In-Depth Topic Review

American Journal of Nephrology

Am J Nephrol 2016;44:92–103
DOI: 10.1159/000447954

Published online: July 14, 2016

Metabolomics in Diabetic Kidney Disease: Unraveling the Biochemistry of a Silent Killer

Manjula Darshi[a–c] Benjamin Van Espen[a, b] Kumar Sharma[a–c]

[a]Institute of Metabolomic Medicine and [b]Center for Renal Translational Medicine, University of California San Diego, and [c]Division of Nephrology-Hypertension, Veterans Affairs San Diego Healthcare System, La Jolla, Calif., USA

Key Words
Metabolomics · Diabetic kidney disease · Chronic kidney disease · GC-MS · Mitochondria

Abstract
The development of new therapies for chronic diseases, such as diabetic kidney disease (DKD), will continue to be hampered by lack of sufficient biomarkers that will provide insights and will be responsive to treatment interventions. The recent application of metabolomic technologies, such as nuclear magnetic resonance and mass spectroscopy, has allowed large-scale analysis of small molecules to be interrogated in a targeted or untargeted manner. Recent advances from both human and animal studies that have arisen from metabolomic analysis have recognized that mitochondrial function and fatty acid oxidation play key roles in the development and progression of DKD. Although many challenges in the technology for clinical chronic kidney disease (CKD) are yet to be validated, there will very likely be ongoing major contributions of metabolomics to develop new biochemical understanding for diabetic and CKD. The clinical application of metabolomics and accompanying bioinformatic tools will likely be a cornerstone of personalized medicine triumphs for CKD.

Introduction

Chronic kidney disease (CKD), a condition characterized by the gradual decline in kidney function over time, has become a major global health concern [1]. According to the National Kidney Disease Fact Sheet, in 2014, more than 10% of the United States adult population have varying stages of CKD with high risks for end-stage renal disease (ESRD), cardiovascular diseases, and death [2]. In 2013, more than 45,000 Americans died of kidney disease and it is important to emphasize that this number is higher than those who have died of breast and prostate cancers. Currently, approximately 500,000 Americans are on dialysis with more than 100,000 among them waiting for a kidney transplant.

Diabetes and hypertension are the 2 major causes of CKD [2, 3]. Approximately 1 in 3 of diabetic and 1 in 5 of hypertensive patients develop CKD progressing to ESRD [2]. In 1995, 4.0% of global population has diabetes and it is estimated that this will rise to 5.4% by the year 2025 [4]. It is estimated that more than 29 million US adults have diabetes and 70 million have high blood pressure [5].

Despite the existing increase in the number of patients with diabetes and diabetic kidney disease (DKD), limited therapeutic options are currently available to slow the progression of CKD. This is partly due to the lack of suit-

KARGER

0250–8095/16/0442–0092$39.50/0

E-Mail karger@karger.com
www.karger.com/ajn

Kumar Sharma
Center for Renal Translational Medicine, University of California San Diego/
Veterans Affairs San Diego Healthcare System, Stein Clinical Research Building
4th Floor, 9500 Gilman Drive, La Jolla, CA 92093-0711 (USA)
E-Mail kumarsharma@ucsd.edu

able approaches for early detection of the DKD for clinical intervention. Estimated glomerular filtration rate (eGFR) based on serum creatinine concentration, age, and gender, and albuminuria are the current gold standard biomarkers to evaluate kidney function [4, 6, 7]. Unfortunately, these biomarkers lack sensitivity and specificity and DKD is often undetected until substantial kidney injury has occurred. Increase in urinary albumin excretion is typically accepted to characterize DKD with microalbuminuria defined as 20–200 μg/min and macroalbuminuria defined as >200 μg/min. However, several studies have shown that even with normal urinary albumin excretion, the eGFR may be severely reduced [8, 9]. On the other hand, serum creatinine, which is used to calculate eGFR, does not significantly increase until the GFR is reduced to 50% of normal levels, thus underestimating ongoing decrease in true GFR. Because of these discrepancies, there is a clear need for improved biomarkers that can predict early stage kidney injury and detect progression of CKD.

Metabolomics or metabonomics is a systems approach for profiling in vivo metabolic status and it offers a promising approach to identify biomarkers in disease [10–13]. Metabolomics identifies and quantifies metabolites that are small molecular mass (<1,500 Da) end products of biochemical processes. Metabolites relay signals from genes to proteins to the environment, hence they are functional readouts of the physiological status of an organism and more closely reflect the phenotype.

Metabolomics has been classified as targeted or untargeted. A targeted approach involves identification of a defined set of metabolites typically focusing on a pathway of interest or metabolites that are previously known to be associated with the disease under observation. This is usually hypothesis driven but may also serve for discovery if multiple classes of molecules or biochemical pathways are surveyed. An untargeted approach provides a comprehensive analysis of all measurable metabolites in a sample without any bias and is typically used only for discovery. There has been some overlap of these terms that has led to confusion in the field. At present, the term untargeted may also refer to survey of molecules that are well characterized by their retention time and mass/charge ratios, but for the sake of throughput and cost, the metabolites are not typically quantified [14]. A statistical likelihood of a particular peak will have to be verified with subsequent targeted analysis often involving inclusion of the metabolite standard, calibration curves and multiple reaction monitoring. For the purpose of this review, an untargeted analysis will be reserved for a shot-gun screening discovery approach where the peaks are truly unknown and a targeted analysis will assume that the peaks of interest are identifiable and quantifiable.

Traditional metabolomic analysis assesses steady-state metabolite levels or levels at a single snap-shot in a targeted or untargeted manner. Even though this analysis detects changes in metabolites that are robust, to gain more knowledge about the mechanisms involved behind such changes, it is necessary to understand the dynamic flux of metabolites through a predefined pathway. Toward this, metabolic flux analysis is necessary [15–17]. Typically, heavy isotope-labeled substrates are added and their incorporation into a pathway of interest along with quantification of enzymes in the metabolic pathway is analyzed at different time points [17]. For example, incorporation of U-13C glucose or 13C-pyruvate is a common method to study perturbation in glycolysis and TCA cycle [17]. As flux analysis methodology has not yet been well developed in animal and human systems, the present review will primarily focus on data generated from cross-sectional or longitudinal metabolomics studies with measurements performed at a single time point.

Metabolomic Technologies

1H or 13C nuclear magnetic resonance (NMR) and mass spectroscopy coupled with gas or liquid chromatography are the 2 common approaches in metabolomics, used to characterize samples, such as urine, blood plasma/serum, tissue, cell extracts or media [10, 18, 19]. The data are analyzed by univariate and multivariate statistical models to identify statistically significant biomarkers associated with the disease. Advances in NMR and mass spectroscopy now offer highly improved specificity and sensitivity and this has enhanced the utility of targeted metabolite analysis [19]. Specific benefits and disadvantages associated with each of these techniques are outlined in table 1.

