Published in final edited form as:

Semin Nephrol. 2018 March ; 38(2): 127–141. doi:10.1016/j.semnephrol.2018.01.004.

Lipidomics and Biomarker Discovery in Kidney Disease

Farsad Afshinnia, MD, MS¹, Thekkelnaycke M. Rajendiran, PhD^{2,3}, Stefanie Wernisch, PhD¹, Tanu Soni, MS², Adil Jadoon, MD¹, Alla Karnovsky, PhD⁴, George Michailidis, PhD^{2,5}, and Subramaniam Pennathur, MD^{1,2,6}

¹Department of Internal Medicine-Nephrology, University of Michigan, Ann Arbor, MI

²Michigan Regional Comprehensive Metabolomics Resource Core, University of Michigan, Ann Arbor, MI

³Department of Pathology, University of Michigan, Ann Arbor, MI

⁴Department of Computational Medicine & Bioinformatics, University of Michigan

⁵Department of Statistics, University of Florida, Gainesville, FL

⁶Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI

Abstract

Technological advances in mass spectrometry-based lipidomic platforms have provided the opportunity for comprehensive profiling of lipids in biological samples and revealed alterations in the lipidome that occur in metabolic disorders. A lipidomic approach serves as a powerful tool for biomarker discovery and gaining insight to molecular mechanisms of disease especially when integrated with other – omics platforms, i.e. transcriptomics, proteomics, and metabolomics, in the context of systems biology. In this review, we describe the workflow commonly applied to the conduct of lipidomic studies including important aspects of study design, sample preparation, biomarker identification and quantification, and data processing and analysis, as well as crucial considerations in clinical applications. We also review some recent studies of the application of lipidomic platforms that highlight the potential of lipid biomarkers and add to our understanding of the molecular basis of kidney disease.

Keywords

Lipidomics; Biomarkers; Mass spectrometry; Metabolomics

Address Correspondence to: Subramaniam Pennathur, Division of Nephrology, 5309 Brehm Center, 1000 Wall Street, University of Michigan, Ann Arbor, MI 48105. Phone: 734-936-5645 spennath@umich.edu.

Financial disclosure and Conflicts of interest: None.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Lipids are the most abundant metabolites in the circulation and are an integral part of cell structure and function. Their alterations are linked with disease severity and outcome.^{1,2} Historically, clinical and epidemiological research on lipids has been limited to measurements of lipoproteins and total triglycerides. However, technological advances in modern mass-spectrometry – based lipidomic platforms have allowed identification and quantification of a large array of lipids.^{3–5} Such advances provide opportunities to expand diagnostic capabilities through biomarker discovery efforts, identify novel alterations in involved pathways, and potentially identify targets amenable to intervention. The utility of large-scale lipidomic profiling has been shown in a number of systemic disorders,^{3–8} and recent, promising data indicate lipid profiling provides a powerful discovery tool in nephrology research as well.^{2,8–11} In this article, we review the processes of lipid identification and quantification with lipidomic platforms, discuss the steps that are commonly applied in biomarker discovery efforts, and summarize some of the newly emerging findings on role of lipids in clinical kidney-related studies.

CHALLENGES

Lipidomics has evolved tremendously in the last decade. However, despite this rapid growth, significant barriers remain to achieving the goal of a precise and comprehensive evaluation of the lipidome and its incorporation into metabolic pathways. The most significant challenge remains the extreme molecular heterogeneity of lipid compounds, which limits comprehensive characterization and quantification of the classes, multiple subclasses, and numerous species of biological lipids.¹² The desired balance between achieving precise quantification and capturing an ever-expanding lipidome determines the most appropriate platform for each lipidomic experiment.

The structural diversity that gives lipids their biological versatility and determines their chemical properties makes an all-inclusive method for their analysis difficult to achieve. Because standardized fragmentation spectra are lacking for lipids, pairing high mass accuracy with retention time is particularly helpful in the identification of lipid species. The current instrumentation (Orbitrap, Fourier Transform)¹³ provides adequate mass resolution and accuracy, but challenges remain in separating lipid classes using hydrophobicity-based criteria. This is further complicated by similar hydrophobicity characteristics of some classes, and recent efforts have employed serial chromatographic steps to achieve compound separation and increase coverage of the lipidome.^{14,15} Currently, the most popular methods for high-throughput lipid analysis are limited in their ability to determine the precise arrangement of fatty acid substituents attached to the lipid-head group and the positions of any double bonds in the structure. Several techniques using alkali metal adducts¹⁶ and ozone- induced dissociation¹⁷ have been employed with mixed results and are complicated by very low ion abundance and the generation of complex data, which can compromise results. Recent experiments using ion-mobility mass spectrometry have shown promising results, and this technology remains at the forefront of method development.¹⁸

There are additional obstacles in data processing for lipidomic analyses due to the absence of robust, platform-independent software for conversion of raw data into quantifiable biological information for analysis. This bioinformatic deficit is further exacerbated by the inability to comprehensively integrate lipidomic data with molecular profiles from other types of “-omic” analyses, which would allow us to validate the lipidomic data through investigation of other -omic modalities and identify pathways or lipids requiring further study to enhance our understanding of metabolic regulation. Our current measurements and analyses provide a snapshot of the lipidome at a given point in time without identifying the determinants of the lipid profile and raising questions about the role of enzymatic activity and genetic regulation. These aspects can be explored further with experiments looking at stable isotope monitoring in flux analysis to determine reaction rates in biochemical pathways and advance our understanding of both human health and disease.

LIPIDOMICS WORKFLOW

Lipidomics workflow may differ from study to study based on the aims and scopes of each project. The steps summarized in Figure 1 are followed in most studies.

Study Design

At the beginning of each lipidomics study, a decision must be made regarding which platform (untargeted or targeted) is most appropriate to answer the study question. Further, application of biological and technical replicates and a plan for quality control and assurance are important study pillars that need to be addressed in the design and are largely determined by the study's questions and aims.

Untargeted and targeted platforms—Lipidomic studies are broadly classified as untargeted or targeted. Table 1 summarizes these two approaches to lipidomics and their most important characteristics.

The untargeted approach is a hypothesis-generating platform that provides a snapshot of all identifiable lipid species in the biosample. In an untargeted analysis, the goal is to identify as many lipids as possible in order to determine differentially regulated lipid panels. Analysis using an untargeted platform is viewed as the discovery phase, and the differentially regulated lipids identified then comprise a panel of candidate biomarkers, which is required for confirmatory downstream studies using targeted platforms. During sample preparation, chromatographic separation, and mass spectrometric detection, there may be preferential detection of certain lipids and signal loss for others, which make the method susceptible to systematic and methodological bias. Measurements are semi-quantitative and are based on relative abundance of peak intensities, because absolute quantification is not feasible. When using untargeted platforms, more rigorous quality control is required to ensure high-quality data. Adequate quality control includes continuous instrument maintenance and calibration and application of various pooled samples, internal standards, and technical replicates. Internal standards are isotopically labeled analogs of the target analytes, which are added to the samples to compensate for losses during sample preparation and ionization, and help with normalization process. In untargeted platforms, generally no more than 1 or 2 internal

standards are used per lipid class, due to the unavailability of internal standards for each endogenous lipid and prohibitive costs upon availability.

