamine as their main source of energy and display a severely limited capacity to use glucose as fuel under normal nutritional condition[47–50]. This inflexibility is thought to occur due to low activities of glycolytic enzymes on proximal tubules, and proximal tubular role in gluconeogenesis[51]. Under normal conditions, kidney uptake of fatty-acids is thought to outpace FAO oxidation; early studies with labeled lipid substrates *in vivo* and kidney sections from various animals demonstrated that most of labeled fatty-acid are stored in the form of diglycerides (DAG), TAGs, phospholipids and cholesterol esters[51]. Lipid transport, particularly long-chain lipids, into the kidney is thought to be mediated mainly by the cluster of differentiation (CD) 36 receptors. However, other transporters are implicated in fatty-acid import into the kidney, as fasted CD36 knock-out mice do not exhibit decreased levels of injected labeled oleate in the kidney compared to the wildtype mice[52]. Fatty-acid transporter-2 (FATP2), which is expressed mainly in proximal tubules, has been demonstrated to be responsible for fatty-acid uptake in polarized proximal tubule culture and micro-dissected proximal tubules[53].

In CKD, dysregulation of lipid oxidation, lipid uptake, and lipogenesis are thought to contribute to disease. Reduced and inefficient FAO has been long thought to be a major mechanism of tubular injury and fibrosis. A landmark study demonstrated that FAO transcripts and its regulatory genes are down-regulated in the tubulointerstitium of CKD patients, and mouse models of tubulointerstitial fibrosis[54]. For cultured podocytes, palmitate toxicity, which is cytotoxic stress and cell death caused by palmitate *in vitro*, is worsened by etomoxir blockage of FAO[55]. Peroxisome proliferator-activated receptor-gamma coactivator (PGC1α) upregulation by drugs or transgenic overexpression is associated with disease amelioration in multiple murine models of kidney disease: aldosterone induced podocyte damage[56], acute kidney injury (AKI)[57], and DKD[58] and the improvement in disease is thought in part to be upregulation in FAO by these regulators, amongst many other mechanisms. A recent study of renal tubular specific conditional

carnitine palmitoyltransferase 1A (CPT1a) over-expression demonstrated reduced fibrosis and disease in UUO, folic-acid induce nephropathy, and adenine-induced nephropathy[59]. CPT1a overexpression improved mitochondrial morphology and improved FAO, supporting the hypothesis that FAO downregulation is a key driver of kidney fibrosis.

Decreased FAO, in addition to causing insufficient ATP production, is also thought to contribute to increased kidney lipid accumulation in CKD. However, increased transport of fatty-acids has also been attributed to increasing lipid uptake by the kidneys. CD36 expression has been shown to be increased in patients with nephrotic syndrome[60] and DKD[61,62]. In addition, CD36 deficiency or blockade of CD36 has been demonstrated to ameliorate disease in models of kidney disease and fibrosis with reduced intracellular lipid deposition [63–66]. Of note, CD36 has been demonstrated to mediate CKD not only via increased uptake of lipids also activation of the NLRP3 inflammasome[67–69], and interactions with partners such as thrombospondin 1[70] and discoidin domain receptor 1[71] in podocytes and Na/K-ATPase[72] in proximal tubules to induce lipotoxicity. CD36 deficient macrophage models of UUO and IR models of kidney disease also demonstrate CD36 role in regulating immune cells in CKD[73]. CD36 binds to numerous ligands, including oxidized lipids, for its activation (see ref [74]) and therefore serves as a relay between circulating lipid profiles and cellular function.

Apical uptake of fatty-acid albumin complexes by proximal tubular FATP2[53,53] and megalin[75] in the setting of CKD are also thought to contribute to lipotoxicity in CKD. A recent study[76] of high-fat diet and streptozotocin and $Lepr^{db/db}$ $eNOS^{-/-}$ FATP2 deficient mouse models of DKD demonstrated a reduction in kidney fibrosis and loss of eGFR, along with reduction in intracellular lipid deposition in the FATP2 deficient DKD mice compared to its controls. However, the FATP2 deficient DKD mice also displayed reduced hyperglycemia and somewhat preserved insulin production compared to FATP2 control DKD mice; further work is necessary to clarify the role of FATP2 in DKD, although evidence suggests it a therapeutic target for CKD.

Increased neutral lipid accumulation in the kidney, particularly TAGs, occurs after many types of hypoxic, ATP-depleting, and inflammatory insults[77–79]. Histological studies of patients with DKD have also found lipid deposits in the glomeruli and tubules[61]. TAGs are widely accepted as biologically inert storage form of lipids, particularly of lipotoxic saturated fatty acids. It is thought that accumulations of bioactive diacylglycerol (DAGs), ceramides, and mitochondrial overloading with fatty-acids leads to cellular toxicity[80]. Upregulation in sterol-regulatory binding element (SREBP) transcripts, which induce increased expression of lipogenic enzymes such as fatty-acid synthase and acetyl-coa carboxylase, is thought to be a maladaptive response in several models of CKD. This increase in SREBP transcripts leads to increased TAG synthesis, but also cholesterol and intracellular fatty-acid synthesis, which likely confer the harmful effects of increased lipid accumulation in CKD[81–83].

In summary, the kidney is a highly energetically demanding organ that relies heavily on FAO for fuel. CKD has been associated with aberrations in FAO, lipid uptake and lipogenesis that lead to intracellular lipid deposition and eventually loss of function and fibrosis.

Lipid biomarkers of CKD

Plasma and serum lipid profiles are highly dysregulated in CKD. In this next section, we will discuss recent lipidomics findings on specific lipid classes, and studies that investigate their mechanism of disease pathogenesis. For information on lipoprotein associated dyslipidemia in CKD as well as cardiovascular disease risk in CKD, we direct readers to a recent review in *Nature Reviews Nephrology* that provides in-depth discussion on this topic: (10.1038/s41581–021-00423–5).

Free fatty acids in CKD

Fatty acids serve as the backbone to all complex lipid synthesis and serve as the substrate for lipid oxidation. Therefore, the circulating free fatty acids or non-esterified fatty acids (NEFAs) may drive the kidney intracellular lipid profile and metabolism in CKD. Plasma and serum profiles of NEFAs in CKD are elevated usually only with advanced CKD (stage 4–5)[84], although the NEFA profile is altered even in early-stage CKD. In many studies, decreased polyunsaturated fatty acid (PUFA) levels in the plasma are associated with CKD, particularly omega 3 and omega 6 fatty acids[85–88], whereas increased monounsaturated fatty acid (MUFA) levels are associated with CKD and worsening CKD[87–89]. Saturated fatty acid (SFA) levels, especially of intermediate and long chain fatty acids are elevated in CKD[85,87]. Analysis of plasma free fatty acids in the CPROBE cohort demonstrated significantly higher abundance of "shorter" chain saturated fatty acids (C20) and lower abundance of "longer" chain unsaturated fatty acids (>C20) by worsening CKD stage from stage 2 to 5[37]. In the Pima American Indian diabetic cohort, a cox regression model adjusted for ACR, GFR found that one standard deviation increase in unsaturated free fatty acids was associated with 0.54 fold (95% CI: 0.36 to 0.79, p=0.002) lower risk of DKD progression[36]. In summary, CKD patients develop altered NEFA profiles that become progressively more enriched in shorter saturated fatty acids (SFAs) and reduced in polyunsaturated fatty acids (PUFAs).