NMR Spectroscopy

NMR spectroscopy is a highly reproducible and quantitative technique with minimum sample preparation. As sample preparation does not require any separation or derivatization, this is ideal for biofluids such as urine, plasma/serum and cell culture media, with minimal alterations to the sample during preparation [20]. Even though one-dimensional 1H NMR spectroscopy is commonly used for metabolic profiling, 2D NMR spectroscopy has been employed for structural assignment albeit with limited profiling applications. A major limitation

Table 1. A comparison of GC-MS, LC-MS and NMR technologies in metabolomics

Technique	Advantages	Disadvantages
GC-MS	High sensitivity Large linear range Abundant libraries Relatively less expensive instrumentation Suitable for less polar metabolites Moderate sample volumes (100–200 μl) Minimal matrix effects compared with LC-MS High chromatographic resolution compared with LC-MS Compatible with gases and liquids	Long sample preparation time Derivatization required Limited to low molecular mass and volatile analytes Molecular ion is often lost during EI, making it difficult to identify the metabolite when the sprectrum is not in the library
LC-MS	High sensitivity Minimal sample processing, typically deproteinization Suitable for polar, non-volatile compounds that cannot be analyzed by GC-MS Small sample volumes (10–100 μl) Produces molecular ion used for identification of the metabolite, hence more suitable for discovery based approaches Compatible with solids and liquids	High matrix effects Suffers from ion suppresion Less chromatogrphic separation as compared with GC-MS No sprectral libraries High instrument cost
NMR	Minimal or no sample processing No ionization involved, non-destructive Requires no chromatographic separation Rapid data acquisition (2–3 min per sample)	Less sensitive compared with MS based approaches Large sample volumes (100–500 μl) High instrument cost

with NMR-based analysis is that its sensitivity is limited to micro-molar concentration; therefore, low-abundant metabolites are difficult to detect [21].

Mass Spectroscopy

Compared to NMR, mass spectroscopy coupled with gas (GC-MS) or liquid chromatography (LC-MS) offers higher sensitivity along with the ability to detect a broad range of metabolites. The 2 platforms offer different sensitivities toward different classes of metabolites. In GC-MS, a carrier gas is used to separate samples through the column after which samples are ionized either by electron or by chemical ionization for detection by the mass spectrometer. While GC-MS is a highly sensitive and specific method, the separation occurs at high temperatures necessitating metabolites to be volatile and thermally stable. For samples to be readily volatile, chemical derivatization is often required. Sample preparation is one of the major drawbacks of the GC-MS because of the prolonged time involved in derivatization, often requiring 2 days. In addition, there are often slight unaccounted variations in sample preparation due to the multiple steps and incomplete derivatization or adduct formation. These steps may affect the reproducibility of quantified measurements. GC-MS also has limitations as it is useful for relatively small molecular mass (molecular ion mass-to-charge ratio, m/z, <800) and is not suitable for less or medium polar compounds. LC-MS has distinct advantages in that sample preparation is minimal and can be used to analyze different classes of metabolites that cannot be separated/fragmented by GC-MS because of their high molecular mass or polarity. For proteinaceous samples such as blood/plasma or tissue, samples are deproteinated using solid phase extraction or solvent precipitation. Urine from healthy individuals contains minimum protein, so often samples are simply centrifuged and diluted with water prior to LC-MS analysis [22, 23]. However, matrix effects are higher in LC-MS than in GC-MS causing ion suppression and interference with spectral resolution. Additionally, the typical instrument cost of an LC-MS is substantially higher than that of a GC-MS.

Data Handling and Statistical Analysis

Similar to other 'omics', metabolomics generate a large amount of data. To answer relevant biological questions, appropriate data handling is necessary. Typically, this includes preprocessing and statistical analyses. Preprocessing includes normalization of data across sample

sets, dealing with missing values, rescaling the data by applying logarithmic transformation to generate symmetrical data. Following preprocessing, statistical analysis is done to test various assumptions either by univariate (one variable analysed at a time) or multivariate (2 or more variables analysed) methods [24]. The t test, analysis of variance, analysis of covariance, and univariate linear regression ($Y = a + bX$) are the most common univariate test methods. The commonly used multivariate methods include multivariate analysis of variance, multivariate linear regression ($Y = a + bX + cX + \ldots$), cluster analysis, partial least squares discriminant analysis and principal component analysis (PCA). The probability of identifying at least one significant result due to chance increases as more hypotheses are tested. However, multiple testing methods often require adjustment for false positives and require more stringent criteria to determine significance than the conventional $p < 0.05$ [25]. The Bonferroni, which corrects for family-wise error rate (FWER) and the Benjamini–Hochberg, which corrects for false discovery rate (FDR) are the 2 most popular methods used to minimize errors in multiple testing approaches. In the Bonferroni method, an adjustment is made to p values by dividing the significance FWER (mostly 0.05) with the number of tests being performed simultaneously on a single data set. The statistical significance of the study is then calculated based on the modified p value. However, with a large number of multiple comparisons, the Bonferoni correction could lead to a high rate of false negatives. In large-scale multiple testings, correcting for FDR, the proportion of false positives among the significant results, is a more suitable method. The Benjamini–Hochberg FDR correction provides less stringent control of type I errors and thus has greater power at the cost of increased rates of type I errors [26]. This method calculates the expected p values under the null hypotheses and compares them against obtained p values to select the hypothesis whose actual p values are lower than expected. The selection criteria for this step are determined in order to control FDR. The calculation of expected p values is based on the assumption that the tested hypotheses are all independent of each other, and therefore this method is suitable for the statistical settings where one can assume that each test is independent of each other.

Metabolomics of Clinical DKD

Several recent studies have sought to identify metabolites that are differentially expressed in DKD using a variety of metabolomic approaches and biosamples. An overall conclusion from recent studies is that robust changes in metabolites of the TCA cycle, lipid metabolism, amino acid metabolism, urea cycle and nucleotide metabolism are associated with DKD (table 2) [8, 27–36].

Several cross-sectional studies have been performed in both DKD and CKD without diabetes [27, 30, 31, 37]. Hirayama et al. [27] used capillary electrophoresis coupled with time-of-flight mass spectrometry to analyze serum metabolites in 78 subjects with different stages of DKD. Nineteen metabolites, out of which 11 were unknown, with no previously assigned chemical structures, were significantly different between DKD with macroalbuminuria and diabetic patients without albuminuria (correlated with albuminurea and eGFR). The 8 assigned metabolites included 4 amino acids, aspartic acid, citruline, symmetric dimethylargine (SDMA) and kynurenine, all of which were increased in DKD. Aspartic acid and citrulline are involved in the production of urea in the urea cycle. SDMA and asymmetric dimethylarginine (ADMA) are formed by the enzymatic methylation of arginine. While kidneys further metabolize ADMA, SDMA is excreted directly into the urine. Tryptophan is metabolized to kynurenine and further metabolized to acetyl-CoA and NAD in the tryptophan-kynurenine pathway. Several of these metabolites have also been identified in CKD without diabetes, suggesting that these could be markers for renal dysfunction [31, 37]. Benito et al. [31] also reported the accumulation of several of these metabolites in plasma samples of pediatric-CKD patients (table 2).