Conversely, the targeted approach is a hypothesis-driven platform. In targeted analyses, the goal is to validate and quantify a predetermined panel of lipids that may have been suggested for further analysis by an untargeted approach. Unlike the susceptibility of untargeted methods to preferential loss of certain lipids, with a targeted platform an analytical method is developed to specifically quantify the putative biomarkers. Quantification is based on reports of absolute concentrations. Lipids are identified in multiple-reaction-monitoring (MRM) mode often with addition of specific internal standards, allowing improvement of the signal-to-noise ratio and facilitating low detection limits. As a result, the measurements are more precise. Because the number of candidate biomarkers is limited, ideally there should be one internal standard for each candidate lipid, unless such a standard is unavailable.

Quality control and quality assurance—A major concern when analyzing a large number of samples is drift in the performance of mass spectrometry over time and across several different batches. There are several strategies to minimize the detrimental effects of alterations in study conditions over time. Upfront, the samples should be randomized into a predetermined number of study batches by a person who is blinded to sample phenotypes or outcome data. This procedure will eliminate bias from allocation of one phenotype or outcome to a batch with different measured values due to drift in performance of mass spectrometry. Quality can also be assured by using at least two sets of pooled samples spiked with internal standards. One set comprises reference pooled samples and the other comprises pooled samples from the study.

Pooled reference samples can be obtained from plasma from a large pool of blood donors, for example from the American Red Cross, which is available in a large quantity and can be stored for long periods of time. Normalization using an external reference pooled sample makes it possible to compare and interpret data obtained at different time points and from various studies, specifically in untargeted platforms that measure relative abundance rather than absolute quantity. We recommend up to 10 sequential runs of the reference pooled samples at the beginning of each batch to confirm reproducibility of the instrument prior to running the actual samples. To further monitor instrument performance, technical replicates are also made by reconstitution of 10 μ L of a dried matrix-free mixture of the internal standards.

A second set of pooled samples (referred to as the test pool) is obtained from actual study samples. It is recommended to run these sample at the beginning of each batch (after the last run of the pooled sample from reference population and prior to the first actual sample), and then after each 10–15 study samples (Figure 2A). The rationale for using a test pool is the need to monitor the lipid extraction process and assess drift in the performance of mass spectrometry over time within each batch and from batch to batch, so that any batch effects can be corrected with a normalization protocol.

Sample Preparation

Two essential components of sample preparation are the addition of internal standards and lipid extraction. Using appropriate internal standards is a critical step to account for any differences in extraction efficiency. By spiking internal standards into the biological matrix prior to lipid extraction, quantification can be achieved by measuring the abundance ratio of sample-derived lipids to the internal standards. Internal standards should have similar physicochemical properties to the lipids being analyzed. Stable isotope-labeled internal standards are an excellent choice, as they are chemically indistinguishable from their unlabeled analogs. Lipids with odd-chain fatty acids, which are found in very low abundance in samples of biological origin, are also frequently employed as internal standards for endogenous lipids belonging to the same class.

Due to the diversity of lipid molecules, achieving complete lipid extraction necessitates application of several polar solvents, such as alcohol, and non-polar solvents, such as chloroform. The majority of the lipidomic profiling techniques depend on lipid extraction methodology developed by either Folch and colleagues¹⁹ or Bligh and Dyer²⁰, often in modified forms. Both extraction methodologies employ different volume ratios of chloroform, methanol, and water. Recently, Matyash and colleagues employed methyl *t*-butyl ether and methanol for extraction, and then water was added to separate two layers.²¹ In some studies, chloroform is replaced with dichloromethane (DCM).²²

As an example, when we prepare samples for lipidomic analysis, we add water, methanol, and DCM with a 2:2:2 ratio to a biofluid at room temperature, followed by spiking with 10 μ L of internal standards comprising of broad array of lipid classes (Table 2). When analyzing biological tissues, we accurately weigh the tissue and then homogenize with methanol and extract the lipids with a 2:2 volume ratio of DCM and water. After equilibration for 5 minutes at room temperature, we centrifuge the extract at 4000 RPM for 5 minutes at 15°C, collect the lower lipid-containing organic phase, and dry it completely under a gentle stream of nitrogen gas. Then, the dried lipid extract is reconstituted in 100 μ L of acetonitrile/isoproterenol/water (10:85:5) containing 10mM ammonium acetate, and we inject 5 μ L for chromatography and subsequent mass spectrometry analysis.

Separation and Detection

The two major strategies employed to carry out global lipidomic profiling are liquid chromatographic separation of lipid extracts and infusion of the extracts into the mass spectrometer. When applied together, the analysis is referred to as liquid chromatography–mass spectrometry (LC-MS).^{23,24} Direct infusion to the mass spectrometer without chromatographic separation is an experimentally simple and rapid method for detecting various classes of lipids in a single experiment.^{25,26} However, ion suppression and challenges associated with resolving isobaric species are major concerns with this approach.^{27,28} In contrast, LC-MS–based methods have advantages over direct infusion techniques including more reliable identification of individual lipid species, even at trace levels, separation of isomers and isobars, reduced ion suppression, and potentially higher sensitivity. Nonetheless, standard LC-MS methods require relatively longer analysis time than direct infusion and can also harbor a risk of sample carryover that must be overcome.²⁹

Further, due to the extremely large number of different molecular lipid species, co-elution of isobaric manifests as a limiting factor.³⁰ To reduce the issue of co-elution, ultra-performance liquid chromatography (UPLC) can be applied.³¹ UPLC operates at a higher flow rate without loss of resolution as compared with conventional high-performance liquid chromatography (HPLC) columns, allowing for a short analysis time with highly efficient separation, and more accurate ion determination when coupled with a high-resolution mass spectrometer.^{32–35}

Identifying different lipids with identical mass-to-charge ratio (m/z) is a common finding in high-throughput mass spectrometry; the application of tandem mass spectrometry may overcome this limitation. Tandem mass spectrometry, also known as MS/MS, involves sequential steps of mass spectrometry in which a precursor is identified in the first mass spectrometry (MS1), and fragmented into product in the second mass spectrometry (MS2).³⁶ Specific fragmentation in MS2 allows accurate identification of unique lipids in MS1. Tandem mass spectrometry can be classified as tandem in-time or tandem in-space.³⁷ With tandem in-time MS, the separation takes place in the same collision cell but through multiple steps over time, while tandem in-space MS identifies precursors and fragments in separate, coupled mass spectrometers. These methods of separating and detecting lipids in biologic samples have proven to be very effective. For example, simple, reversed-phase, UPLC-quadrupole time of flight (QTOF) MS/MS-based comprehensive lipidomic profiling of plasma from patients with chronic kidney disease (CKD) is capable of identifying >500 molecular lipid species from various classes of lipids including phospholipids, sphingolipids, and glycerolipids.^{2,9}

Data Handling

Data processing—The data processing for lipidomics analyses is done in a semi-targeted, recursive fashion. Global lipid profiling allows us to look for all possible identifiable lipids present in the samples. Once the lipids are identified, the next step is to target all identified lipids using m/z and retention time for quantification. The raw data processing pipeline is a 3- step process: identification, library creation, and quantification.

• **Identification:** Identification of lipids is accomplished by either data-dependent³⁸ or data-independent³⁹ MS/MS product ion analysis of lipid species in both positive and negative ionization modes.^{40–43} In data-dependent mode, a specific intensity threshold is used to trigger the acquisition of a precursor, with selection of a highly abundant MS peak, discarding all other peaks outside the threshold.^{37,38} This provides information on the nature of the head group or neutral loss of the head group from the molecular ion adducts.⁴⁴ The subsequent product-ion matching algorithm uses traditional similarity scoring of remaining candidates. Reverse search scores are used in cases of impurities or unexplained peaks.⁴⁵ Identification is based on matching unique combinations of precursor ions and fragments to a database of lipids using LipidBlast, an *in silico* mass spectral library resource that contains 212,516 spectra covering 119,200 compounds from 26 lipid-compound classes including phospholipids, glycerolipids, bacterial lipoglycans, and plant glycolipids.⁴⁶ Each *in-silico* spectrum has an assigned, accurate mass precursor ion that is used for precursor filtering during the database matching process. After the precursor filter is passed, each spectrum is

searched using a dot-product and probability search-based method. A library hit score is obtained for all candidates in a manner analogous to a mass spectral library search.