Increased levels of SFA, in particular palmitate, are associated with lipotoxicity, whereas unsaturated lipids are known to be protective against SFA toxicity. Addition of monounsaturated fatty acid (MUFA) oleate in addition to palmitate, is known to protect cultured proximal tubules and other cell cultures from palmitate lipotoxicity[90–94]. In addition, overexpression of stearoyl-coa desaturase (SCD) in cultured podocytes and proximal tubules is protective against palmitate toxicity[95,96]. This protection by MUFAs is thought to be conferred by increased palmitate partitioning to TAGs synthesis, instead of being metabolized into bioactive lipids such as ceramides, and also from increased competition for these cytotoxic pathways. The exact mechanism for why unsaturated fatty acids can induce fatty acid remodeling of TAGs is not fully understood, but some putative mechanisms may include insulin sensitivity improvement[97] and favorable alteration of fasting appetite hormones[98]. Protein activity assays of diacylglycerol O-acyltransferase 1 and 2 (DGAT1 and DGAT2), which catalyze diacylglycerols and fatty-acyl CoAs to TAGS, demonstrate that oleyl-CoAs are preferred substrates to palmitoyl-CoAs[99]. In addition, oleic acid and oleoyl-CoA, in addition to other MUFAs are stronger ligands and conformational activators of PPARα in comparison to palmitate and palmitoyl-CoA[100]. Studies have also demonstrated that triglyceride "loading" with oleate before cellular injury can protect

against cytotoxic stress that leads to increases in saturated fatty acids. An early study with HK-2 cells demonstrated that oleic acid supplementation prior to iron-mediated oxidative stress or addition of calcium inonophore with antimycin A and 2-deoxyglucose resulted in decreased cell death that was dependent on the concentration of oleate added to the media[77]. A recent study of clear renal cell carcinoma (ccRCC) demonstrated that triglyceride loading with oleate was protective against hypoxia likely due to hormone-sensitive lipase mediated excretion of oleate from triglyceride pool protected against SFA partitioning into forming harmful lipids such as ceramides and acylcarnitines[101].

PUFAs also serve to protect against SFA toxicity like MUFAs, by serving as ligand activators of PPARα and PPARγ[102] and also mediating conversion of DAGs to TAGs[90,103]. Additionally, PUFAs are known regulators of blood pressure and inflammation, and higher levels omega-3 fatty acids in particular are associated with decreased risk for CVD[104]. Recently, a study has proposed a role for PUFA in regulating redox balance. Kim *et. al.,* demonstrated that PUFA can serve as substrates to generate highly unsaturated fatty acids by delta-desaturase 5 and 6 (D5D, D6D) with consumption of NADH to generate NAD+. This mechanism seems to be driven by decreased NAD+/NADH ratio. *In vivo* treatment of mice with rotenone to block aerobic respiration increased TAG production, of increased highly-unsaturated fatty acid (HUFA) backbone in multiple organs. Interestingly, kidney production of HUFA incorporated TAGs outmatched those of skeletal muscle, liver, and heart in rotenone treated mice[105].

Dietary FFA profiles have been demonstrated to impact mitochondrial membrane profiles in the liver[106–113]. The relative abundance of saturated or unsaturated dietary FA has been associated with levels of such fatty-acyl residues in mitochondrial phospholipids, which in turn is thought to control cardiolipin acyl-chain profiles[114]. Excessive dietary saturated FA has been associated with increased mitochondrial oxidative stress and reduced mitochondrial oxygen consumption capacity[106,109]. On the other hand, increased PUFA content, particularly of long chain omega 3 and omega 6 fatty acids has been demonstrated to be able to replace linoleic acid content of membrane phospholipids[110], and is associated with delayed Ca^{2+} induced opening of the mitochondrial permeability transition pore for apoptosis[115], improve mitochondrial sensitivity to ADP levels[111], and has been demonstrated to be beneficial in the context of heart-failure[108,112]. However, how dietary FFA affect renal mitochondrial phospholipid composition in the context of CKD remains to be investigated.

Of note, a Cochrane review of PUFA intake clinical studies concluded that there is slight reduction in cardiovascular events with increased dietary PUFA, but does not significantly impact cardiac mortality[116], although this conclusion is controversial[117]. Other beneficiary effects of PUFA diet include improvement of insulin resistance[97], decreased synthesis of cholesterol and lipoproteins[118], and favorable alteration of fasting appetite hormones[98].

In summary, fatty acids serve as the precursor to all complex lipids. Circulating lipid profiles become progressively more saturated and shorter in length as eGFR declines in CKD patients. While circulating FFA itself can mediate damage and have recently been implicated in regulating redox balance, FFAs also serve as the basic building blocks for all complex

lipids, including mitochondrial membranes. Enrichment for saturated short FFA may dictate the level and lipid profile of complex lipids that contribute to CKD pathogenesis; we will discuss some of those complex lipids in the following sections.

Plasma acylcarnitines

Acylcarnitines are metabolites of interest in CKD because they represent fatty acids or branch-chain amino acids designated for mitochondrial oxidation and mobilization across cellular membranes. Numerous metabolomic studies of CKD patient plasma acylcarnitines have shown that circulating acylcarnitines accumulate to higher levels in CKD[119–121] or hemodialysis[122,123] patients compared to healthy controls, and that levels of acylcarnitines increase with worsening CKD status. Studies of diabetic have also found higher levels of plasma/serum acylcarnitines in DKD patients compared to diabetic patients without kidney disease[119,120,124], and predicted DKD progression[119,124–126]. Large scale metabolomic studies of the general population have also established that circulating levels of acylcarnitines are significantly associated with eGFR: study of KORA F4 and Twins UK population found acylcarnitine, in particular glutarylcarnitine, inversely correlated with eGFR[127] and another study of Chinese adults with GFR 60 found acylcarnitines of various lengths also inversely correlate with decrease in eGFR[128].

Incomplete FAO leads to acylcarnitines accumulation[129] in tissues that become eventually reflected in the plasma. Apart from measuring the absolute levels of acylcarnitines, another marker of mitochondrial inefficiency is the relative abundance of various acylcarnitines by chain length, including short, medium, and long chain acylcarnitines. In the CPROBE cohort, relatively lower levels of long chain acylcarnitines is associated with advanced stages of CKD and (C16-C20)/(C5-C14) ratio, which is the ratio of the levels of long-chain to medium-chain acylcarnitines, were associated with worsening CKD stage[42]. Of note, the CPROBE cohort exhibited a graded increase in relative abundance of short and medium chain saturated FFA with increasing CKD stage, consistent with similar alteration in FFAs with the same carbon number, suggesting that FFA profiles may contribute to acylcarnitine profiles as FFA serve as the substrate for medium to long acylcarnitine synthesis and FAO. This association of relatively low levels of long-chain acylcarnitine and high levels of medium and short-chain acylcarnitine and CKD status was also found to be associated with DKD progression (defined as decrease in iGFR by at least 40%) in the American Pima Indian cohort at baseline before onset of iGFR reduction[31]. Lower levels of long-chain acylcarnitines were associated with decreased expression of FAO genes in the glomerular compartment and genes regulating FAO in the tubulointerstitial compartment[36], suggesting inefficient β-oxidation of longer chain fatty acids with CKD progression and in ESKD.

Acylcarnitines are filtered by the kidney and is excreted into the urine and therefore decreasing eGFR levels likely contribute to increased short- to medium-chain plasma acylcarnitine levels[130]. With progression to ESKD and reliance on dialysis, the abundance of longer chain acylcarnitines increase[123,131], which is primarily attributed to the inefficient of filtration of acylcarnitines greater than 8 acyl-chain carbons through dialyzer membranes[130]. Increased levels of long-chain acylcarnitines in ESKD patients has been demonstrated to predict increased cardiovascular mortality[123] and reduced physical function[132]. A study of

111 hemodialysis patients found that C2/(C16+C18:1) serum acylcarnitine ratio, which can also represent FAO efficiency, predicted mortality; patients with higher ratio and therefore better predicted mitochondrial FAO efficiency had lower morality rates[133]. To explain these results, researchers have suggested that the accumulation of plasma acylcarnitine is likely mitochondrial overload and dysfunction that occurs in CKD due to increased free-fatty acid levels and insulin resistance from CKD and its comorbidities, and therefore serve as a biomarker of overall patient health in CKD.