Ng et al. [28] analyzed urinary metabolites in Chinese type 2 diabetes (T2D) patients with low eGFR but no proteinuria. By using GC-MS, the authors identified 11 metabolites with strong association with low eGFR (table 2). By using LASSO logistic regression models, octanol, oxalic acid, phosphoric acid, benzamide, creatinine, 3,5-dimethoxymandelic amide and N-acetylglutamine were selected as the best predictors of eGFR decline. Further, using LC-MS measurements, the study identified 19 metabolites including indoxyl sulfate, a well-known uremic toxin, with significant correlations with low eGFR (table 2).

Amino acids and acyl carnitines have been identified as predictors of progression of DKD in several studies. Elevated concentrations of branched chain and aromatic amino acids were identified as predictors of diabetes 12 years prior to the onset of kidney disease in a longitudinal LC-MS-based plasma metabolomic analysis [38]. Van der kloet et al. [29] identified acyl carnitines, acyl-glycines and intermediates of tryptophan metabolism (table 2) to be correlated with changes in albuminuria and associated

Am J Nephrol 2016;44:92–103
DOI: 10.1159/000447954

Table 2. Summary of metabolites affected in DKD identified by clinical metabolomic analysis

Sample type and ref.	Analysis platform	Study population	Significantly different metabolites	Pathways affected
Serum, Hirayama et al. [27]	CE/TOF-MS	Non DN (n = 20); micro DN (n = 32); macro DN (n = 26)	Creatinine (↑), aspartic acid (↑), γ-butyrobetaine (↑), citruline (↑), symmetric dimethyl arginine (↑), kynurenine (↑), azelaic acid (↓) and galactaric acid (↓)	Urea cycle, and tryptophan-kynurenine pathway
Urine, Ng et al. [28]	GC-MS and LC-MS	T2D patients with non proteinurea and low eGFR (n = 44); healthy controls (n = 44)	GC-MS: oxalic acid (↓), octanol (↑), 3,5-dimethoxymandelic amide (↓), N-acetylglutamine (↑), creatinine (↑), benzamide (↓), phosphoric acid (↓), 2-hydroxyadipic acid (↑), ribonic acid (↑), hydroxy phenylacetic acid (↑), sarcosine (↑) and salicyluric acid (↑) LC-MS: 4-methoxyphenylacetic acid (↑), N-6-acetyl-L-lysine (↑), chondroitin sulphate (↑), citric acid (↑), phenylacetyl-L-glutamine (↑), 2-deoxyuridine (↑), deoxypyridinoline (↑), dehydrotestosterone glucuronide/retinyl-β-glucuronide (↑), N-acetylspermine (↑), creatinine (↑), sphingosine (↑), 3,7-dimethyluric acid (↑), 10-nitrolinoleic acid (↑), 2,6-dimethylheptanoyl carnitine nonanoyl carnitine (↑), hyocholic acid/cholic acid/ursocholic acid (↑), phosphoribosyl-formylglycine amidine (↑), IDP (↑), androsterone glucuronide/etiocholanolone glucuronide (↑), and indoxyl sulphate (↑)	
Urine, van der Kloet et al. [29]	GC-MS and LC-MS	T1D progressed to micro albuminurea (n = 26); T1D non-progressors/normal albuminurea (n = 26)	GC-MS: 4-oxoproline (↓), pseudouridine (↑), 3,4,5-trihydroxypentanoic acid (↓), deoxyfructose (↑), 3-hydroxy-3-(3-hydroxyphenyl) propanoic acid (↓), L-valine (↑), 2,3-dihydroxy-3-methylbutanoate (↓), 5-hydroxymethyl-2-furancarboxylic acid (↓), galactonic acid (↓), 2-hydroxyvaleric acid (↑), N-formylproline or N-ethylproline (↓), 2-hydroxyglutaric acid (↑), N-(3-hydroxybenzoyl) glycine (↑), arabinose (↓), benzoic acid (↓), glucuronide compound (↓), D-glutamic acid (↑), gluconic acid (↓), glycolic acid (↑), and L-cystine (↓) LC-MS: tryptophan (↑), salicyluric acid (↑), substituted carnitine (↑), S-(3-oxododecanoyl) cysteamine (↑), hippuric acid (↓), N-methyl guanosine (↑), kynurenic acid (↑), 2-(2-phenylacetoxy) propionylglycine (↑), indoleacetic acid (↓), 3-methylcrotonylglycine (↑), and heptanoyl carnitine (↑)	Fatty acid oxidation and tryptophan metabolism
Plasma, Han et al. [32]	GC-MS	T2D with DN (n = 90), T2D (n = 30), healthy controls (n = 30)	Arachidonic acid (↑) and non-esterified fatty acids (↑)	Inflammation
Plasma and urine, Pena et al. [39]	LC-MS	T2D (n = 90); hypertension (n = 150)	Plasma: butenoyl carnitine (↑) and histidine (↓); urine: hexose (↓), glutamine (↓) and tyrosine (↓)	Fatty acid oxidation and amino acid metabolism
Plasma, Benito et al. [31]	LC-QTOF-MS	Pediatric CKD (n = 32); pediatric (n = 24)	Glycine (↑), dimethylglycine (↑), citrulline (↑), creatinine (↑) and asymmetric (↑) and symmetric dimethylarginine (↑)	Amino acid and arginine-creatinine metabolism

Table 2. (continued)

Sample type and ref.	Analysis platform	Study population	Significantly different metabolites	Pathways affected
Plasma, Niewczas et al. [33]	GC-MS	T2D progressed to ESRD (n = 40); T2D non progressors (n = 40)	p-cresol sulfate (↑), phenylacetylglutamine (↑), myo-inositol (↑), pseudouridine (↑), indoxyl sulfate (↑), hippurate (↑), C-glycosyltryprophan (↑), acyl carnitines (↑), 2 oxo isoleucine (↓), and 2 oxo isocaproate (↓)	Gut microbiome, fatty acid and amino acid metabolism
Urine, Posada-Ayala et al. [35]	NMR (discovery) and LC-MS (validation)	CKD and healthy controls (n = 15 CKD and 15 healthy controls in discovery and 15 CKD and 15 healthy controls in validation, 2 DN in each phase)	Glutamate (↑), guanidoacetate (↑), α-phenylacetylglutamine (↑), trimethylamine N-oxide (↑), 5-oxoproline (↓), taurine (↓) and citric acid (↓)	
Urine and plasma, Sharma et al. [36]	GC-MS	Diabetic with CKD (n = 61); diabetes without CKD (n = 73); FSGS (n = 12); healthy controls (n = 24)	3-hydroxy isovalerate (↓), aconitic acid (↓), citric acid (↓), 2-ethyl 3-OH propionate (↓), glycolic acid (↓), homovanillic acid (↓), 3-hydroxy isobutyrate (↓), 2-methyl acetoacetate (↓), 3-methyl adipic acid (↓), 3-methyl crotonyl glycine (↓), 3-hydroxy propionate (↓), tiglylglycine (↓) and uracil (↓)	Organic anion transport, amino acid metabolism and TCA cycle

Metabolite changes in cases with respect to controls are shown as (↑) for increase or (↓) for decrease.

with the progression of DKD in urine samples of T1D patients. Plasma fatty acid concentrations, both esterified and non-esterified fatty acids, were also shown to be associated with disease progression of DKD in T2D patients [32]. Plasma metabolites histidine, butenoylcarnitines, and urine metabolites hexose, glutamine and tyrosine were identified as predictors for progression to micro- and macro-albuminurea in T2D and hypertensive patients [39]. In a recent study, Kang et al. [40] have showed that defective fatty acid oxidation in tubular cells plays a major role in kidney fibrosis in both humans and animal models. The study showed that tubular epethelial cells preferentially use fatty acid oxidation for their fuel requirement, and the fatty acid oxidation is considerably compromised in animal and cell culture models of fibrosis. Several enzymes related to the fatty acid oxidation including carnitine palmitoyl transferase 1 (CPT1) and CPT2, the fatty acid transporters, are reduced in the human and mouse fibrotic kidneys, suggesting impaired fatty acid uptake resulting in the accumulation of fatty acids [40].