In data-independent mode, the instrument fragments all peaks irrespective of the peak height, creating an enriched collection of MS2 spectra that lacks the precursor information.³⁷ One advantage of the data-independent approach is that all molecules, even the very low intensity precursor ions, undergo fragmentation. The primary disadvantage is that the direct link between the fragmented ion and the precursor is broken.³⁷ Specific software (MS-DIAL) is required to reconstruct the precursor ion.⁴⁷

• **Alignment and library creation:** The MS/MS identification results from all the files are combined to create a library for target search and peak integration. The data are aligned and corrected for retention time differences to allow identification from different samples creating a master library with names of the lipid species, target m/z , and retention time.

• **Lipid quantification and batch-effect normalization:** All raw MS data files are searched against a library of identified lipids based on mass and retention time using vendor specified software such as Multiquant 1.1.0.26 software (ABSciex, Concord, Canada).⁴⁸ Quantification is done using MS1 data. To quantify lipid levels, the individual molecular ion-peak intensities are normalized using an internal standard for each lipid class. After completion of data acquisition, a coefficient of variation of each internal standard in the reference pooled plasma, as well as in the test pool, is calculated within and between various batches. Negligible batch-to-batch variability of pooled plasma and test pools distributed in 4 batches in a lipidomic study of 214 patients with CKD is shown in Figures 2B and 2C.² Variability of up to 15% in the internal standards within each batch and up to 20% between batches is acceptable. If variability is large, further investigation is required to determine whether or not the variability is random or comes from an identifiable systematic alteration in measurements. In case of a large random variability, the corresponding internal standard is excluded from the downstream normalization processes. If the high variability is not random, the corresponding bias and its direction can be identified, quantified, and corrected using various normalization procedures. Such procedures may be as simple as removal of the pooled samples (example: in the case of measuring an empty vial in the last measurement) or model-based normalization in cases of systematic drift. Examples of such normalization procedures include quality control (QC) sample-based Loess normalization and scaler adjustment of intensities between batches. In Loess normalization, QC samples are used to assess the drift and train the model to get the span parameter used for smoothing on the biological samples.⁴⁹ In scaler adjustment of intensities, QC samples are used in a regression model to estimate the value for adjustment for different batches. For the studies with big shifts in intensities due to changes in instrument conditions, QC-based scaler adjustments work better than Loess normalization. Because there is no consensus on the best normalization method in lipidomic platforms, the investigator may be required to go through several rounds using trial and error to identify the most appropriate method that best corrects the batch-to-batch variabilities in their data.

• **Imputation of missing values:** Deletion of lipids with over 40% missing values at random from further analysis is recommended, unless the missing data is exclusively observed in

only one of the study subgroups, in which case the corresponding lipid could be a valuable candidate biomarker. Common causes of missing data are values below the detection limit or ion suppression during MS. Missing data due to quantities below the detection level are suggested by a high number of missing values in the presence of very low intensities in samples with measured values. An appropriate imputation for this condition would be imputation by half the minimum value. Otherwise imputation by the k-nearest neighbor method is an acceptable method that is successfully used in the analysis of other -omics data.⁵⁰

• **Normalization of Data Distribution:** Many statistical models (e.g. regression models) rely on the assumption of normality. Because parametric tests are preferred under the normal distribution assumption, a log transformation of the data can be useful to bring the distribution of the data closer to satisfying the normal assumption. Non-parametric tests can be applied for analysis when the data distribution is not normal.

Statistical analysis—The type of analyses and choice of statistical tests may vary from study to study depending on the aims, design, and study questions. The most commonly applied procedures are compound-by-compound comparisons to determine differentially regulated lipids, modeling for classification and risk estimation, dimension reduction, identification of subgroups of lipids or participants, and assessment of classification power.

• **Compound-by-compound comparison:** As a high-throughput data generating platform, hundreds of data points per sample may be generated by the mass spectrometer. The first task is identification of lipids with differential abundance across study subgroups by testing their nominal statistical significance. This can be achieved using a t-test for comparison of abundance of lipids between two groups and by using analysis of variance (ANOVA) when there are more than 2 groups. Multiple measurements lead to multiplicity of Type-I error requiring adjustment for multiple comparisons. Historically, a Bonferroni correction has been used to adjust the p value for multiple comparisons.⁵¹ The Bonferroni correction sets the statistical threshold for rejection of the null hypothesis as $(0.05/n)$, n being the number of comparisons. This adjustment controls the familywise error rate of all n tests and does not require their independence. However, in the presence of the many lipids produced by the platform, which is in the 100s, a Bonferroni correction becomes exceedingly conservative, thus making it nearly impossible to identify any differentially regulated lipids even in studies with 100s of samples. To that end, the alternative Benjamini–Hochberg procedure that controls the false discovery rate (FDR)—the expected proportion of false discoveries—provides a more flexible a mechanism for addressing the multiple-comparisons problem. It is less stringent in controlling Type-I error, and therefore decreases the inflation of false negatives as compared with the Bonferroni correction.⁵² However, the basic version of the Benjamini-Hochberg procedure is only applicable under the assumption that the underlying tests (lipids) are independent. In the presence of positive dependence, an alternative is provided by the Benjamini-Yekutieli procedure.⁵³ More recently, estimating the ‘effective number’ ($M(\text{eff})$) of independent tests for control of correlated tests was shown to be a useful method with better control of the error rate and increased study power when there are numerous correlated measurements.⁵⁴

• **Modeling:** The purpose of modeling is either estimating the risk of an outcome by alterations of lipid levels or classification of subjects by an outcome of interest, for example classification of patients with CKD into progressors to end-stage kidney disease or non-progressors. Multivariable adjustments may be used to control for potential confounders and to improve the accuracy of the models. In cross-sectional or case-control designs, logistic regression models are used to provide the odds of an outcome by alteration of each one unit of the corresponding lipid, whereas in longitudinal cohorts, where the outcome variable of interest is time to event (e.g. biochemical recurrence in cancer studies), Cox proportional hazards models are applied to estimate the risk of an outcome by a change in lipid levels. For classification purposes, forward inclusion of predictive covariates and backward elimination of non-predictive covariates have been used to select the significant predictors in a panel. This approach is limited by selection of covariates that are strongly associated with outcome in certain occasions and not necessarily in a reproducible manner, leading to model over-fitting. In 1996, the Least Absolute Shrinkage and Selection Operator (LASSO) method was proposed to both select the predictive covariates and to regularize prediction accuracy by minimizing over-fitting.⁵⁵ LASSO itself also has limitations. For example, when the number of significant predictive lipids is greater than the number of available samples, the maximum number of lipids selected by LASSO is approximately equal to the sample size. The method may also select no more than one lipid from a subset of highly correlated lipids. In 2005, the elastic net method was proposed to overcome some of the limitations of LASSO by adding an ℓ^2 penalty (ridge) term.⁵⁶ Addition of ℓ^2 penalty term decreases the prediction error of LASSO by enhancing the grouping effect of highly correlated lipids.⁵⁶ Another popular variant is to use prior information (e.g. lipid classes) in the modeling and use a group LASSO penalty.⁵⁷ This penalty will result in a whole lipid class to be selected and included in the model or to be excluded. Such penalized regularizations can easily be extended to many statistical models, such as generalized linear models, logistic regressions, and proportional hazard models, providing a subset of predictors with better predictive accuracy and less over-fitting. Further, these models can be used to build a scoring system with a threshold by which the discrimination of study phenotypes or the outcome of interest is maximized. In addition to logistic regression models, random forests (RF), partial least square-discriminant analysis (PLS-DA), and support vector machines (SVM) are standard supervised classification methods for building models for classification purposes.