Researchers have proposed the idea that increased acylcarnitines export into plasma and elimination in urine may be an adaptive method of dealing with mitochondrial overload, preventing fatty acid mediated cellular damage, and freeing CoA from acyl-CoAs to participate in other energy generating capacities especially in early CKD. Studies of carnitine-acetyltransferase (CrAT) demonstrates the important role of acylcarnitine, especially of acetylcarnitine, efflux out of the mitochondria. Muscle-specific deletion of CrAT, which serves to remove short-chain acetyl-CoAs out of the mitochondria by conjugation to carnitines, leads to decreased glucose tolerance and increased insulin resistance, attributed due to the increased inhibition of pyruvate dehydrogenase (PDH) by accumulating acetyl-CoA[134]. A recent study of proximal tubular cell-specific CrAT deficient mice demonstrated that CrAT function is indispensable to the normal function of proximal tubules. CrAT deficient mice develop tubulointerstitial fibrosis and secondary glomerulosclerosis, which is worsened by a high-fat diet challenge. CrAT deficient kidneys accumulated long-chain acylcarnitines and decreased mitochondrial oxygen-consumption rate. Interestingly, these CrAT deficient mice secreted less short chain and medium chain acylcarnitines in the urine, supporting the notion that efflux of short and medium chain efflux into plasma and urine aid in mitochondrial homeostasis with FAO[135]. Of note, carnitine supplementation, especially in ESKD where patients are prone to dialysis related free-carnitine loss, has not yielded clear improvements in disease parameters such as serum lipid levels, hemoglobin levels, and skeletal muscle weakness[136–138]. However, there is lack of research assessing for whether carnitine supplementation improves insulin resistance or improves mitochondrial function in CKD.

Plasma acylcarnitines are thought to be mainly derived from skeletal muscle[139], as it is the largest reservoir of carnitine and acylcarnitine in the body, although the liver also contributes to circulating acylcarntines[140]. In the models of muscle specific and proximal tubule specific CrAT mice[134,135], there was no effect on plasma acylcarnitine levels despite dysregulated tissue acylcarnitine levels, suggesting that multi-organ changes to acylcarnitine metabolism may be necessary to affect plasma acylcarnitine levels. In summary, increase in plasma acylcarnitines in CKD patients likely represents the run-off of systemic mitochondrial FAO overload, decreased excretion by the kidney, and may serve as an adaptive response to combat mitochondrial stress in CKD.

Triacylglycerols

It is well established that CKD is associated with increased levels of circulatory triglycerides (TAG) thought to be due to decreased catabolism and increased hepatic production of TAG lipoproteins[141]. A higher TAG/HDL ratio, which has been proposed as a measure

of increased insulin resistance, have been found to be associated with increased CKD risk[142,143] and predictive of CKD development and progression[144,145]. Hypertriglyceridemia is also associated with increased risk of atherosclerotic disease and events. A recent secondary analysis of the Study of Heart and Renal Protection trial found increased risk of atherosclerotic events with increase in triglyceride, TAG/HDL ratio, and triglyceride associated lipoproteins: apo-B and triglyceride-rich lipoprotein cholesterol[146]. In ESKD patients treated with hemodialysis, however, the total TAG levels have been reported to be lower than in healthy patients[147] and a study found elevated TAG/HDL associated with positive cardiovascular and overall mortality[148], although the finding has not been recapitulated with peritoneal dialysis patients[149].

Recent studies have improved our understanding of the compositions of TAGs in CKD. Analysis of triglycerides fatty-acyl chain compositions in the CPROBE cohort found enrichment of TAG species with longer acyl chain length and higher degree of saturation with increasing CKD severity[37]. Another cross-sectional study of 44 hemodialysis patients found TAGs of medium chain length (52–56) to be relatively increased while shorter TAGs were relative decreased compared to TAG profiles of health controls[147]. In the Pima Native America cohort, clinical progressors had significantly higher levels of polyunsaturated TAGs, and Cox regression included main effect term of short and lower double-bond TAG species levels to predict DKD progression[36]. A mechanism for the relative enrichment of TAGs with high double-bond and length in CKD, is likely due to the up-regulation of elongation and desaturation of shorter more toxic fatty acids to their less toxic polyunsaturated counterparts. The incorporation of the relatively less toxic polyunsaturated long into TAGs may be a compensatory mechanism due to the alteration of mitochondrial fuel preference for shorter-chain fatty acids (C10), which do not depend on the carnitine for mitochondrial transmembrane transport[150], in the setting of mitochondrial dysfunction and carnitine deficiency in ESKD. Analysis of the (C16-C20)/(C5-C14) ratio, aforementioned in the acylcarnitine section, found an inverse association with this ratio with high double-bond longer-chain TAGs in the CPROBE and Pima cohorts[36,37], suggesting a relationship between mitochondrial FAO fuel profiles and TAGs profiles. In summary, TAG profiles in late-stage CKD and ESKD are enriched for longer and saturated fatty acids and may be due to an increased mitochondrial demand for shorter and saturated fatty acid for fuel.

Of note, fibrates, which reduce circulating cholesterol and triglyceride levels, have not been strongly indicated for lipid management in CKD. Clinical trials and meta-analyses have demonstrated that fenofibrates can reduce cardiovascular disease events and death[151,152], decrease eGFR reductions and microalbuminuria in DKD patients[152,153], but seem to confer no reduction in CKD incidence and progression to ESKD[152]. In summary, further studies are required to fully understand how fatty acyl profiles of TAGs are altered in CKD at different stages of disease, and how the TAG profiles affect TAG function and role in CKD.

Phosphatidylethanolamines

Phosphatidylethanolamines have been identified in multiple clinical studies to be associated with CKD. A secondary analysis of serum samples from two clinical studies: African American Study of Kidney Disease and Hypertension (AASK) and the Modification of Diet

in Renal Disease (MDRD) found six PE species that correlated significantly with proteinuria in both AASK and MDRD cohorts[154]. Pathway enrichment analysis of all metabolites associated significantly with proteinuria found PEs overrepresented in the analysis. In the CRIC cohort, intra-class mean of PEs were higher in the progressors as compared to the non-progressors[42]. In the Pima Native American cohort, higher polyunsaturated PEs were independently associated with higher risk of DKD progression in type 2 diabetes; a Cox regression model, which included unsaturated PEs as a main effector term, found that each 1 SD higher unsaturated fatty acids was associated with 2.57 times higher risk of DKD progression[36]. Lipidomic analysis of CPROBE patients found higher levels of PCs and PEs in CKD patients who experienced stroke compared to CKD patients who did not[155].

PEs are also complex lipids, like TAGs, whose acyl-chain profiles become progressively longer and unsaturated with increasing CKD severity in the CPROBE cohort. Analysis of the (C16-C20)/(C5-C14) ratio in the CPROBE and Pima cohorts found an inverse association with this ratio with high double-bond longer-chain PEs[36,37]; similar to TAGs, it is possible that mitochondrial fuel demands for shorter and saturated fatty acids lead to PE profiles with high abundance of high double-bond longer-chain PEs and consumption of PEs with low double-bond and shorter-chain PEs.

PEs are phospholipids involved in numerous cellular functions. For mammalian cells, major *de novo* synthesis of PEs occurs in the ER (Kennedy pathway) and in the mitochondrial inner membrane by the action of phosphatidylserine (PSD1)[156]. PEs can further be processed via the Kennedy pathway to generate phosphatidylcholines (PCs) or via the action of PEMT specifically in the liver[157]. Maintaining normal levels and production of PEs in mitochondria is indispensable to mitochondrial morphology, fission and fusion, and respiration[158–160]. Clinical studies have associated increased PC:PE ratio with decreased insulin sensitivity, and exercise has been demonstrated to increased PC and PE levels in skeletal muscles[161] and PC:PE ratio in the context of long-term exercise[162]. Deficiency of phosphatidylethanolamine N-methyltransferase (PEMT), a liver specific enzyme that converts PE to PC, resulted in increased levels of mitochondrial PE and therefore lower PC:PE ratio, and was associated with increased hepatocyte mitochondrial respiration[163]. Of interest, PEMT −/− mice exhibited reduced DKD severity when injected with STZ despite unchanged PC:PE ratio in the kidney, suggesting hepatic, and therefore the systematic, PC and PE levels may influence kidney disease[164]. The authors proposed that reduction in ER stress with downregulation of PEMT contributes to DKD amelioration.