Accumulation of uremic toxins in plasma is one of the key features identifying the decline of kidney function in CKD and progression to ESRD. Uremic toxins are the retention molecules normally excreted by kidneys in healthy individuals [41]. Based on their physical properties they are further classified as low-molecular mass solutes (<0.5 kD), such as urea, oxalate, uric acid and guanidines, protein-bound solutes, the most common bein indoxyl sulfate and p-cresol sulfate, and middle molecules such as beta2-microglobulin (0.5–60 kD). Niewczas et al. [33] identified increased concentration of uremic solutes in plasma by using global mass spectrometry analysis in T2D patients with normal renal function followed at the Joslin Clinic, half of which progressed to ESRD in 8–10 years of follow-up. Elevated concentrations of uremic solutes and amino acid-derived acyl carnitines, and low concentrations of essential amino acids were identified in the progressors in baseline plasma metabolites (table 2). Current evidence suggests that uremic solutes including ρ-cresol and indoxyl sulfate are produced by the gut microbiome [42]. Even though it is unclear if accumulation of uremic toxins precedes or follows renal damage, it may be associated with disturbances in the gut microbiome in patients with diabetes and CKD [43].

A recent study used both LC-MS and NMR in urine samples of patients with advanced CKD [35]. This study applied NMR-based metabolomics in the discovery phase in 15 patients with CKD stages 3–5 (2 with DKD), compared with 15 healthy control subjects, and identified 29 differentially expressed metabolites. Validation of the 29

metabolites in an independent cohort of 16 CKD patients (2 with DKD) and 15 healthy controls using LC-MS and NMR identified 5-oxoproline, glutamate, guanidoacetate, α-phenylacetylglutamine, taurine, citrate, and trimethylamine N-oxide (TMNO) as a urinary metabolomic signature of advanced CKD. Among the 7 metabolites, glutamate, guanidoacetate, α-phenylacetylglutamine and TMNO were increased and 5-oxoproline, taurine and citrate were reduced in patients with CKD (table 2) [35].

Metabolomics Coupled with Bioinformatic and Systems Biology Tools Reveals Mitochondrial Dysfunction as Characteristic of DKD

Metabolomic analysis coupled with bioinformatics can provide major insights into complex diseases such as DKD. In recent studies, we have employed targeted metabolomics together with systems biology tools to establish that human DKD is associated with mitochondrial dysfunction [36]. Patients with established DKD and reduced eGFR have a characteristic panel marked by changes in organic anions, TCA cycle and amino acid metabolites [36]. We identified 13 metabolites as the urinary metabolomic signature of DKD (table 2) [36]. In a targeted approach, we quantified, by GC-MS, 94 organic acid metabolites that had been previously associated with a variety of inborn errors of metabolism, in timed human urine samples of diabetic patients with and without CKD, nondiabetic CKD and healthy controls [36]. Our screening cohort included 24 patients from the San Diego region with a diagnosis of T2D and CKD stages 3–4 (mean eGFR 35.5 ± 10.9 ml/min/1.73 m^2). The validation cohort comprised 61 subjects with diabetes and CKD, and 73 subjects with diabetes without CKD (both T1D and T2D) from Philadelphia, Minnesota and Washington, D.C. as well as from Finland. An additional age- and gender-matched control group included 73 subjects with diabetes without CKD (both T1D and T2D). In the screening cohort, we identified 17 metabolites to be significantly different compared with healthy controls, after multiple testing and corrections for FDR. We observed a high degree of consistency of the urinary metabolite pattern in the validation cohort as 13 of the 17 metabolites remained statistically and significantly different (table 2). All the 13 urine metabolites were significantly lower in DKD compared with healthy controls.

To identify potential contribution of diabetes to the changes in metabolites, we compared the 13 metabolites in diabetic patients with CKD to patients with diabetes and without CKD. After adjusting for potential confounders, including age, gender, body mass index, mean arterial pressure, hemoglobin A1C, and duration of diabetes, 12 of the 13 metabolites were statistically significant, even after correction for multiple testing. Furthermore, to determine the specificity of the signature of diabetic CKD, we analyzed urinary metabolites in an independent cohort with FSGS, another type of CKD. Comparison of metabolomic signature of patients with FSGS with that of diabetic CKD identified 5 out of 13 metabolites to be significantly different between the 2 groups. These 5 metabolites specific for DKD included 2-methyl acetoacetate, 3-methyl adipic acid, 3-methyl crotonyl glycine, 3-hydroxy propionate and tiglylglycine. Eight metabolites overlapped with FSGS and could potentially serve as signature of general CKD. The overlapping metabolites included TCA cycle metabolites (citric acid, aconitic acid), and short chain fatty acids (SCFAs) (2-ethyl 3 hydroxy propionate, 3-hydroxy isobutyrate). Five out of the 13 metabolites (3-hydroxy isovalerate, aconitic acid, glycolic acid, uracil and citric acid) showed significant correlations with eGFR and an independent set of 3 metabolites from the 13 correlated with albuminuria (2 methyl acetoacetate, 3-methyl crotonyl glycine, and 3-methyl adipic acid).

Since several metabolites identified in our analysis were organic anions, we hypothesized that organic anion transporters (OAT), which were involved in the elimination of these organic anions via the kidney, might be affected in DKD [44]. This hypothesis was supported, as there was a greater than twofold reduction in the gene expression levels of OAT1 and OAT3 in kidney biopsy samples from patients with diabetic nephropathy compared with that of non-diseased kidney tissue. Furthermore, studies with OAT1 knockout mice revealed a similar reduction in the urine of many of the same organic acids that were reduced in the patients with DKD [45].