• **Unsupervised Dimension Reduction:** Unlike supervised methods in which the outcome or the clinical phenotype is used to build a model for classification, data reduction using unsupervised methods is independent of the clinical phenotype. Principal component analysis (PCA) is a method in which a highly correlated subset of variables are aggregated into secondary variables (principal components) aimed at capturing the maximal variance of a dataset using a small number of variables.⁵⁸ The principal components are weighted combinations of the original variables (e.g. lipids). Usually, several principal components are derived from an analysis, and each one captures a decreasing amount of the total variance in the data. The optimal number of generated principal components may be derived from the cumulative amount of variance explained, or by a pre-specified threshold required to be exceeded by the variance of each principal component (eigenvalue). The generated secondary variables then will be used in downstream analysis in lieu of the initial covariates.

K-means clustering and hierarchical clustering are additional unsupervised methods aimed at obtaining groups (clusters) composed of similar members.⁵⁹ In k-means clustering, the samples are classified into k clusters; each sample belongs to a cluster to which its measurements are closest to the mean of the measurements in the cluster. In hierarchical clustering, grouping can be achieved by an agglomerative or a divisive approach. In the agglomerative or bottom-up approach, clustering starts at the level of each sample, so that similar samples merge to form new clusters and then pairs of similar clusters merge to move up in the hierarchy. The procedure stops when all samples are merged to a single cluster. In the divisive approach, all samples start as one cluster and then are split as the procedure moves down the hierarchy and stops when each sample occupies its own sample. The selection of clusters is usually based on visual inspection by the practitioner, although a silhouette plot⁶⁰ provides a useful, visual diagnostic. Clustering can be achieved at the patient level, at the lipid level, or both. New clusters can be used as secondary variables in downstream analyses.

• **Assessment of Classification Power:** The classification power of candidate biomarkers is assessed by the extent the panel can discriminate disease from non-disease. Candidate biomarkers should have a reasonably high sensitivity and specificity to gain clinical applicability. An ideal binary classifier is a test with 100% sensitivity and specificity, characteristics that are lacking from most tests in clinical practice, and therefore a suboptimal panel with classification power superior to conventional biomarkers may still be of value. For continuous variables that are directly associated with the likelihood of the disease, the sensitivity increases at the expense of specificity as the level of a biomarker decreases. On the other hand, higher levels of the biomarker are associated with a higher specificity at the expense of sensitivity. The receiver operating characteristic (ROC) curve is applied as a graphical tool to identify the classification power of a continuous variable across various levels of the classifier by plotting sensitivity against “1-specificity” and calculating the corresponding area under the curve. A perfect classification is the (0,1) point where sensitivity and specificity are both 100% and the area under ROC curve (c-statistic) is 1. A non-informative classifier is one with an area under the ROC curve of 0.5. In practice, most tests do not have a c-statistic of 1, and therefore their impact on improving classification can be tested when added to a panel of traditionally used biomarkers. The improvement in the c-statistic of the new panel over that of a traditional panel can be tested using available statistical methods.⁶¹ Interpretation of the statistical significance of the improvement imparted by a new panel as compared with a traditional panel should be made with caution as a very large sample size may drive the statistical significance when only a very small change in c-statistic is achieved, or the c-statistic may be hardly moved by a good biomarker when the baseline model consists of several strong predictors.⁶² To address some of these limitations, the Net Reclassification Improvement (NRI) and Integrated Discrimination Improvement (IDI) have gained popularity as additional methods to assess improvement of classification.^{62,63} The event NRI is the net percentage of events that are correctly classified, and the non-event NRI is the percentage of non-events that are correctly classified by the biomarker. Overall, NRI is the sum of the event NRI and the non-event NRI and ranges from “-2 to 2” with zero being no net improvement in classification.⁶⁴ Similarly, event IDI is improved sensitivity by the biomarker across different levels of predicted risk,

while non-event IDI is improvement of specificity across different risk levels. Overall, the IDI is the sum of event and non-event IDI.⁶⁵ Despite the popularity of NRI- and IDI-driven comparisons, recent reports provide some recognition of their limitations.^{66,67} For example, Pepe and colleagues showed that even a poorly calibrated biomarker with no predictive value may show an improvement in classification when tested using NRI⁶⁷, an observation that calls for extra caution when using and interpreting this technique.

• **Validation:** The biomarkers in any lipidomic analysis should have internal and external validity to ensure applicability in clinical practice. If the sample size of the study is adequately large, the cohort should be randomly divided into training and test sets. The training set is used as a discovery subcohort, and the resulting proposed biomarkers are then validated on the test set. However, if the sample size of the study is not very large, cross-validation strategies can be employed. In k-fold cross validation, the original sample is randomly divided into k equal subsamples, of which k-1 subsamples are used for training and the other subsample is applied for validation. Finally, the biomarker or biomarkers identified with high-throughput lipidomic methodologies should undergo external validation by replication of the findings in independent study populations.

Bioinformatics—Bioinformatics analysis is aimed at enabling biological data interpretation by highlighting important trends in the data and placing them into a relevant biological context. The most common bioinformatics approach for the analysis of -omics data relies on the application of existing pathways available in biological knowledge databases (e.g. the KEGG database). Over the past decade, a number of tools have been developed to help map and visualize experimentally measured polar metabolites and lipids in known biochemical pathways.^{68–70} However, the scope of application of the existing tools is limited to known compounds from large, well-annotated pathways. In contrast, a large proportion of lipids identified in lipidomic analyses are either unknown or not mapped to a certain pathway, and therefore fall outside the current capabilities of bioinformatics tools. One reason for this discrepancy stems from the fact that pathway databases provide a genome-centric view of the lipidome. In contrast, experimental lipidomics provide a chemistry-centric sampling defined by a chosen analytical method. The area of overlap of these two views typically includes primary metabolic pathways, while the coverage of secondary and lipid metabolism is scarce.⁷¹ Further, most pathway databases describe lipid metabolism at the class level, whereas modern lipidomic analysis methods can provide more elaborate information about the length of the carbon chains and their level of saturation. Therefore, pathway-based mapping techniques have limited utility in analysis of lipidomics data.

Data-driven, network-based methods are an alternative to knowledge-based pathway-mapping approaches. A wide array of methods has been applied to create network context for experimentally measured polar metabolites and lipids. One method incorporates building *correlation-based networks* followed by the identification of differentially correlated clusters.⁷² Because correlation networks capture both direct and indirect statistical associations, the output of such analyses results in dense network modules where metabolite/lipid nodes are connected by multiple edges, which can be challenging to interpret.