Ceramides

Sphingolipids are of large interest not only in CKD, but many other diseases such as diabetes, and cardiovascular disease because they are a large group of lipids with important regulatory functions. Incorporation of fatty acids into generating these signaling and regulatory molecules may therefore impact CKD pathogenesis. At the center of sphingolipid synthesis are ceramides, which can be further modified to generate sphingomyelin, glycosphingolipids, or broken down to generate sphingosine-1-phosphate[165]. Previous studies of sphingolipid levels in plasma of CKD patients report on few specific sphingolipids of specific length and saturation, which makes understanding the dynamics of

sphingolipid metabolism difficult. Different acyl-chain lengths, saturation and lipid species of sphingolipids can have different and sometimes opposing impacts on biology. Of note, sphingolipids are highly associated with DKD because substrates for sphingolipid and glycosphingolipid synthesis such as fatty acids and glucose derived substrates (lactose, galactose, etc) are increased in diabetes[166].

Of particular interest amongst sphingolipids in CKD are ceramides. Ceramides are second messengers involved in promotion of apoptosis, amongst many of its function. Studies have demonstrated that the ceramide carbon number and degree of unsaturation can alter this activity. Ceramide synthases 1–6 (CerS) catalyze ceramides of differential carbon lengths. In HeLa cells, overexpression of CerS 5, which preferentially uses palmitoyl-CoA to generate C16:0 containing ceramides, increased irradiation (IR) induced apoptosis, whereas overexpression of CerS2, which preferentially uses long-chain fatty acids to generate C22:0-C24:0 containing ceramides, protected cells from IR apoptosis[167]. A recent study using a method of *in situ* selective ceramide synthesis called "traceless ceramide ligation", found that synthesis of ceramide containing saturated fatty acid backbone (16:0, 18:0) reduced viability in HeLa cells but not when synthesis occurred with monounsaturated fatty acids (24:1, 18:1)[168].

CKD patients generally exhibit an increase in plasma/serum levels of ceramides. In a recent study of 415 patients who were evaluated for ischemic heart disease, researchers found increased levels of Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/24:0), and Cer(d18:1/24:1). The levels of these ceramides also correlated with CKD stage, increasing with CKD progression. Adjustment for risk factors for CKD, such as diabetes, body mass index, and hypertension did not alter the significance of association of these ceramides and CKD status[169]. These results are corroborated by other studies: ceramide levels of these species were increased in a study of juvenile patients with CKD compared to healthy controls[170], and in a study of patients with T2DM DKD compared to patients with T2DM without DKD for Cer(18:1/16:0) and Cer(18:1/16:1)[120]. On the other hand, in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) cohort, researchers found that very long chain (C20 – 26) ceramides were significantly decreased in T1DM DKD patients with micro and macroalbuminuria as compared to T1DM without albuminuria. Baseline plasma ceramide levels from the DCCT/EDIC patients collected before patients progressed to micro or macroalbuminuria, demonstrated that increase in very-long ceramides were associated with decreased odds of developing macroalbuminuria[171].

It is unclear how circulating levels of ceramides levels reflect kidney tissue levels; a metabolomics study of ceramides in the BKS-*db/db* mice demonstrated elevated plasma but decreased kidney levels of ceramides and glucosylceramides, which suggest disparity in plasma and kidney ceramide regulation[172]. Although kidney levels of sphingolipid levels in CKD are relatively unknown, transcriptomics data and inhibition of sphingolipid enzymes through drug or genetic engineering suggest that sphingolipid metabolism is dysregulated in CKD, and accumulation of kidney ceramides seem to mediate damage. Blockage of ceramide synthesis by *de novo* synthesis with myrocin or conversion from

sphingomyelin to by amitriptyline has been demonstrated to improve high-fat induced murine nephropathy[173,174] and cisplatin induced AKI[175].

Studies of CerS deficient obese mice have demonstrated tissue-specific CerS subtype and ceramide subspecies relevance. For example, in HFD fed mice, skeletal muscles were found to accumulate C18:0 fatty acid backbones preferentially, mediated by the action of CerS1, and its absence in the skeletal muscle leads to reduction in C18:0 accumulation and improvement in glucose handling in obese mice, whereas CerS5 and CerS6 skeletal muscle deficiency does not result in significant alterations to the C16:0 ceramide pool and glucose metabolism[176]. Study of CerS2 haplo-insufficient mice demonstrated insulin signaling impairment in the liver associated with the accumulation of C16:0 through the action of CerS6[177]. While it is unclear whether ceramides and which ceramide species mediate insulin resistance in CKD, recent studies of sphingomyelin phosphodiesterase acid-like 3b (SMPDL3b) lipid raft enzyme have uncovered ceramide-1-phosphate (C1P) as a regulatory sphingolipid of podocyte insulin signaling. SMPDL3b is a lipid-raft enzyme that binds to and dephosphorylates C1P to ceramide, in addition to binding ceramide kinase and modulating its activity[178,179]. SMPDL3b expression is increased the glomeruli patients with DKD and *db/db* mice[178,180], and overexpression of SMPDL3b in podocytes hinders insulin receptor B association with caveolin-1 and downstream Akt phosphorylation[178]. Proper insulin signaling is restored in SMPDL3b OE podocytes and *db/db* mice when supplemented with exogenous C1P, suggesting a lipid driven regulatory role in podocyte insulin signaling in diabetes[180]. Of note, SMPDL3b has been shown to also play a role in focal segmental glomerulosclerosis (FSGS); levels of SMPDL3b is decreased in FSGS patients in contrast to DKD patients, and this decrease leads potentially to increased suPAR-mediated β3 integrin activation[180]. It is interesting that differential SMPDL3b expression is associated with different CKD etiologies, and further work is necessary to uncover SMPDL3b and ceramide role in CKD.

While the roles of specific CerS proteins and ceramide species in CKD have not yet been fully elucidated, increase in short and saturated NEFA plasma levels may affect intracellular ceramide profiles to generated shorter and unsaturated C16:0 and C18:0 ceramides that enhance apoptosis, insulin resistance, and decreased mitochondrial FAO, amongst many other cytotoxic mechanisms. In the kidney, C1P deficiency has emerged as a potential underlying mechanism for insulin resistance in the podocytes. For future studies, further comprehensive lipidomics analysis of the sphingolipid pathway may find additional sites of dysregulation important to CKD.

Bile acids

Bile acids (BA) are synthesized from hepatocytes and play numerous roles in lipid emulsification and digestion, in addition to serving as signaling molecules. Although clinical lipidomics of BAs in the CKD population is very limited, current studies concur in that serum BA levels in CKD patients are elevated, due to decreased eGFR, and its compositions are significantly altered CKD[181–185]. Secondary BAs are primary BAs modified through the action of gut microbiome. A study reported increased share of deoxycholic acid (DCA), an unconjugated second, as a percent of total bile acids in

CKD patients compared to the control group[181]. In another study of ESKD patients, researchers found an increase in the proportion of conjugated BAs and a decrease in the proportion of unconjugated BAs in ESKD patients[182]. A study of microbiome-derived metabolites in ESKD patients demonstrated significantly elevated levels of conjugated secondary BAs relative to conjugated primary BAs in CKD patients, and microbiome analysis demonstrated an enrichment for bacterial species generating secondary bile acids in the disease population[185]. A study of CKD stage 3b to 4 patients demonstrated a positive correlation with the levels of DCA with vascular calcification[183]. Of note, while serum BA levels can be reduced through dialysis in ESKD patients, the composition of BAs is not normalized[181].