Pathway analysis of the metabolites from the signature of DKD revealed the TCA cycle (citrate, aconitate), pyrimidine (uracil), amino acid (3-hydroxy isovaleric acid, 3-methyl crotonyl glycine, tiglylglycine), fatty acid (2-ethyl 3-hydroxy propionate, 3-hydroxy propionate, 3-hydroxy isobutyrate, 3-methyl adipic acid), and oxalate metabolism (glycolic acid) to be significantly affected pathways. We also observed high prevalence of SCFAs in the metabolomic signature of DKD. In humans, SCFAs, acetate, propionate, and butyrate are produced from gut microbiome through the fermentation of fibrous food [46–48]. As there was a reduction in all 3 SCFAs, we speculate that DKD is associated with alterations in gut microbiome leading to both a reduction in beneficial metabolites (SCFAs), and accumulation of toxic metabolites (uremic toxins) as observed in several previous studies [33].

We also constructed an approach to overlay metabolites with their associated enzymes using the Cytoscape software. Protein–protein interactions were also incorporated into the analysis to reveal novel underlying networks. Interestingly, 12 of the 13 metabolites were connected within one large network. The majority of the 13 metabolites or the enzymes producing metabolites were localized or transported into mitochondria, thus implicating mitochondrial dysfunction as a major feature associated with DKD. Further analysis of kidney biopsy samples and urinary exosomes from patients with diabetic nephropathy revealed the reduction in mitochondrial DNA and proteins in addition to the reduction in PGC1α, the master regulator of mitochondrial biogenesis [49, 50]. Together, these studies implicate reduced mitochondrial biogenesis and function as a likely dominant characteristic of DKD and progressive CKD.

Animal Studies of Metabolomics in Kidney Disease

In addition to the above-mentioned clinical studies, researchers have applied transgenic animal models to identify mechanisms and signature metabolite changes associated with different stages of renal dysfunction. Developing a common biomarker suitable for animal and clinical studies is extremely important, as it would help researchers to facilitate basic research to understand mechanistic aspects and for preclinical study design. Metabolomic studies in animal models have provided evidence that alterations in TCA cycle, fatty acid oxidation and amino acid metabolism are the major pathways affected in DKD similar to that of clinical studies (table 3) [51–54].

Studies from our laboratory have identified that the TCA cycle metabolite fumarate plays a key role in mediating the effects of NADPH oxidase isoform 4 (NOX4) in DKD [51]. This NOX isoform has previously been reported as elevated in DKD [55] and our study showed that podocyte-specific induction of NOX4 can induce characteristic changes observed in DKD (glomerular hypertrophy, mesangial matrix accumulation, glomerular basement membrane thickening, albuminuria, and podocyte dropout) in transgenic F1 Akita mice. To investigate the potential role and mechanism of NOX4 in DKD, we administered the F1 Akita mice with a NOX1/NOX4-specific inhibitor (GKT137831) for 4 months and analyzed changes in urinary metabolites. Urinary metabolomic analysis in diabetic F1-Akita mice identified an increase in several TCA cycle metabolites compared with F1 control mice. Interestingly, the NOX4 inhibitor treatment resulted in significant reduction of one of the TCA cycle intermediates, fumarate, with no significant changes in succinate, the upstream metabolite which is oxidized by succinate dehydrogenase (SDH) to generate fumarate. In the TCA cycle, fumarate can be accumulated because of the (i) inhibition of fumarate hydratase (FH), which converts fumarate to malate or the (ii) stimulation of SDH. Our further studies revealed that FH protein is reduced in diabetic mouse kidney and treatment with NOX4 inhibitor can stimulate FH expression and activity, thus providing evidence that FH is a major target for NOX4 in DKD. FH protein was also found to be reduced in biopsy samples from patients with diabetic nephropathy.

In a recent study, Liu et al. [53] analyzed serum, urine, and renal extracts from the streptozotocin (STZ)-induced DN rats by 1H NMR-based metabolomics and identified elevated allantoin and uric acid in the DN rats suggesting impaired purine metabolism in DN. Further, the authors showed that in DN, the activity of xanthine oxidase is elevated causing an increase in intracellular ROS, inflammation and oxidative damage. The above-mentioned studies further highlight that metabolomics could serve as a powerful approach to identify metabolic changes and target mechanisms toward therapeutic interventions.

Stec et al. [54] assessed urinary metabolites of T1D and T2D mouse models of DN, STZ-eNOS$^{-/-}$ C57BLKS and eNOS$^{-/-}$ C57BLKS db/db, respectively, by 1NMR spectroscopy. eNOS$^{-/-}$ C57BLKS db/db mouse serves as model for advanced stage diabetes. Six urinary metabolites– 3-indoxyl sulfate, cis-aconitate, 2-oxoisocaproate, N-phenyl-acetylglycine, 4-hydroxyphenyl acetate, and hippurate, corresponding to TCA cycle and amino acid catabolism– were significantly reduced in both T1D and T2D animals compared to controls. Further, 4-hydroxyphenyl acetic acid and hippuric acid showed the strongest inverse correlation with the albumin-to-creatinine ratio (table 3).

To investigate time-related metabolic changes associated with diabetes and to identify potential biomarkers associated with early-stage DKD, Li et al. [56] used the db/db mouse model of T2DM. The authors analyzed urine and serum metabolites at 6, 8, 10, 12, and 16 weeks in db/db mice and db/m mice using GC-TOF-MS. Together with PCA, the study identified distinct metabolomic profiles between db/db and db/m mice, which further differed with progression from diabetes to early, medium and late stages of DKD. The TCA cycle, glycolysis, lipid metabolism and amino acid turnover were the primary pathways associated with progression from diabetes to DN (table 3). In a similar study, Wei et al. [52] recently

Table 3. Summary of metabolomic alterations in animal models of DKD

Sample type and ref.	Analysis platform	Study population	Identified metabolites	Potential pathways affected
Urine, You et al. [51]	GC-MS	F1 control (n = 12) and F1 control treated with Nox4 inhibitor (n = 27) F1 Akita (n = 12) and F1 Akita treated with Nox4 inhibitor (n = 24)	TCA cycle metabolites: citrate (↑), malate (↑), isocitrate (↑), 2-oxoglutarate (↑), aconitate (↑), succinate (↑) and fumarate (↓) upon Nox4 inhibitor treatment	TCA cycle and fumarate hydratase
Serum, urine and kidney, Liu et al. [53]	1H-NMR	Male Sprague–Dawley rats control (n = 10) DN (n = 10)	Allantoin (↑) and uric acid (↑)	Purine metabolism
Urine, Stec et al. [54]	1H-NMR	Type 1 (STZ-eNOS(–/–) C57BLKS) (n = 11) and type 2 (eNOS(–/–) C57BLKS db/db) (n = 11) diabetic mouse models of DN	TCA cycle metabolites (↓), 3-indoxyl sulfate (↓), cis-aconitate (↓), 2-oxoisocaproate (↓), N-phenyl-acetylglycine (↓), 4-hydroxyphenyl acetate (↓) and hippurate (↓)	TCA cycle and aromatic amino acid catabolism
Serum and urine, Li et al. [56]	GC/TOF-MS	C57Bl/KS mice db/db (n = 40) db/m (n = 40)	Serum: fumarate (↑), citrate (↑), α-ketoglutarate (↑), malate (↑), cis-aconitate (↑), 3-hydroxybutyrate (↓), glycerate (↓), Lysine (↓), isoleucine (↑), valine (↑), 5-hydroxy proline (↑), hexadecanoic acid (↑), tetradecanoic acid (↑), eicosatetraenoic acid (↑), 9-octadecanoic acid (↑), octadecadienoic acid (↑), arginine (↓) and methionine (↓) urine: succinate (↑), malate (↑), cis-aconitate (↓), 3-hydroxybutyrate (↑), 2-oxocaproic acid (↓), octadecanoic acid (↓), glucaronate (↓), glutarate (↓), erythronate (↓), ethanolamine (↓), glycerate (↓), gluconate (↓) and azelate (↓)	TCA cycle, glycolysis, lipid and amino acid metabolism
Renal cortex, Zhao et al. [57]	GC/TOF-MS UPLC/ TOF-MS	Wistar rats control (n = 16) diabetic (n = 32)	Almost all amino acids (↓), hippurate (↑), polyols (↑), carbohydrates (↑), acyl carnitine (↑) and glucuronides (↑)	Amino acid metabolism and uremic toxins