Alternatively, *partial correlation networks* can help address this challenge through their ability to differentiate between direct and indirect associations; however, their application is also limited to very large data sets.⁷³

To unravel meaningful associations in high-throughput data generated by studies with relatively small sample sizes, sparse modeling methods have been introduced to overcome limitations imposed by sample size.⁷⁴ We mapped the interrelationships between various triacylglycerols in a group of 214 patients with CKD from the Clinical Phenotyping Resource and Biobank Core (CPROBE) cohort² by applying sparse modeling methodology.⁷⁴ We used the debiased sparse partial correlation (DSPC) method described by Basu and colleagues and visualized it using Metscape (Figure 3)⁷⁴ and noticed at least 2 distinct subgroups of triacylglycerols that differed from each other in their correlation pattern. One subgroup consisted of shorter triacylglycerols (C52) with a lower number of double bonds, and the other subgroup included longer triacylglycerols (C54) with a higher number of double bonds. Within each subgroup, there are numerous triacylglycerols that are correlated with each other, but the number of correlated triacylglycerols between the subgroups is small. This observation suggests that in patients with CKD, there may be at least 2 different types of triacylglycerols that differ from each other in their biological characteristics and possibly in their clinical implications. The insight generated from the data-driven approach, when combined with elements of prior knowledge, holds promise to advance the analysis of lipidomics data sets and helps in better understanding underlying biological processes.

Clinical Application of Lipid Biomarkers

Once a panel of potential biomarkers is identified using a lipidomic approach, further validation is required in large cohorts and clinical trials prior to gaining clinical acceptance. There are several important considerations of how to use and interpret a new lipid biomarker in clinical practice. One consideration is whether or not the biomarker is predictive of a future outcome or if it is diagnostic for a current disease. Another consideration is if the marker is the key regulator of downstream adverse events, in which case its modification may mitigate the disease process and monitoring of its level can be used as a tool for clinical response and disease activity. A biomarker may also be a surrogate marker of pathway activities, in which case its modification may not necessarily translate to an improved outcome but it may be of value for risk-factor stratification and monitoring. A cost-benefit analysis is required to understand the extent to which a new test or therapy based on the biomarker may improve clinical practice (enhanced diagnostic capabilities, diminished adverse outcomes, cost saving) or generate harm (adverse outcome from exposure to additional tests or interventions, unjustified costs) as a consequence of clinical application. This analysis may or may not justify application in daily clinical practice. All these issues regarding the clinical application of biomarkers identified in lipidomic analyses can be the subject of extensive downstream clinical studies.

APPLICATION OF LIPIDOMICS IN NEPHROLOGY

There have been several recent studies using lipidomic analysis to better understand alterations in lipid metabolism in patients with kidney disease. Reis and colleagues reported

a case-control study in which 10 patients with stage 4 or 5 CKD were studied along with 10 individuals with normal kidney function and compared the lipidomic profile of low-density lipoprotein (LDL) particles between the two groups.¹¹ LDL was isolated using density-gradient ultracentrifugation and an untargeted platform was applied using LC/MS. In total, 352 lipid species were found. The content of triacylglycerol and N-acyltaurines was significantly increased, while the content of phosphatidylcholines, plasmalogen ethanolamines, sulfatides, ceramides, and cholesterol sulfate were significantly decreased in the LDL particles of CKD patients despite similarities in oxidized LDL, LDL concentration, and particle size between patients with CKD and patients with normal kidney function.¹¹ In a similar observational study, Rhee and colleagues compared the metabolic profile of >350 metabolites in the plasma of 44 dialysis patients before and after dialysis and the metabolic profile of 10 individuals with normal kidney function.⁸ The relative abundance of triacylglycerols with 40 to 48 carbons in dialysis patients was significantly lower than triacylglycerols with higher carbon numbers as compared with the control group.⁸ In a cross-sectional study, the plasma lipidomes of 214 patients with the entire spectrum of CKD, including 36 with stage 1 or 2 CKD, 99 with stage 3 CKD, 61 with stage 4 CKD, and 18 with stage 5 CKD, were compared.² The relative abundance of saturated C16-C20 free fatty acids increased by worsening stage of CKD (Figure 4A). In parallel, there was a significant decrease in the long-chain-to-intermediate-chain acylcarnitine ratio from stage 2 CKD to stage 5 CKD (Figure 4B), which suggested progressive impairment of β -oxidation during more severe stages of CKD. Similar to Rhee's observation, the abundance of triacylglycerols with low carbon numbers and low numbers of double bonds decreased from stage 2 to stage 5, while there was a progressive increase in abundance of polyunsaturated triacylglycerols with a higher number of double bonds from stage 2 to 5 (Figure 4C). This pattern was similarly observed in cholesteryl esters, diacylglycerols, phosphatidylethanolamines, plasmalogen-phosphatidylethanolamines, plasmalogen-phosphatidylcholines, phosphatidylinositols, sphingomyelins, and cardiolipins using a principle component analysis. The increase in polyunsaturated triacylglycerols with high carbon numbers was explained in part by a decline in the ratio of long-chain-to-intermediate-chain acylcarnitine. Together, these findings suggested that increased abundance of saturated free fatty acids coupled with progressive impairment of β -oxidation during progression of CKD may provide a mechanistic explanation for the increases in polyunsaturated complex lipids (specifically triacylglycerols). The lower abundance of short, complex lipids may signify their preferred utilization in catabolism, because they may be less dependent on mitochondrial shuttling in the absence of efficient β -oxidation.² In line with this study, Chen and colleagues also found a higher abundance of saturated free fatty acids in 180 patients at stage 4 or stage 5 CKD when compared with 120 healthy individuals.¹⁰

In a longitudinally ascertained outcome study of end-stage kidney disease, we frequency matched 79 patients who progressed to end-stage kidney disease (progressors) with 121 patients with CKD who did not progress to end-stage disease in a sub-cohort of Chronic Renal Insufficiency Cohort (CRIC)⁹. The sub-cohort was randomly divided into training and test sets with a ratio of 2 to 1. Overall, 510 lipid species were identified in samples from these patients. At a class level, cholesteryl esters, diacylglycerols (DAG) and phosphatidylethanolamines were enriched in progressors as compared with non-progressors

with overrepresentation of differentially lower cholesteryl esters and diacylglycerols but higher phosphatidylethanolamines in progressors.⁹ In the training set, a panel consisting of DAG32:0, DAG36:0, and monoacylglycerol (MAG)16:0 was independently associated with progression.⁹ The c-statistic of the corresponding panel was significantly higher when added to estimated glomerular filtration rate (eGFR) and urine protein-creatinine ratio (UPCR) as compared with the c-statistic of eGFR and UPCR alone, an observation which was replicated in the test set ($p < 0.05$, Figure 5).⁹ Similarly, when the panel of DAG32:0, DAG36:0, and MAG16:0 was added to eGFR and UPCR analysis, there was improvement of overall NRI and IDI (Figures 6 and 7). These observations need to be replicated in independent cohorts. All together, these studies suggest that lipidomic platforms are powerful tools with great potential for biomarker discovery and the capability to give mechanistic insights into disease biology in the context of systems biology.

FUTURE DIRECTIONS AND CONCLUSIONS

Over the past 3 decades, methods for mass spectrometry-based data acquisition have continuously improved. Such technological advances along with improved computational capabilities (both in analytic methods and hardware infrastructures) have provided exciting opportunities to couple lipidomic platforms with rational scientific questions to explore untapped territories. Integration of lipidomics with other -omics platforms, across different cell lines, in response to various stressors, and at different time points in samples with various phenotypes of interest will maximize its utility. The challenges to successful lipidomic analyses are also numerous and include, but are not limited to, the ever-expanding number of lipids identified in human lipidome, the need for development of new methods for quantification of specific classes of lipids in targeted approaches, and the optimization of analytic and bioinformatics capabilities in response to the new challenges. Additionally, there is a need for extensive clinical outcome studies to assess the clinical utility of proposed biomarkers. We conclude that lipidomics has not only the utility in biomarker discovery but also as a powerful discovery tool to reveal the systemic alterations underlying metabolic disorders in context of system biology.