The strongest evidence for the importance of BAs in CKD comes from studies of bile-acid receptors farnesoid X receptor (FXR) and membrane-bound G protein coupled receptor TGR5. FXR deficient STZ type 1 diabetic mice exhibit significant kidney fibrosis, albuminuria, lipid-deposition, as compared to the WT STZ mice[186]. FXR activation with various agonists (INT-747, GW4064, cholic acid) in *db/db*, STZ type 1 or HFD-induced murine models of kidney dysfunction demonstrated improvement in disease parameters through modulation of SREBP-1 expression which leads to reduction in lipogenesis genes, reduction in fibrosis and proinflammatory cytokine signaling pathways, and increase in oxidative stress handling mechanisms[187–190]. TGR5 activation with agonist INT-777 in *db/db* mice improve disease by upregulating lipid-oxidation and mitochondrial biogenesis genes[186,187]. Of note, activation of these receptors leads to an improvement in circulating lipid panels: lower serum total cholesterol, mostly due to LDL reduction, and serum triglyceride contents, and this improvement likely contributes to amelioration of DKD[186,188,189]. However, FXR and TGR5 agonism on cultured mesangial cells and podocytes, respectively, also activate or deactivate same set of pathways as *in vivo*, suggesting direct impact of FXR and TGR5 action on kidney tissues[186,189].

Studies of patient kidney biopsies have demonstrated decreased FXR transcript and protein expression in glomeruli and tubules of patients with DKD and obesity[188]. TGR5 transcripts were reduced in DN patients and its levels correlated with degree of proteinuria, glomerular sclerosis, and eGFR measurements[186]. These clinical data suggest FXR and TGR5 relevance in disease. Of note, FXR and TGR5 agonist Nidufexor is currently under investigation as a potential therapeutic for DKD[191] (NCT03804879).

From a lipidomics standpoint, it is unclear whether the alterations in serum BAs in CKD affect FXR and TRG5 signaling. Primary BA chenodeoxycholic acid (CDCA) is the strongest activator of FXR, whereas secondary BA lithocholic acid (LCA) is the strongest activator of TRG5[192]. It is possible that a shift in the levels and composition in BAs in CKD could lead to changes in FXR and TRG5 function. More work is necessary to uncover the regulatory function of CKD BAs in disease.

Discussion and future perspectives

Lipids are a structurally diverse group of molecules that, despite belonging to the same class, have major differences and sometimes opposing biological functions depending

on its chemical composition. Technological improvements in chromatography and mass spectrometry have generated new insights into the altered lipid profiles in CKD, with the most recent comprehensive lipidomics studies reporting on lipid subclass information on carbon number, degree of unsaturation, and hydroxylation. Further structural (e.g. isomer) lipid deconvolution, which is an ongoing area of research, will grant further insights into how lipid profiles are altered in CKD.

Lipidomics of plasma and serum from CKD patients have demonstrated both an increase in the overall concentration and alterations of the structural lipid species in numerous lipid classes. NEFA levels of short and saturated fatty acids has been associated with late-stage CKD and has also been predictive of disease progression, whereas decreased PUFA levels have been associated with early-stage CKD and have been demonstrated to have cardioprotective effects in dietary supplementation studies. Short and saturated fatty acids have been demonstrated to be weak PPAR activating ligands, and their shunting into acylcarnitines and ceramides is thought to be cytotoxic, whereas long and unsaturated fatty acids are protective against the actions of short and saturated NEFAs. In turn, complex lipids such as TAGs and PEs become enriched for their long-chained and saturated counterparts, potentially due to mitochondrial substrate preference for shorter chain-fatty acids due to mitochondrial stress and dysfunction in CKD (Figure 5).

Clinical lipidomics studies have been limited to measurement of circulating lipid profiles as markers of CKD, but the extent of the effect of CKD on peripheral lipid profiles and levels require further investigation. To our knowledge, the only study that systematically compared plasma and kidney (also liver, retina, and nerve) lipid profiles across different lipid classes in a model of kidney disease found that liver lipid composition most closely reflected plasma lipid composition[193]. Interestingly, this was much more pronounced in nondiabetic control mice than in mice with diabetes. Furthermore, the majority of lipid subclasses that were similarly coregulated between diabetic plasma and diabetic liver tissue were also similar between control plasma and control liver tissue. This association was not as pronounced between plasma and the other tissues (kidney, nerve, and retina), where there were fewer similarities across the control and diabetic conditions for each plasma/ tissue comparison. This was particularly true between plasma and kidney, as only one chain-length lipid subclass and two saturation lipid subclasses were commonly regulated in both control and diabetic conditions. To examine how lipid levels were associated within and across classes, we performed correlation analyses in control and diabetic plasma, kidney, nerve, and retina using the 364 lipid features identified in all samples. In this analysis, lipids in plasma and kidney displayed much more correlation than did nerve and retina. Other murine studies examining the relationship between serum and kidney lipid compositions demonstrate highly class-specific responses between the two compartments. A lipidomics study of aging mice demonstrated concurrent changes in TAG and DAG levels, but disparate changes in many phospholipids (many of which are not abundant in the serum)[194]. In the aforementioned proximal tubular specific CrAT knock-out mice[135], which developed tubulointerstitial fibrosis, circulating acylcarnitine levels did not significantly change. Therefore, changes in circulating lipids in CKD likely represent systematic effects and multi-organ dysfunction as a result of CKD.

The relationship between circulating lipid profiles and mitochondrial dysfunction, and furthermore, exactly which processes contribute to CKD requires further investigation. Exogenous lipids likely affect mitochondrial function either by serving as substrates for oxidation or altering mitochondrial lipid composition. Excessive fatty acid load, much like excess glucose load is thought to also contribute to increased reactive oxygen species production in mitochondria. Cardiolipin peroxidation due to such reactive oxygen species production is thought to alter cardiolipin association with oxidative phosphorylation machinery and cue the cell for apoptosis[195]. In murine models of diabetic kidney disease (DKD), reduction in poly-unsaturated cardiolipin and increases in saturated and mono-unsaturated cardiolipin species occur and is attributed to the increased peroxidation of cardiolipins[196,197]. On the other hand, pharmacological approaches that target cardiolipin peroxidation such as Elamipretide[195,198–201] or transgenic upregulation of FAO with CPT1[202] have been successful in ameliorating disease in various murine models of CKD, suggesting that targeting mitochondrial dysfunction, regardless of dyslipidemia status, is sufficient for disease prevention. In conclusion, it is likely that circulating dyslipidemia and mitochondrial dysfunction are two inter-linked processes that form a vicious cycle that contributes to CKD progression.

More research is necessary in understanding how unfavorable circulating lipid profiles directly contribute to tissue dysfunction in CKD of various etiologies, and recent efforts in integrating lipidomics and tissue transcriptomics have been able to identify FAO and de novo lipogenesis as dysregulated pathways in CKD. Many of the studies discussed in this review address CKD of metabolic etiologies, but transcriptomic profiling studies of kidney biopsy in patients from the Nephrotic Syndrome Study Network (NEPTUNE) cohort demonstrated that genes regulating cholesterol transport and metabolism are upregulated[203] and are potentially dysregulated in the presence of apolipoprotein L1 allele variants G1 and G2[204], suggesting that lipid dysregulation also contributes to CKD of non-metabolic etiologies. The various CKD cohorts such as NEPTUNE and Kidney Precision Medicine Program (KPMP)[205] provide the opportunity to integrate, multi-omics approaches (transcriptomics, lipidomics, proteomics) since the kidney biopsy, blood and urine samples used to generate these different types of molecular data are frequently obtained during routine clinical care. The ultimate goal is to develop an integrated molecular classification which will confer insight into individual renal disease specific targets of lipid dysregulation.