Metabolite changes in cases with respect to controls are shown as (↑) for increase or (↓) for decrease.

used 1H-NMR and analyzed age-dependent metabolite changes in urine and kidney tissue extracts of db/db and control mice. The authors proposed cis-aconitate and cis-allontoin to be potential biomarkers for diagnosis of DN.

In another study, Zhao et al. [57] profiled renal cortical tissue metabolites associated with DKD induced by STZ in rat models before and following treatment with fosinopril, a pharmacological inhibitor of angiotensin II converting enzyme. A combination of GC/TOF-MS and UPLC/TOF-MS approaches identified a significant increase in uremic toxins along with changes in amino acids, carbohydrates, poly-ols, lysophospholipids, glucoronides, and glucotoxicity-associated metabolites. Several of these metabolites showed correlations with 24 h urinary protein levels and tubulointerstitial injury index (table 3).

The implication of the metabolite changes in the animal models are of unclear significance as the degree of renal dysfunction is variable. Further studies are required to determine which stage of DKD is being modeled in relation to human DKD and to establish whether there are similar patterns in animal models of DKD with human DKD.

Concluding Remarks

With the omics revolution fully underway and changing the way that medicine is being practiced in many clinical specialties, nephrology too can greatly benefit. In particular, metabolomics analysis in well-designed preclinical and clinical studies is beginning to show robust patterns of altered metabolites. There have been many technical advances in the past few years; however, careful attention needs to be paid to issues of sample handling, sample preparation and quantitative output in order to develop

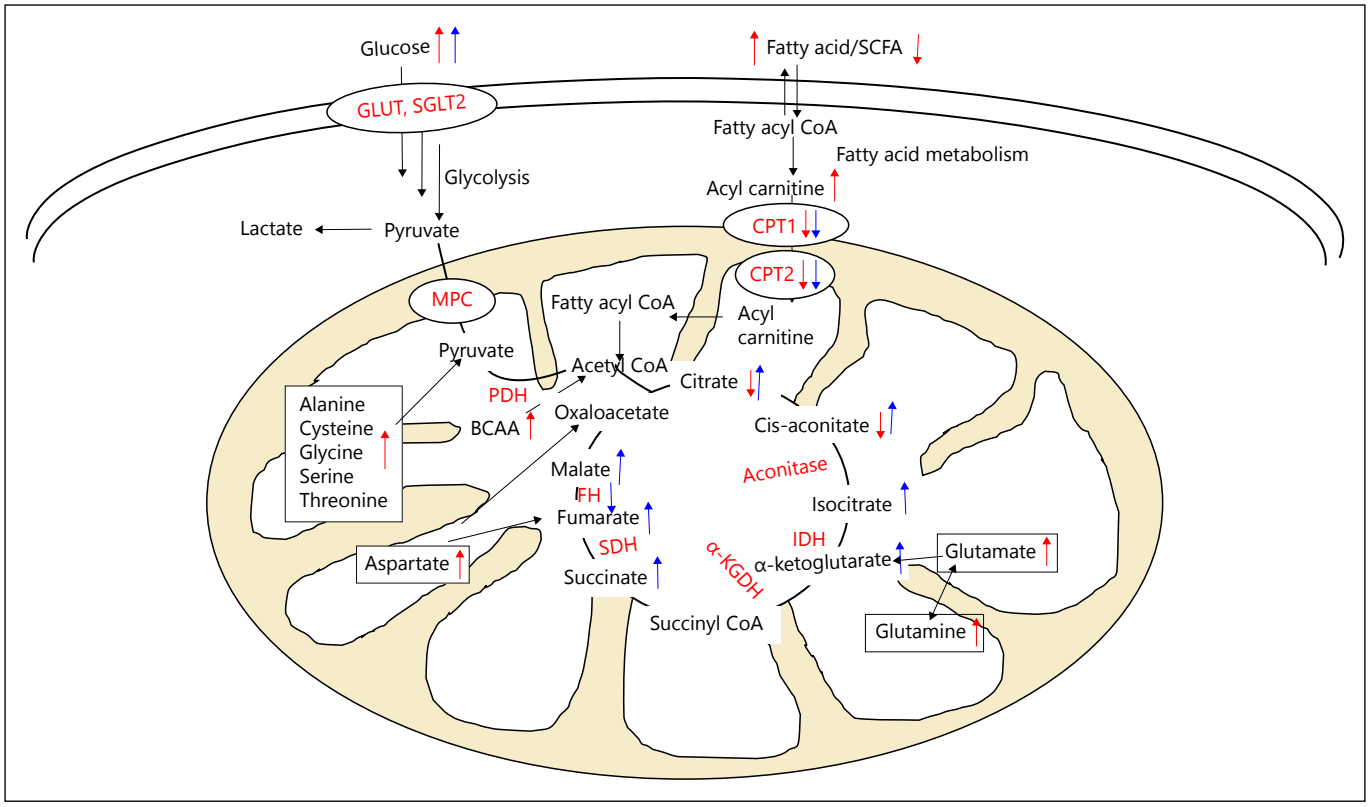

Fig. 1. Overview of urinary metabolite changes in TCA cycle, fatty acid and amino acid metabolism, and affected enzymatic pathways in DKD clinical and preclinical studies. MPC = Mitochondrial pyruvate carrier; PDH = pyruvate dehydrogenase complex; IDH = isocitric dehydrogenase; α-KGDH = alpha ketoglutarate dehydrogenase. Metabolites associated with clinical studies are indicated with red arrows and preclinical studies with blue arrows.

consistent data across metabolomic platforms. Heterogeneity of various classes of metabolites and their abundance in body fluids pose a major challenge for the metabolomics research community. There is no single technique that can measure all of the different classes of metabolites. With a targeted, quantitative approach, consistent patterns are emerging to indicate important alterations of a variety of TCA metabolites, lipid metabolism and amino acid metabolism from both preclinical and clinical studies (fig. 1). However, it is interesting to note that despite similarities in pathways, there are considerable differences in metabolite expression patterns between clinical and preclinical studies (fig. 1). For example, while studies in human samples have consistently showed a decrease in several TCA cycle metabolites in diabetic CKD, several animal model studies have indicated an increase in these metabolites. This may be related to the disease stage, differences in animal models and type of analysis. Limited studies are currently available comparing early and advanced stage DKD animal models (fig. 1). While future studies should focus on this aspect, novel pathways and exciting fundamental discoveries have certainly been made based on metabolomic results. Also, as there is now precedence in using metabolites as responsive markers of novel therapies, there will likely be many new studies that will guide the path to personalized medicine approaches for DKD in the near future.