References

1. Quehenberger O, Dennis EA. The human plasma lipidome. The New England journal of medicine. 2011; 365:1812–23. [PubMed: 22070478]
2. Afshinnia F, Rajendiran TM, Soni T, et al. Impaired beta-Oxidation and Altered Complex Lipid Fatty Acid Partitioning with Advancing CKD. Journal of the American Society of Nephrology : JASN. 2017
3. Kontush A, Lhomme M, Chapman MJ. Unraveling the complexities of the HDL lipidome. Journal of lipid research. 2013; 54:2950–63. [PubMed: 23543772]
4. Graessler J, Schwudke D, Schwarz PE, Herzog R, Shevchenko A, Bornstein SR. Top-down lipidomics reveals ether lipid deficiency in blood plasma of hypertensive patients. PLoS One. 2009; 4:e6261. [PubMed: 19603071]
5. Stegemann C, Drozdov I, Shalhoub J, et al. Comparative lipidomics profiling of human atherosclerotic plaques. Circulation Cardiovascular genetics. 2011; 4:232–42. [PubMed: 21511877]
6. Pietilainen KH, Sysi-Aho M, Rissanen A, et al. Acquired obesity is associated with changes in the serum lipidomic profile independent of genetic effects--a monozygotic twin study. PLoS One. 2007; 2:e218. [PubMed: 17299598]

7. Rhee EP, Cheng S, Larson MG, et al. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. *The Journal of clinical investigation*. 2011; 121:1402–11. [PubMed: 21403394]
8. Rhee EP, Souza A, Farrell L, et al. Metabolite profiling identifies markers of uremia. *Journal of the American Society of Nephrology : JASN*. 2010; 21:1041–51. [PubMed: 20378825]
9. Afshinnia F, Rajendiran TM, Karnovsky A, et al. Lipidomic Signature of Progression of Chronic Kidney Disease in the Chronic Renal Insufficiency Cohort. *Kidney Int Rep*. 2016; 1:256–68. [PubMed: 28451650]
10. Chen H, Chen L, Liu D, et al. Combined Clinical Phenotype and Lipidomic Analysis Reveals the Impact of Chronic Kidney Disease on Lipid Metabolism. *Journal of proteome research*. 2017; 16:1566–78. [PubMed: 28286957]
11. Reis A, Rudnitskaya A, Chariyavilaskul P, et al. Top-down lipidomics of low density lipoprotein reveal altered lipid profiles in advanced chronic kidney disease. *Journal of lipid research*. 2015; 56:413–22. [PubMed: 25424003]
12. Fahy E, Subramaniam S, Brown HA, et al. A comprehensive classification system for lipids. *Journal of lipid research*. 2005; 46:839–61. [PubMed: 15722563]
13. Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Graham Cooks R. The Orbitrap: a new mass spectrometer. *Journal of mass spectrometry : JMS*. 2005; 40:430–43. [PubMed: 15838939]
14. Hein EM, Blank LM, Heyland J, Baumbach JI, Schmid A, Hayen H. Glycerophospholipid profiling by high-performance liquid chromatography/mass spectrometry using exact mass measurements and multi-stage mass spectrometric fragmentation experiments in parallel. *Rapid communications in mass spectrometry : RCM*. 2009; 23:1636–46. [PubMed: 19408252]
15. Hu C, van Dommelen J, van der Heijden R, et al. RPLC-ion-trap-FTMS method for lipid profiling of plasma: method validation and application to p53 mutant mouse model. *Journal of proteome research*. 2008; 7:4982–91. [PubMed: 18841877]
16. Hsu FF, Turk J. Elucidation of the double-bond position of long-chain unsaturated fatty acids by multiple-stage linear ion-trap mass spectrometry with electrospray ionization. *Journal of the American Society for Mass Spectrometry*. 2008; 19:1673–80. [PubMed: 18692406]
17. Thomas MC, Mitchell TW, Harman DG, Deeley JM, Murphy RC, Blanksby SJ. Elucidation of double bond position in unsaturated lipids by ozone electrospray ionization mass spectrometry. *Analytical chemistry*. 2007; 79:5013–22. [PubMed: 17547368]
18. Fenn LS, Kliman M, Mahsut A, Zhao SR, McLean JA. Characterizing ion mobility-mass spectrometry conformation space for the analysis of complex biological samples. *Analytical and bioanalytical chemistry*. 2009; 394:235–44. [PubMed: 19247641]
19. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry*. 1957; 226:497–509. [PubMed: 13428781]
20. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*. 1959; 37:911–7. [PubMed: 13671378]
21. Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *Journal of lipid research*. 2008; 49:1137–46. [PubMed: 18281723]
22. Cequier-Sanchez E, Rodriguez C, Ravelo AG, Zarate R. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. *Journal of agricultural and food chemistry*. 2008; 56:4297–303. [PubMed: 18505264]
23. Houjou T, Yamatani K, Imagawa M, Shimizu T, Taguchi R. A shotgun tandem mass spectrometric analysis of phospholipids with normal-phase and/or reverse-phase liquid chromatography/ electrospray ionization mass spectrometry. *Rapid communications in mass spectrometry : RCM*. 2005; 19:654–66. [PubMed: 15700236]
24. Laaksonen R, Katajamaa M, Paiva H, et al. A systems biology strategy reveals biological pathways and plasma biomarker candidates for potentially toxic statin-induced changes in muscle. *PloS one*. 2006; 1:e97. [PubMed: 17183729]