Acknowledgments

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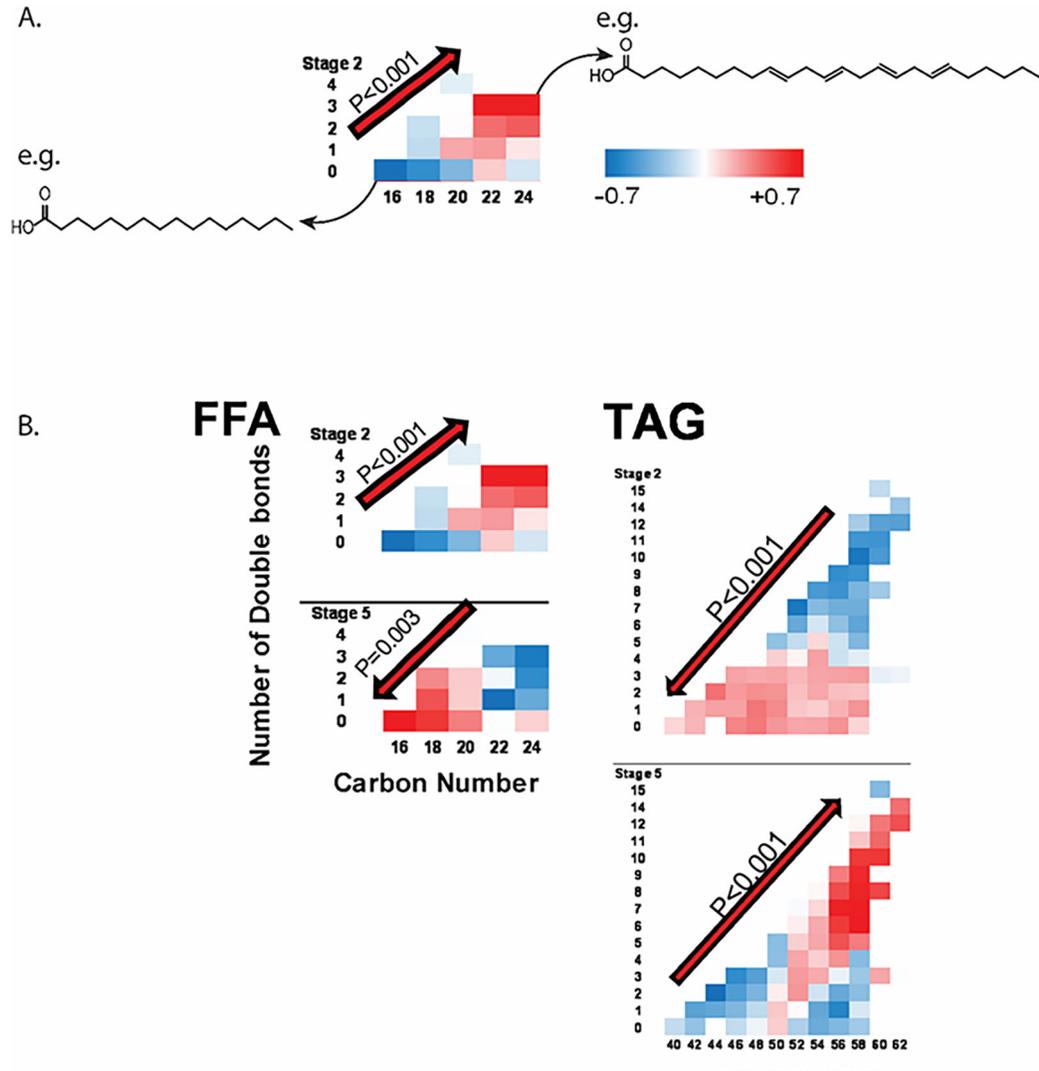
Major points:

- Lipidomic analyses remain a challenge due to the numerous species that compose each class; techniques that reduce analytical complexity while retaining important structural information about lipid structure is useful.

- A key challenge with profiling technologies including lipidomics is the ability to integrate data to gain insights into biological systems, as well as disease onset and progression mechanisms. We discuss framework for systems integration to identify critical nodes for mechanistic and therapeutic interrogation.

- Fatty acid profiles enriched in shorter and more saturated species are associated with later stage of CKD and predictive of CKD progression.

- Due to the immense structural diversity of lipids, lipidomics analyses are guided by choice of untargeted and targeted analysis. Sample preparation, extraction and separation methods may require optimization for specific classes of interest.

- Acylcarnitine profiles are a marker of mitochondrial function. A lower long-chain to medium-chain acylcarnitine ratio represents increased mitochondrial inefficiency that is associated with worsening CKD.

- Complex lipids such as triacylglycerols (TAGs) and phosphatidylethanolamines (PEs) become enriched in longer and more unsaturated fatty acyl side-chains in late-stage CKD and end-stage kidney disease (ESKD). Inverse association of mitochondrial efficiency and high double-bond longer-chain TAGs and PEs suggest a mechanistic link between fatty-acid oxidation and TAG and PE fatty acid profiles.
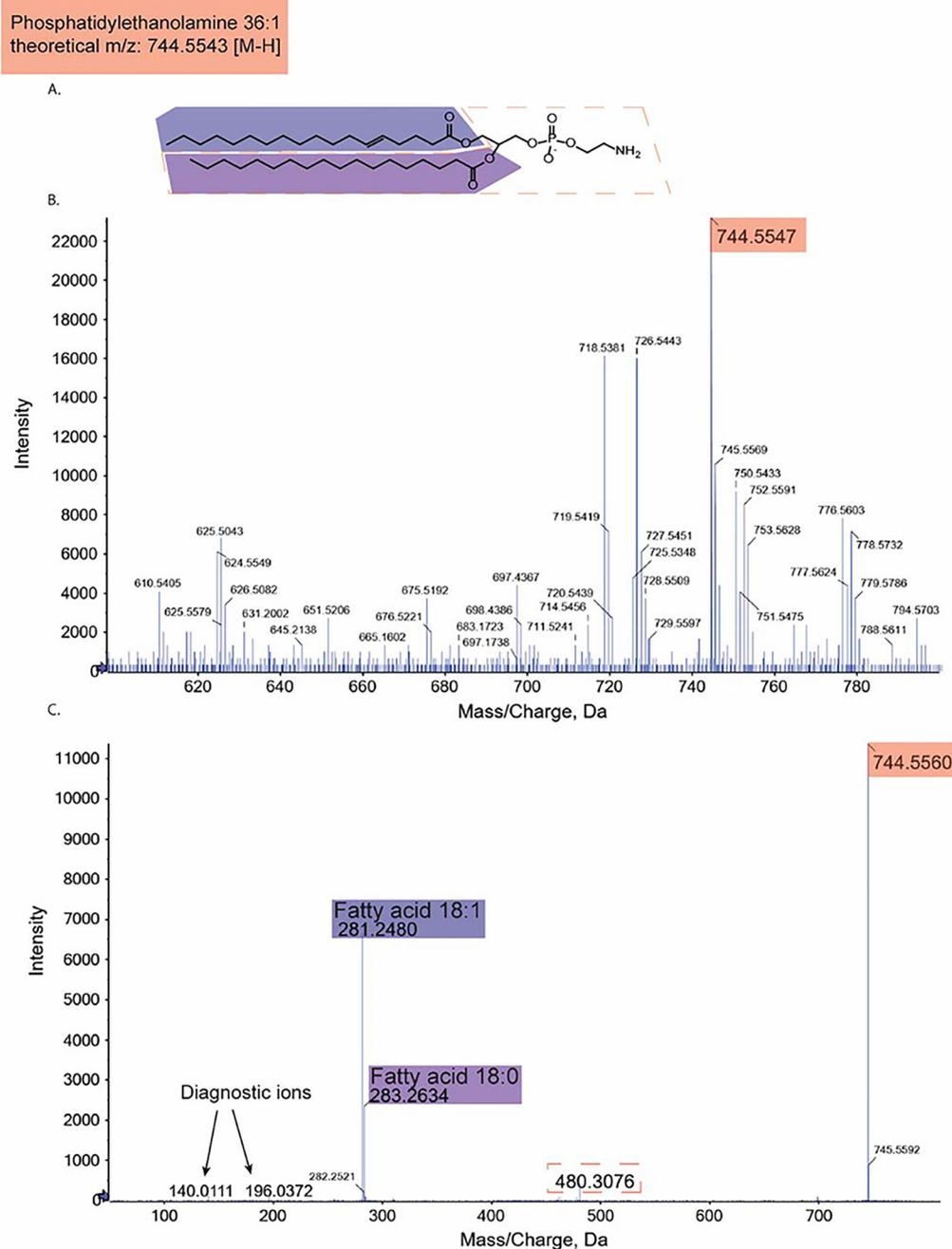
Figure 1:

Diagram of the MS and MS/MS spectra acquired from a triple-TOF for phosphatidylethanolamine (PE) 36:1 with theoretical *m/z* 744.5543 for its [M-H] precursor ion. A) Chemical structure of PE 36:1. Fragments identified in the following spectra are highlighted. B) MS spectra identifying the [M-H] precursor ion with its *m/z* highlighted. C) MS/MS spectra identifying fragments of PE 36:1. Each *m/z* is highlighted with colors that correspond to its fragments highlighted in A). Diagnostic ions for the PE class are labeled.