Acknowledgment

Funding for parts of the work discussed in this article is provided by NIDDK DP3-DK094352 and JDRF Network grants to K.S.

Disclosure Statement

Dr. Sharma receives grant support from Merck, Inc. and Boerhinger-Ingleheim. He is the founder of ClinMet, Inc. and was on the Board of Directors, a scientific adviser and held an equity interest. Dr. Sharma's spouse was a co-founder and was the President and Chief Operating officer and held an equity interest.

References

1 Jha V, Garcia-Garcia G, Iseki K, Li Z, Naicker S, Plattner B, Saran R, Wang AY, Yang CW: Chronic kidney disease: global dimension and perspectives. Lancet 2013;382:260–272.
2 Centers for Disease Control and Prevention (CDC): National Chronic Kidney Disease Fact Sheet: General Information and National Estimates on Chronic Kidney Disease in the United States. Atlanta, US Department of Health and Human Services, Centers for Disease Control and Prevention, 2014.
3 Afkarian M, Sachs MC, Kestenbaum B, Hirsch IB, Tuttle KR, Himmelfarb J, de Boer IH: Kidney disease and increased mortality risk in type 2 diabetes. J Am Soc Nephrol 2013;24:302–308.
4 King H, Aubert RE, Herman WH: Global burden of diabetes, 1995–2025: prevalence, numerical estimates, and projections. Diabetes Care 1998;21:1414–1431.
5 Statistics About Diabetes: National Diabetes Statistics Report. American Diabetes Association, 2014.
6 Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T: Diabetic nephropathy: diagnosis, prevention, and treatment. Diabetes Care 2005;28:164–176.
7 Rheinberger M, Böger CA: [Diabetic nephropathy: new insights into diagnosis, prevention and treatment]. Dtsch Med Wochenschr 2014;139:704–706.
8 Krolewski AS, Niewczas MA, Skupien J, Gohda T, Smiles A, Eckfeldt JH, Doria A, Warram JH: Early progressive renal decline precedes the onset of microalbuminuria and its progression to macroalbuminuria. Diabetes Care 2014;37:226–234.
9 Tabaei BP, Al-Kassab AS, Ilag LL, Zawacki CM, Herman WH: Does microalbuminuria predict diabetic nephropathy? Diabetes Care 2001;24:1560–1566.
10 Dumas ME: Metabolome 2.0: quantitative genetics and network biology of metabolic phenotypes. Mol Biosyst 2012;8:2494–2502.
11 Du F, Virtue A, Wang H, Yang XF: Metabolomic analyses for atherosclerosis, diabetes, and obesity. Biomark Res 2013;1:17.
12 Breit M, Weinberger KM: Metabolic biomarkers for chronic kidney disease. Arch Biochem Biophys 2016;589:62–80.
13 Gao R, Cheng J, Fan C, Shi X, Cao Y, Sun B, Ding H, Hu C, Dong F, Yan X: Serum metabolomics to identify the liver disease-specific biomarkers for the progression of hepatitis to hepatocellular carcinoma. Sci Rep 2015;5:18175.
14 Cajka T, Fiehn O: Toward merging untargeted and targeted methods in mass spectrometry-based metabolomics and lipidomics. Anal Chem 2016;88:524–545.
15 Sims JK, Manteiga S, Lee K: Towards high resolution analysis of metabolic flux in cells and tissues. Curr Opin Biotechnol 2013;24:933–939.
16 Vacanti NM, Divakaruni AS, Green CR, Parker SJ, Henry RR, Ciaraldi TP, Murphy AN, Metallo CM: Regulation of substrate utilization by the mitochondrial pyruvate carrier. Mol Cell 2014;56:425–435.
17 Chouchani ET, Pell VR, Gaude E, Aksentijević D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord EN, Smith AC, Eyassu F, Shirley R, Hu CH, Dare AJ, James AM, Rogatti S, Hartley RC, Eaton S, Costa AS, Brookes PS, Davidson SM, Duchen MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T, Murphy MP: Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 2014;515:431–435.
18 Dieterle F, Riefke B, Schlotterbeck G, Ross A, Senn H, Amberg A: NMR and MS methods for metabonomics. Methods Mol Biol 2011;691:385–415.
19 Emwas AH: The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. Methods Mol Biol 2015;1277:161–193.
20 Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK: Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat Protoc 2007;2:2692–2703.
21 Pan Z, Raftery D: Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem 2007;387:525–527.
22 Want EJ, Wilson ID, Gika H, Theodoridis G, Plumb RS, Shockcor J, Holmes E, Nicholson JK: Global metabolic profiling procedures for urine using UPLC-MS. Nat Protoc 2010;5:1005–1018.
23 Bruce SJ, Tavazzi I, Parisod V, Rezzi S, Kochhar S, Guy PA: Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry. Anal Chem 2009;81:3285–3296.
24 Saccenti E, Hoefsloot HCJ, Smilde AK, Westerhuis JA, Hendriks MMWB: Reflections on univariate and multivariate analysis of metabolomics data. Metabolomics 2014;10:361–374.
25 Broadhurst DI, Kell DB: Statistical strategies for avoiding false discoveries in metabolomics and related experiments. Metabolomics 2006;2:171–196.
26 Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B Met 1995;57:289–300.
27 Hirayama A, Nakashima E, Sugimoto M, Akiyama S, Sato W, Maruyama S, Matsuo S, Tomita M, Yuzawa Y, Soga T: Metabolic profiling reveals new serum biomarkers for differentiating diabetic nephropathy. Anal Bioanal Chem 2012;404:3101–3109.
28 Ng DP, Salim A, Liu Y, Zou L, Xu FG, Huang S, Leong H, Ong CN: A metabolomic study of low estimated GFR in non-proteinuric type 2 diabetes mellitus. Diabetologia 2012;55:499–508.
29 van der Kloet FM, Tempels FW, Ismail N, van der Heijden R, Kasper PT, Rojas-Cherto M, van Doorn R, Spijksma G, Koek M, van der Greef J, Mäkinen VP, Forsblom C, Holthöfer H, Groop PH, Reijmers TH, Hankemeier T: Discovery of early-stage biomarkers for diabetic kidney disease using MS-based metabolomics (FinnDiane study). Metabolomics 2012;8:109–119.
30 Duranton F, Lundin U, Gayrard N, Mischak H, Aparicio M, Mourad G, Daurès JP, Weinberger KM, Argilés A: Plasma and urinary amino acid metabolomic profiling in patients with different levels of kidney function. Clin J Am Soc Nephrol 2014;9:37–45.
31 Benito S, Sánchez A, Unceta N, Andrade F, Aldámiz-Echevarria L, Goicolea MA, Barrio RJ: LC-QTOF-MS-based targeted metabolomics of arginine-creatine metabolic pathway-related compounds in plasma: application to identify potential biomarkers in pediatric chronic kidney disease. Anal Bioanal Chem 2016;408:747–760.
32 Han LD, Xia JF, Liang QL, Wang Y, Wang YM, Hu P, Li P, Luo GA: Plasma esterified and non-esterified fatty acids metabolic profiling using gas chromatography-mass spectrometry and its application in the study of diabetic mellitus and diabetic nephropathy. Anal Chim Acta 2011;689:85–91.
33 Niewczas MA, Sirich TL, Mathew AV, Skupien J, Mohney RP, Warram JH, Smiles A, Huang X, Walker W, Byun J, Karoly ED, Kensicki EM, Berry GT, Bonventre JV, Pennathur S, Meyer TW, Krolewski AS: Uremic solutes and risk of end-stage renal disease in type 2 diabetes: metabolomic study. Kidney Int 2014;85:1214–1224.
34 Pena MJ, de Zeeuw D, Mischak H, Jankowski J, Oberbauer R, Woloszczuk W, Benner J, Dallmann G, Mayer B, Mayer G, Rossing P, Lambers Heerspink HJ: Prognostic clinical and molecular biomarkers of renal disease in type 2 diabetes. Nephrol Dial Transplant 2015;30(suppl 4):iv86–iv95.
35 Posada-Ayala M, Zubiri I, Martin-Lorenzo M, Sanz-Maroto A, Molero D, Gonzalez-Calero L, Fernandez-Fernandez B, de la Cuesta F, Laborde CM, Barderas MG, Ortiz A, Vivanco F, Alvarez-Llamas G: Identification of a urine metabolomic signature in patients with advanced-stage chronic kidney disease. Kidney Int 2014;85:103–111.
36 Sharma K, Karl B, Mathew AV, Gangoiti JA, Wassel CL, Saito R, Pu M, Sharma S, You YH, Wang L, Diamond-Stanic M, Lindenmeyer MT, Forsblom C, Wu W, Ix JH, Ideker T, Kopp JB, Nigam SK, Cohen CD, Groop PH, Barshop BA, Natarajan L, Nyhan WL, Naviaux RK: Metabolomics reveals signature of mitochondrial dysfunction in diabetic kidney disease. J Am Soc Nephrol 2013;24:1901–1912.

37 Fleck C, Janz A, Schweitzer F, Karge E, Schwertfeger M, Stein G: Serum concentrations of asymmetric (ADMA) and symmetric (SDMA) dimethylarginine in renal failure patients. Kidney Int Suppl 2001;78:S14–S18.
38 Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE: Metabolite profiles and the risk of developing diabetes. Nat Med 2011;17:448–453.
39 Pena MJ, Lambers Heerspink HJ, Hellemons ME, Friedrich T, Dallmann G, Lajer M, Bakker SJ, Gansevoort RT, Rossing P, de Zeeuw D, Roscioni SS: Urine and plasma metabolites predict the development of diabetic nephropathy in individuals with type 2 diabetes mellitus. Diabet Med 2014;31:1138–1147.
40 Kang HM, Ahn SH, Choi P, Ko YA, Han SH, Chinga F, Park AS, Tao J, Sharma K, Pullman J, Bottinger EP, Goldberg IJ, Susztak K: Defective fatty acid oxidation in renal tubular epithelial cells has a key role in kidney fibrosis development. Nat Med 2015;21:37–46.
41 Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A; European Uremic Toxin Work Group: Normal and pathologic concentrations of uremic toxins. J Am Soc Nephrol 2012;23:1258–1270.
42 Ramezani A, Raj DS: The gut microbiome, kidney disease, and targeted interventions. J Am Soc Nephrol 2014;25:657–670.
43 Knip M, Siljander H: The role of the intestinal microbiota in type 1 diabetes mellitus. Nat Rev Endocrinol 2016;12:154–167.
44 Nigam SK, Bush KT, Martovetsky G, Ahn SY, Liu HC, Richard E, Bhatnagar V, Wu W: The organic anion transporter (OAT) family: a systems biology perspective. Physiol Rev 2015;95:83–123.
45 Eraly SA, Vallon V, Vaughn DA, Gangoiti JA, Richter K, Nagle M, Monte JC, Rieg T, Truong DM, Long JM, Barshop BA, Kaler G, Nigam SK: Decreased renal organic anion secretion and plasma accumulation of endogenous organic anions in OAT1 knock-out mice. J Biol Chem 2006;281:5072–5083.
46 den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM: The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 2013;54:2325–2340.
47 Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A, Kimura I: Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. Nutrients 2015;7:2839–2849.
48 Canfora EE, Jocken JW, Blaak EE: Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinol 2015;11:577–591.
49 Liang H, Ward WF: PGC-1alpha: a key regulator of energy metabolism. Adv Physiol Educ 2006;30:145–151.
50 LeBleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, de Carvalho FM, Damascena A, Domingos Chinen LT, Rocha RM, Asara JM, Kalluri R: PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. Nat Cell Biol 2014;16:992–1003, 1–15.
51 You YH, Quach T, Saito R, Pham J, Sharma K: Metabolomics reveals a key role for fumarate in mediating the effects of NADPH oxidase 4 in diabetic kidney disease. J Am Soc Nephrol 2016;27:466–481.
52 Wei T, Zhao L, Jia J, Xia H, Du Y, Lin Q, Lin X, Ye X, Yan Z, Gao H: Metabonomic analysis of potential biomarkers and drug targets involved in diabetic nephropathy mice. Sci Rep 2015;5:11998.
53 Liu J, Wang C, Liu F, Lu Y, Cheng J: Metabonomics revealed xanthine oxidase-induced oxidative stress and inflammation in the pathogenesis of diabetic nephropathy. Anal Bioanal Chem 2015;407:2569–2579.
54 Stec DF, Wang S, Stothers C, Avance J, Denson D, Harris R, Voziyan P: Alterations of urinary metabolite profile in model diabetic nephropathy. Biochem Biophys Res Commun 2015;456:610–614.
55 Gorin Y, Block K, Hernandez J, Bhandari B, Wagner B, Barnes JL, Abboud HE: Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. J Biol Chem 2005;280:39616–39626.
56 Li M, Wang X, Aa J, Qin W, Zha W, Ge Y, Liu L, Zheng T, Cao B, Shi J, Zhao C, Wang X, Yu X, Wang G, Liu Z: GC/TOFMS analysis of metabolites in serum and urine reveals metabolic perturbation of TCA cycle in db/db mice involved in diabetic nephropathy. Am J Physiol Renal Physiol 2013;304:F1317–F1324.
57 Zhao T, Zhang H, Zhao T, Zhang X, Lu J, Yin T, Liang Q, Wang Y, Luo G, Lan H, Li P: Intrarenal metabolomics reveals the association of local organic toxins with the progression of diabetic kidney disease. J Pharm Biomed Anal 2012;60:32–43.