25. Han X, Gross RW. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass spectrometry reviews*. 2005; 24:367–412. [PubMed: 15389848]
26. Schwudke D, Liebisch G, Herzog R, Schmitz G, Shevchenko A. Shotgun lipidomics by tandem mass spectrometry under data-dependent acquisition control. *Methods in enzymology*. 2007; 433:175–91. [PubMed: 17954235]
27. Carrasco-Pancorbo A, Navas-Iglesias N, Cuadros-Rodríguez L. From lipid analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part I: Modern lipid analysis. *TrAC Trends in Analytical Chemistry*. 2009; 28:263–78.
28. Haynes CA, Allegood JC, Park H, Sullards MC. Sphingolipidomics: methods for the comprehensive analysis of sphingolipids. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences*. 2009; 877:2696–708. [PubMed: 19147416]
29. Berg M, Vanaerschot M, Jankevics A, Cuypers B, Breitling R, Dujardin JC. LC-MS metabolomics from study design to data-analysis - using a versatile pathogen as a test case. *Computational and structural biotechnology journal*. 2013; 4:e201301002. [PubMed: 24688684]
30. Pere-Trepát E, Lacorte S, Tauler R. Solving liquid chromatography mass spectrometry coelution problems in the analysis of environmental samples by multivariate curve resolution. *Journal of chromatography A*. 2005; 1096:111–22. [PubMed: 16301074]
31. Nordstrom A, O'Maille G, Qin C, Siuzdak G. Nonlinear data alignment for UPLC-MS and HPLC-MS based metabolomics: quantitative analysis of endogenous and exogenous metabolites in human serum. *Analytical chemistry*. 2006; 78:3289–95. [PubMed: 16689529]
32. Bird SS, Marur VR, Sniatynski MJ, Greenberg HK, Kristal BS. Lipidomics profiling by high-resolution LC-MS and high-energy collisional dissociation fragmentation: focus on characterization of mitochondrial cardiolipins and monolysocardiolipins. *Analytical chemistry*. 2011; 83:940–9. [PubMed: 21192696]
33. Chen S, Hoene M, Li J, et al. Simultaneous extraction of metabolome and lipidome with methyl tert-butyl ether from a single small tissue sample for ultra-high performance liquid chromatography/mass spectrometry. *Journal of chromatography A*. 2013; 1298:9–16. [PubMed: 23743007]
34. Gallart-Ayala H, Courant F, Severe S, et al. Versatile lipid profiling by liquid chromatography-high resolution mass spectrometry using all ion fragmentation and polarity switching. Preliminary application for serum samples phenotyping related to canine mammary cancer. *Analytica chimica acta*. 2013; 796:75–83. [PubMed: 24016586]
35. Kaufmann A, Butcher P, Maden K, Widmer M. Ultra-performance liquid chromatography coupled to time of flight mass spectrometry (UPLC-TOF): a novel tool for multiresidue screening of veterinary drugs in urine. *Analytica chimica acta*. 2007; 586:13–21. [PubMed: 17386691]
36. McLafferty FW. Tandem mass spectrometry. *Science*. 1981; 214:280–7. [PubMed: 7280693]
37. Kind T, Tsugawa H, Cajka T, et al. Identification of small molecules using accurate mass MS/MS search. *Mass spectrometry reviews*. 2017
38. Benton HP, Ivanisevic J, Mahieu NG, et al. Autonomous metabolomics for rapid metabolite identification in global profiling. *Analytical chemistry*. 2015; 87:884–91. [PubMed: 25496351]
39. Broeckling CD, Heuberger AL, Prince JA, Ingelsson E, Prenni JE. Assigning precursor–product ion relationships in indiscriminant MS/MS data from non-targeted metabolite profiling studies. *Metabolomics*. 2013; 9:33–43.
40. Hsu FF, Turk J. Characterization of phosphatidylethanolamine as a lithiated adduct by triple quadrupole tandem mass spectrometry with electrospray ionization. *Journal of mass spectrometry : JMS*. 2000; 35:595–606. [PubMed: 10800048]
41. Kerwin JL, Tuininga AR, Ericsson LH. Identification of molecular species of glycerophospholipids and sphingomyelin using electrospray mass spectrometry. *Journal of lipid research*. 1994; 35:1102–14. [PubMed: 8077849]
42. Murphy RC, Fiedler J, Hevko J. Analysis of nonvolatile lipids by mass spectrometry. *Chemical reviews*. 2001; 101:479–526. [PubMed: 11712255]
43. Pulfer M, Murphy RC. Electrospray mass spectrometry of phospholipids. *Mass spectrometry reviews*. 2003; 22:332–64. [PubMed: 12949918]

44. Meikle P, Barlow C, Weir J. Lipidomics and Lipid Biomarker Discovery. *Australian biochemist*. 2009; 40:12–6.
45. Stein S. Mass spectral reference libraries: an ever-expanding resource for chemical identification. *Analytical chemistry*. 2012; 84:7274–82. [PubMed: 22803687]
46. Kind T, Liu KH, Lee DY, DeFelice B, Meissen JK, Fiehn O. LipidBlast in silico tandem mass spectrometry database for lipid identification. *Nature methods*. 2013; 10:755–8. [PubMed: 23817071]
47. Tsugawa H, Cajka T, Kind T, et al. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nature methods*. 2015; 12:523–6. [PubMed: 25938372]
48. Ejsing CS, Duchoslav E, Sampaio J, et al. Automated identification and quantification of glycerophospholipid molecular species by multiple precursor ion scanning. *Analytical chemistry*. 2006; 78:6202–14. [PubMed: 16944903]
49. Dunn WB, Broadhurst D, Begley P, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nature protocols*. 2011; 6:1060–83. [PubMed: 21720319]
50. Hudak AT, Crookston NL, Evans JS, Hall DE, Falkowski MJ. Nearest neighbor imputation of species-level, plot-scale forest structure attributes from LiDAR data. *Remote Sensing of Environment*. 2008; 112:2232–45.
51. Bonferroni CE. Teoria statistica delle classi e calcolo delle probabilità. *Pubblicazioni del R Istituto Superiore di Scienze Economiche e Commerciali di Firenze*. 1936; 8:3–62.
52. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B*. 1995; 57:289–300.
53. Benjamini Y, Yekutieli D. The control of the false discovery rate in multiple testing under dependency. *The Annals of Statistics*. 2001; 29:1165–88.
54. Li J, Ji L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity*. 2005; 95:221–7. [PubMed: 16077740]
55. Tibshirani R. Regression Shrinkage and Selection via the lasso. *Journal of the Royal Statistical Society Series B (methodological)*. 1996; 58:267–88.
56. Zou H, Hastie T. Regularization and Variable Selection via the Elastic Net. *Journal of the Royal Statistical Society Series B (statistical Methodology)*. 2005; 67:301–20.
57. Ming Y, Yi L. Model Selection and Estimation in Regression with Grouped Variables of the Royal Statistical Society Series B (statistical Methodology). 2006; 68:49–67.
58. Pearson K. On Lines and Planes of Closest Fit to Systems of Points in Space. *Philosophical Magazine*. 1901; 2:559–72.
59. Rokach, L., Maimon, O. Clustering Methods. In: Maimon, O., Rokach, L., editors. *Data Mining and Knowledge Discovery Handbook*. Boston, MA: Springer; 2005. p. 321–52.
60. Rousseeuw PJ. Silhouettes: a Graphical Aid to the Interpretation and Validation of Cluster Analysis. *Computational and Applied Mathematics*. 1987; 20:53–65.
61. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics*. 1988; 44:837–45. [PubMed: 3203132]
62. Pencina MJ, D'Agostino RB Sr, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. *Statistics in medicine*. 2011; 30:11–21. [PubMed: 21204120]
63. Pencina MJ, D'Agostino RB Sr, D'Agostino RB Jr, Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Statistics in medicine*. 2008; 27:157–72. discussion 207–12. [PubMed: 17569110]
64. Leening MJ, Vedder MM, Witteman JC, Pencina MJ, Steyerberg EW. Net reclassification improvement: computation, interpretation, and controversies: a literature review and clinician's guide. *Annals of internal medicine*. 2014; 160:122–31. [PubMed: 24592497]
65. Parikh CR, Thiessen-Philbrook H. Key concepts and limitations of statistical methods for evaluating biomarkers of kidney disease. *Journal of the American Society of Nephrology : JASN*. 2014; 25:1621–9. [PubMed: 24790177]