Figure 2:
Schematic demonstrating the structural diversity of triglyceride 44:2: which has a precursor ion *m/z* of 764.7 and product ion of 493.4 and mass loss of 253.2 that corresponds to fatty acid (FA) 16:1. Four isomers of the original structure (FA 16:1, FA 14:0, FA14:1) are proposed (A-D). FA 16:1 is highlighted in every structure. A) TAG 44:2 with 16:1 and sn-2 and sn-3 acyl-chain carbon isomers. B) TAG 44:2 with FA 16:1 double-bond position isomer. C) TAG 44:2 with FA 16:1 trans double-bond isomer. D). TAG 44:2 with16:1 sn-2 positional isomer.

Figure 3:

Analysis of lipid classes by their secondary characteristics in the CPROBE cohort. Lipid species for specific class are plotted by their carbon number (x-axis) and double-bond number (y-axis), and color coded (blue – low, red – high) to represent standardized measured abundance for CKD stages 2 through 5. A) Example of secondary characteristic plot for free-fatty acids (FFA) in CPROBE CKD stage 2 patients. Each box represents mean standardized abundance for FFA species with the denoted carbon and double-bond number; structures for saturated 16-carbon fatty acid and 24-carbon fatty acid with four double bonds are drawn as representatives of their respective boxes. The interaction term between carbon and double bond number are noted with its p-value and the red arrow denotes the directionality of lipid accumulation with regards to carbon and double bond number. B) CPROBE FFA and triacylglycerol (TAG) secondary characteristic plots demonstrate significant interaction terms for CKD stage 2 and 5 with opposite directionality of lipid accumulation with regards to carbon and double bond number for each stage: at stage 2, FFA demonstrate increased levels of high carbon number and double bond number,

whereas tags demonstrate increased level of low carbon number and double bond number; this directionality is reversed for stage 5. Figure adapted with permission from Journal of American Society of Nephrology.
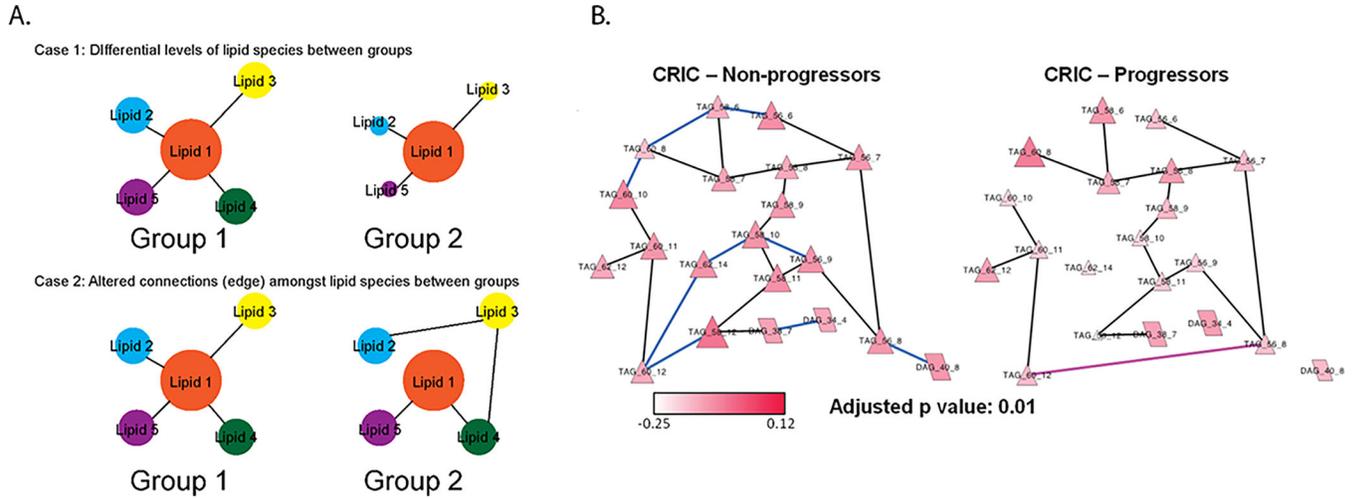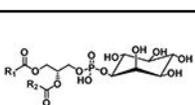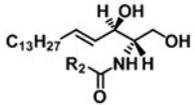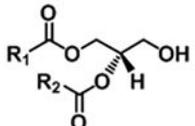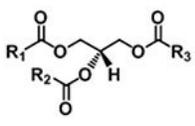
Figure 4:
Differential network enrichment analysis (DNEA) for lipidomics data between CKD groups.
A) DNEA is equipped to differentiate networks that are differentiated by differences
in lipid abundance (Group 2 vs Group 1 in Case 1) or altered correlations or edges
(Group 2 vs Group 1 in Case 2) or both. B) DNEA for non-progressors and progressors
for CRIC patients for triacylglycerols (TAGs) and diacylglycerols (DAGs) in CPROBE.
Nodes represent specific lipid species. Black edges represent correlations present in both
progressors and non-progressors, blue edges represent correlations more likely to be
present in non-progressors and early-stage CKD, and pink edges represent correlations
more likely to be present in progressors and late-stage CKD. Higher abundance of longer
polyunsaturated TAGs in CKD stages 4 and 5 with new edges in neighboring lipids specific
to progressors aligns with upregulation of elongation and desaturation of longer chain fatty
acids and their incorporation in synthesis of longer chain polyunsaturated TAGs in advanced
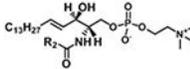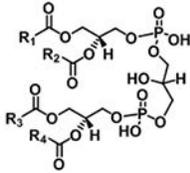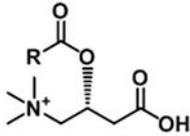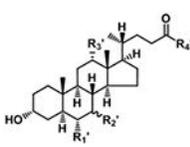CKD. Figure adapted with permission from *Bioinformatics*.
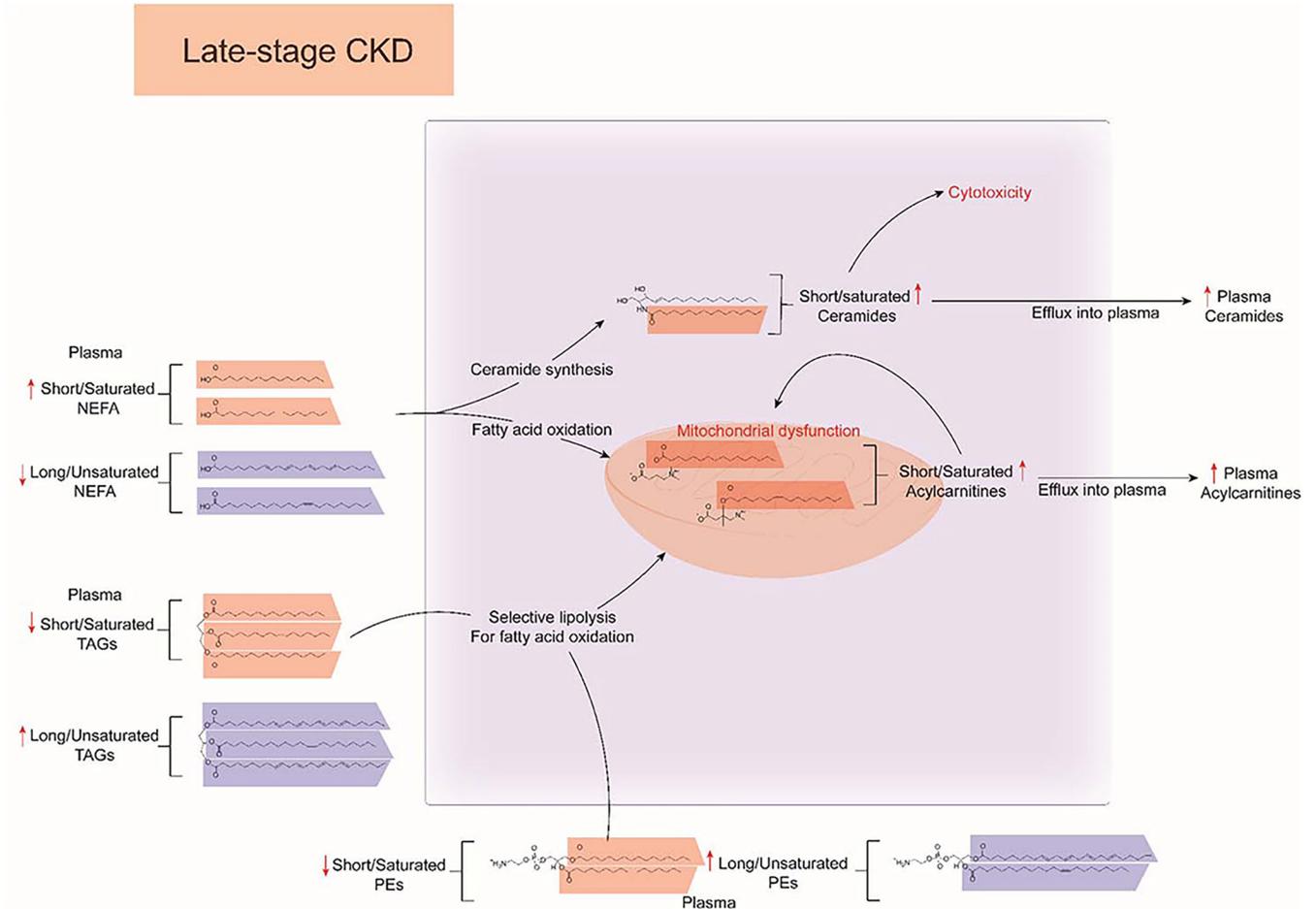
Figure 5:
Overview of dyslipidemia in late-stage CKD. Short/saturated non-esterified fatty acids (NEFAs) are highlighted in orange and and long/unsaturated NEFAs are highlighted in blue. Increased levels of circulating short/saturated NEFA in CKD serve as substrates for fatty-acid oxidation and ceramide synthesis. Increase in short/saturated acylcarnitines contribute to mitochondrial overload and dysfunction, while increased production of short/saturated ceramides contribute to cellular cytotoxicity. Acylcarnitine and ceramides are transported outside the cell and their levels and profiles reflected in the plasma. In turn, mitochondrial overload leads to selective lipolysis of complex lipids such as triacylglycerols (TAGs) and phosphatidylethanolamines (PEs) with short/saturated fatty acid side-chains as they can bypass the carnitine shuttle. This leads to consumption of plasma level short/saturated complex lipids and relative increase in long/unsaturated TAGs

Table 1:

Table of lipid classes, their head groups, most prevalent precursor ion, and class-specific diagnostic fragments generated from the lipid head group.

Lipid Class	Chemical Structure	Precursor Ion	Diagnostic Fragments
Fatty acid (FA)		$[M–H]^-$	(see note) **
Phosphatidylcholine (PC)		$[M+H]^+$	m/z 184.0733 NL 183.0661
		$[M+CH_3COO]^-$	NL 74.0368
Phosphatidyl-ethanolamine (PE)		$[M+H]^+$	NL 141.0191
		$[M–H]^-$	m/z 140.0118 m/z 196.0380
Phosphatidylserine (PS)		$[M+H]^+$	NL 185.0089
		$[M–H]^-$	m/z 152.9958 NL 87.0320
Phosphatidylglycerol (PG)		$[M+NH_4]^+$	NL 189.0402
		$[M–H]^-$	m/z 152.9958 m/z 171.0063
Phosphatidic acid (PA)		$[M+NH_4]^+$	NL 115.0035
		$[M–H]^-$	m/z 152.9958
Phosphatidylinositol (PI)		$[M+NH_4]^+$	NL 277.0563
		$[M–H]^-$	m/z 241.0119 m/z 223.0013 m/z 152.9958 m/z 259.0224
Ceramide with d18:1 backbone		$[M+H]^+$	m/z 264.2685
Diacylglycerol (DAG)		$[M+NH_4]^+$	NL (FA+NH$_3$)
Triacylglycerol (TAG)		$[M+NH_4]^+$	NL (FA+NH$_3$)
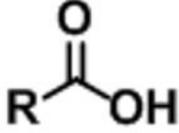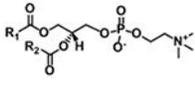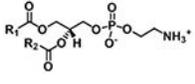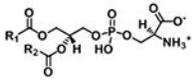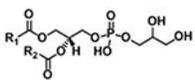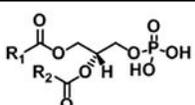
Lipid Class	Chemical Structure	Precursor Ion	Diagnostic Fragments
Sphingomyeline with d18:1 backbone (SM)		$[M+H]^+$	*m/z* 184.0733
		$[M+CH_3COO]^-$	*m/z* 168.0431 NL 74.0368
Cardiolipin		$[M–H]^-$	*m/z* 152.9958
Acylcarnitine		$[M+H]^+$	*m/z* 85.0284 NL 59.0735
Bile Acid		$[M–H]^-$	$R_{1'}$, $R_{2'}$, $R_{3'}$ = H or OH If $R_{4'}$ = OH, fragment varies If $R_{4'}$ = NHCH$_2$CO$_2$H, *m/z* 74.0248 If $R_{4'}$ = NH(CH$_2$)$_2$SO$_3$H, *m/z* 124.0074 *m/z* 79.9574

NL = neutral loss.

*
Functional group R is saturated or unsaturated acyl chain.

**
Free fatty acids are generally not fragmented in common collision-induced dissociation (CID) experiments. For the convenience of multiple reaction monitoring detection, precursor ion mass is also used as fragment ion mass, i.e. for palmitate, FA 16:0, use transition: 255.2 → 255.2.

Table is organized from resources: [206–208]

Table 2:

Relative advantages and disadvantages of alternative lipidomic analytical methods to LC-MS.

Method	Advantages	Disadvantages
Nuclear magnetic resonance	Uniquely suited to be able to identify the chemical locations of isotopically labeled nuclei in isotopic labeling studies (fluxomics)	Low sensitivity
Matrix-assisted laser desorption ionization-time of flight mass spectrometry	Localize measured metabolites to sub-tissue structures; it has been used to provide glomerular and tubulointerstitial metabolite information in the kidney	Can be poorly reproducible; results variable depending on prepared matrix
Direction-infusion (shotgun) lipidomics	Average more mass scans to achieve better signal-to-noise ratio and accomplish more sophisticated structural analysis of lipids	Lack of chromatographic separation means loss of retention time information and matrix effects, which occurs in complex biological samples
Gas-chromatography tandem mass spectrometry (GC-MS)	High-reproducibility	Extensive sample preparation and derivatization, which is time-consuming and potentially results in irreproducibility.
Ion-mobility spectrometry (IMS)	Orthogonal separation method to GC and LC, can be used to separate lipid isomers Can be added to other separation workflows as an additional separation method	Depending on the IMS method and design, low sensitivity due to the ion-mobility compartment
Liquid-chromatography tandem mass spectrometry (LC-MS)	Relatively minimal sample preparation, high sensitivity	Reproducibility can depend on chromatography methods

Table created with references: [209–217]