66. Kerr KF, Wang Z, Janes H, McClelland RL, Psaty BM, Pepe MS. Net reclassification indices for evaluating risk prediction instruments: a critical review. *Epidemiology*. 2014; 25:114–21. [PubMed: 24240655]
67. Pepe MS, Fan J, Feng Z, Gerds T, Hilden J. The Net Reclassification Index (NRI): a Misleading Measure of Prediction Improvement Even with Independent Test Data Sets. *Statistics in biosciences*. 2015; 7:282–95. [PubMed: 26504496]
68. Paley SM, Karp PD. The Pathway Tools cellular overview diagram and Omics Viewer. *Nucleic acids research*. 2006; 34:3771–8. [PubMed: 16893960]
69. Garcia-Alcalde F, Garcia-Lopez F, Dopazo J, Conesa A. Paintomics: a web based tool for the joint visualization of transcriptomics and metabolomics data. *Bioinformatics*. 2011; 27:137–9. [PubMed: 21098431]
70. Kuo TC, Tian TF, Tseng YJ. 3Omics: a web-based systems biology tool for analysis, integration and visualization of human transcriptomic, proteomic and metabolomic data. *BMC systems biology*. 2013; 7:64. [PubMed: 23875761]
71. Barupal DK, Haldiya PK, Wohlgemuth G, et al. MetaMapp: mapping and visualizing metabolomic data by integrating information from biochemical pathways and chemical and mass spectral similarity. *BMC bioinformatics*. 2012; 13:99. [PubMed: 22591066]
72. Laye MJ, Tran V, Jones DP, Kapahi P, Promislow DE. The effects of age and dietary restriction on the tissue-specific metabolome of *Drosophila*. *Aging cell*. 2015; 14:797–808. [PubMed: 26085309]
73. Krumsiek J, Suhre K, Illig T, Adamski J, Theis FJ. Gaussian graphical modeling reconstructs pathway reactions from high-throughput metabolomics data. *BMC systems biology*. 2011; 5:21. [PubMed: 21281499]
74. Basu S, Duren W, Evans CR, Burant CF, Michailidis G, Karnovsky A. Sparse network modeling and metscape-based visualization methods for the analysis of large-scale metabolomics data. *Bioinformatics*. 2017; 33:1545–53. [PubMed: 28137712]

Figure 1.
Summary of typical workflow when using lipidomic platforms for biomarker discovery. LC-MS, liquid chromatography–mass spectrometry; GC-MS, gas chromatography–mass spectrometry.

Figure 2.

Application of “reference pools” (pooled plasma) and “test pools” for quality control. Panel A. A hypothetical batch alignment for a study including 80 samples distributed into 2 batches, with a run of 10 reference pools (pooled plasma), followed by a run of “test pools” prior to actual samples and then after each 10 samples. Start of the run is from left to right. Panel B. Low batch-to-batch variability of internal standards in the test pools. Panel C. Low batch-to-batch variability of internal standards in the reference pools. From the Lipidomics in CPROBE study (with permission from the Journal of the American Society of Nephrology).

Figure 3.

Application of the debiased sparse partial correlation (DSPC) method to visualize the relationships between various species of triacylglycerols. The method reveals 2 distinct subgroups of triacylglycerols with lower carbon numbers and lower numbers of double bonds (blue) and the subgroup with higher carbon numbers and higher numbers of double bonds (red). Edges represent significant correlations adjusted using the Benjamini-Hochberg procedure ($p < 0.001$). This observation suggests that triacylglycerols may be differentially regulated in CKD. Data is from the CPROBE cohort lipidomic study.²

Figure 4. Reciprocal alterations in the abundance of free fatty acids (FFAs) and triacylglycerols (TAGs) by stage of CKD. A. Increased abundance of saturated C16–C20 FFA from CKD stage 2 to 5. B. Decreased long-chain-to-intermediate-chain acylcarnitine ratio from stage 2 CKD to stage 5 CKD, indicative of impaired β -oxidation with advancing CKD stage. C. Increased abundance of polyunsaturated triacylglycerols with higher carbon numbers, but decreased abundance of triacylglycerols with lower carbon numbers and a lower number of double bonds from stage 2 to stage 5 CKD (with permission from the Journal of the American Society of Nephrology).

Figure 5.

Receiver operating characteristic (ROC) curves as a graphical tool to demonstrate the classification power of biomarkers. A panel consisting of DAG32:0, DAG36:0, and MAG16:0 was used to discriminate between patients who progressed from stage 3 CKD to end-stage kidney disease and patients with stage 3 CKD who did not progress. The panel showed an improved c-statistic when added to baseline eGFR and urine-protein creatinine ratio, a model which was developed in the training set ($p<0.05$) and validated in the test set ($p<0.05$).⁹

Figure 6.

Graphical demonstration of the net reclassification improvement (NRI). Overall, the number of correctly classified progressors to end-stage kidney disease and non-progressors using a panel of biomarkers consisting of DAG32:0, DAG36:0 and MAG16:0 and eGFR and urine protein-creatinine ratio (UPCR) in patients with CKD stage 3 from the Chronic Renal Insufficiency Cohort was higher than what was observed with classification using eGFR and UPCR or the panel alone (overall NRI: 1.441, $p < 0.001$).⁹

Figure 7.

Graphical demonstration of integrated discrimination improvement (IDI). The IDI progression is defined as the difference between the area under the curve of sensitivity versus predicted risk obtained with a new marker from that obtained with an old marker. Similarly, IDI non-progression is defined as the difference between the area under the curve of '1-specificity' versus predicted risk obtained from a new marker from that of the old marker. The overall IDI by a panel of biomarkers consisting of DAG32:0, DAG36:0 and MAG16:0, which was examined with eGFR and urine protein-creatinine ratio (UPCR), improved classification of progressors to end-stage kidney disease vs. non-progressors ($p < 0.001$).⁹

Table 1

Overview of characteristics of untargeted and targeted lipidomics.

	Untargeted lipidomics	Targeted Lipidomics
Application	- Hypothesis generating - Discovery phase	-Hypothesis driven -Validation phase
Goal	- To identify as many lipids as possible	- To quantify predetermined, limited number of lipids
Output, data structure	-Relative abundance	-Absolute concentration
Internal standards	- 1 to 2 per lipid class	- Ideally, 1 per candidate lipid
MS features	- High-accuracy MS - Full scan MS/MS analysis	- High-selectivity MS - MRM mode
Advantages	- High throughput data generation - Provides the big picture	- High sensitivity and specificity, - More precise
Limitations	- Susceptible to systematic errors - Higher likelihood of false positives - Less precise - Absolute quantification not	- Unavailability of authentic and internal standards for every lipid

Table 2

Proposed internal standards per major lipid class

Class	Internal Standard	MW
MAG	1-heptadecanoyl-rac-glycerol 17:0	344.293
DAG	1-palmitoyl-2-oleoyl-sn-glycerol 16:0–18:1	594.522
TAG	1,2,3-triheptadecanoyl-glycerol Triheptadecanoate 17:0/17:0/17:0	848.783
gDAG	1,3(d5)-dinonadecanoyl-2-hydroxy-glycerol d5-(19:0/0:0/19:0)	657.632
gTAG	Glyceryl tri(palmitate-d31) d31	899.040
CE	cholest-5-en-3 β -yl heptadecanoate 17:0 cholesteryl ester	632.800
PC	1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (17:0/17:0)	761.593
LPC	1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (17:0/0:0)	509.348
PE	1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (17:0/17:0)	719.547
Ceramide	N-heptadecanoyl-D-erythro-sphingosine C17 Ceramide (d18:1/17:0)	551.528
SM	N-heptadecanoyl-D-erythro- sphingosylphosphorylcholine 17:0 (d18:1/17:0)	716.583
PA	1,2-diheptadecanoyl-sn-glycero-3-phosphate (sodium salt) 17:0	698.486
PG	1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) 17:0	772.523
PS	1,2-diheptadecanoyl-sn-glycero-3-phospho-L-serine (sodium salt) (17:0/17:0)	785.518
PI	1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1'-myo-inositol) 17:0–20:4	987.142

The internal standard stock solutions are stored in chloroform/methanol/water at a concentration of 1 mg/mL at –20°C, and further diluted to 100 pmol/uL prior to spiking. CE, cholesteryl ester; DAG, diacylglycerol; gDAG, glycosyldiacylglycerol; gTAG, glycosylmonoacylglycerol; LPC, lysophosphatidylcholine; MAG, monoacylglycerol; MW, molecular weight (g/mole); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol.