Review Article

Metabolome and Microbiome in Kidney Diseases

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ABSTRACT. Despite several decades of intensive research and hard work in nephrology, a void exists in the availability of markers for identifying at-risk individuals, diagnosing diseases at incipient stage, and predicting treatment response. Most of the current widely available diagnostic tools such as creatinine, urine analysis, and imaging studies are quite insensitive such that about half of the kidney function is lost before perceivable changes are observed with these tests. In addition, these parameters are affected by factors other than renal, questioning their specificity. Renal biopsy, though specific, is quite expensive, risky, and invasive. The recent surge in the knowledge of small molecules in the tissue and body fluids, “metabolomics,” thanks to the Human Metabolome Database created by the Human Metabolome Project, has opened a new avenue for better understanding the disease pathogenesis and, in parallel, to identify novel biomarkers and druggable targets. Kidney, by virtue of its metabolic machinery and also being a major handler of metabolites generated by other tissues, is very much amenable to the metabolomic approach of studying its various perturbations. The gut microbiome, characterized by the Human Microbiome Project, is one of the principal players in metabolomics. Changes in metabolite profile due to alterations in gut microbiome can occur either as a cause or consequence of renal diseases. Unmasking the renal–metabolome–microbiome link has a great potential to script a new era in the diagnosis and management of renal diseases.

Introduction

Kidney diseases are plagued by lack of sensitive and specific markers, right since the inception of nephrology. Salvageable renal function is lost in most situations prior to diagnosis with the currently available diagnostic tools. There is an unmet need for markers for prediction, risk stratification, early diagnosis, treatment response, and prognostication. Metabolomics, although still in its infancy, can potentially fill this void. Microbiome, “second human genome,” one among the various influences on metabolomics, is gaining importance as it provides a new avenue for potential novel treatment targets for many kidney diseases for which no curative treatment is available at present.

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Metabolome

Metabolomics is the study of all small molecules (exogenous and endogenous with molecular weight <1000 Da) in biological fluids and tissue of an organism. Among the available “omics” technologies constituting systems biology, metabolomics represents the most distal and the one which is close to the phenotype. Human phenotype blueprint is coded in the genome which is transcribed into proteins (mostly enzymes), resulting in the generation of metabolites.

Why metabolomics

Genomics has the problem of posttranscriptional modification and also that not all genes are transcribed. Posttranslational modification of proteins and lack of correlation between the level and activity of enzymes pose hurdles in proteomics. In contrast, metabolomics represents the actual real-time events happening in an organism, and it also captures the exogenous influences such as diet and gut flora, which the upstream “omics” lack.

Application of metabolomics

Human diseases produce alteration in the metabolites by virtue of changes in the gene expression, protein activity, or exogenous influences such as drugs or microbes. Metabolomics can be used to identify biomarkers which could be used to detect, prognosticate, and identify treatment responders and to study disease pathways and identify novel druggable targets. By detecting the abnormal pattern of metabolite peaks specific to a disease, a metabolic “fingerprint” could be generated which can be used in diagnosis. By identifying the specific metabolites altered, they could be placed in metabolic pathways with the help of software such as pathway analysis which helps elucidating disease pathogenesis and also, by identifying the enzyme which gets dysregulated, novel targets for treatment could be identified. For example, if the metabolites altered in a specific disease in question cluster in a single metabolic pathway, then the specific enzyme upstream to the metabolites could be identified and modulators of the enzyme could be tested.

Techniques

The analytical technologies used in metabolomics could be used in a targeted or non-targeted approach. In the targeted or “slow-lane approach,” the detected metabolite signal peaks are chemically identified and in the non-targeted or “fast-lane approach”, pattern identification of metabolite signals without chemical identification is done. Nuclear magnetic resonance spectroscopy and mass spectroscopy with their latest improvisations are the techniques used in metabolomics. Nuclear magnetic resonance spectroscopy utilizes the atomic properties to identify metabolites. Its principal advantages are high reproducibility, better structure elucidation, no prior sample processing, and the ability to use on tissue biopsies, whereas high sample volume, relative insensitivity (detects only metabolites with high abundance), and the ability to detect fewer metabolites are its disadvantages. Mass spectroscopy utilizes mass-to-charge ratio to characterize metabolites. It requires prior sample processing with chromatographic techniques such as gas chromatography, liquid chromatography, or capillary electrophoresis, which may result in metabolite loss. In addition, the reproducibility is poor compared to nuclear magnetic resonance spectroscopy. On the other hand, it can detect thousands of metabolites in a single performance and also has comparatively high sensitivity for detection.

Limitations of metabolomics

Having said that metabolomics is “real-time” events occurring in an organism and that it incorporates the exogenous influences which other “omics” technologies lack, it does have certain limitations in its present state, including:

1. Incomplete coverage: Currently, 8000 metabolites are characterized, but the entire metabolome has not yet been elucidated in contrast to genomics where the entire genome has been studied
2. Nonoverlapping: Available techniques, even
when applied to same sample, result in differential metabolite identification due to their inherent limitations$^{5,6}$

3. No standardization in sample collection, processing, and normalization, resulting in difficulty in comparison across studies$^5$.

4. Validity: Metabolite alterations currently identified have not gone beyond discovery stage except for a few. Large multicenter studies are required to validate before they can be applied in clinical practice.

These are not actual limitations, rather hurdles which could be overcome in future.

**Metabolomics and Kidneys**

Metabolomics is not a new concept in nephrology as it has been used from ancient times to detect diseases based on urine odor and color and also in our day-to-day practice when we use urine dipsticks.$^1$ Kidney, next to the heart in mitochondrial content, is ideally suited for metabolomics approach for studying its various perturbations. Kidneys influence metabolite patterns in various ways:$^7$

1. By glomerular filtration, tubular secretion, or reabsorption, they influence metabolite levels produced by various tissues
2. Cellular constituents of the kidney possess enzyme activity which gets altered in various disease states
3. Enzyme activity alterations in other tissues might contribute to kidney diseases.

**Chronic kidney disease**

Current diagnosis of chronic kidney disease (CKD) relies on structural or functional decline in kidney function. However, there are no markers for risk prediction, early diagnosis, and identification of progression. Metabolomics could potentially cater to these needs. Metabolic alterations related to CKD include amino acids, steroids, purine, nitric oxide, tryptophan, oxidative stress, and lipids.$^7$ Indole 2, 3-dioxygenase activity on tryptophan-generating kynurenine and kynurenic acid is upregulated in CKD, resulting in reduced serum levels of tryptophan with increase in kynurenine.$^8$ Acyl carnitines generated from esterification of acyl coA with L-carnitine accumulate due to impaired clearance. Cardiovascular disease remains the leading cause of death in CKD patients. In addition to the traditional risk factors, asymmetric dimethyl arginine (ADMA) derived from L-arginine, an inhibitor of endothelial nitric oxide synthase, is found to accumulate in patients with CKD.$^9$ Accumulation of ADMA occurs due to increased proteolysis of arginine residues in proteins and reduced activity of dimethylarginine dimethylaminohydrolase, an enzyme in the kidney which normally degrades ADMA to citrulline. Apart from causing endothelial dysfunction, ADMA has been shown to cause tubulo-interstitial fibrosis by upregulating collagen deposition and transforming growth factor-$\beta$ expression, causing progression of CKD.$^9$ Metabolites accumulating due to altered gut flora as uremic retention solutes are discussed later. A study on urinary metabolic profile to identify CKD has revealed the presence of 5-oxoproline, glutamate, guanidoacetate, phenylacetylglutamine, taurine, citrate, and trimethylamine N-oxide in the urine of these patients.$^7$ Adequacy of dialysis techniques is now being determined with the traditional urea clearance calculation with inherent limitations. Metabolomics dealing with small molecules could provide better markers for such purpose in future.

**Renal transplantation**

Renal biopsy remains the major diagnostic modality in renal transplant patients, as other blood/urine markers are insensitive; but, it is invasive, expensive, and carries risk.$^{10}$ Markers of CKD are also associated with allograft dysfunction. A study demonstrated higher pre-transplant kynurenine levels to be associated with presensitization status and longer dialysis vintage, although without prognostic value.$^7$ Another study showed a panel of 10 metabolites to identify T-cell-mediated rejection.

**Diabetic kidney disease**

Diabetes continues to be the *numero uno* cause for CKD worldwide despite significant improvements in diabetes management. This is
due to lack of biomarkers to identify diabetic nephropathy quite early. High serum levels of saturated fatty acids and low levels of high-density lipoproteins were found to be associated with accelerated progression. An increase of γ-butyrobetaine, citrulline, symmetric dimethyl arginine, and kynurenine and decrease in azelaic acid in serum were found to predict progression to macroalbuminuria. A recent study using urinary metabolomics showed that metabolite alterations linked to organic anion transporter-1 and -3 and mitochondrial dysfunction (by connecting the identified metabolites through network analysis for organelle localization) occur in diabetes. They also validated their findings by demonstrating reduced expression of OAT1 and OAT3 in tubules of biopsy-proven diabetic nephropathy and also showed reduced expression of cytochrome C oxidase in biopsy specimens with reduced urinary mitochondrial DNA exosome in diabetic nephropathy pa-
tients. Going further to identify the cause for mitochondrial dysfunction, they demonstrated reduced activity of peroxisome proliferator-γ co-activator 1α in biopsy specimens, thereby identifying a potential target to modulate for this otherwise unabated disease.

**Glomerular diseases**

IgA nephropathy, despite being the most common glomerulonephritis, is often diagnosed late and also, there is no effective treatment to halt its progression. A study identified a distinct metabolic signature to differentiate IgA patients from controls, but not low-risk from high-risk patients. In another study using fecal metabolomics, high levels of total free amino acids, glucose, alanine, aspartate, valine, leucine, and proline and low level of keto-
glutamate were associated with disease progression. Urine metabolomics in patients with membranous nephropathy identified significantly increased excretion of dicarboxylic acids, threonine, quinoline, cholesterol, and phenolic acids to be associated with higher protein excretion, indicating greater oxidative stress.

**Acute kidney injury**

Studies of metabolomics in acute kidney injury (AKI) are sparse. A pilot study in AKI identified increase in acylcarnitines, methionine, homocysteine, phenylalanine, and ADMA and a reduction in serum levels of arginine and several lysophosphatidylcholines, representing deranged lipid and nitric oxide metabolism and increased oxidative stress. Animal studies on nephrotoxic AKI due to aminoglycosides, cisplatin, and doxorubicin yielded similar urinary metabolic profile, indicating proximal tubular dysfunction including glucose, amino acids, lactate, and ketones.

**Renal cell carcinoma**

Metastatic renal cell carcinoma despite newer drugs has a dismal prognosis and hence an urgent need for biomarkers for early detection and novel treatment targets. Metabolomic studies have shown increased glycolytic flux and reduced oxidative phosphorylation, consistent with hypoxia-inducible factor-1 activating pyruvate dehydrogenase kinase, which inhibits pyruvate dehydrogenase complex by phosphorylation and thereby impeding pyruvate entering into Kreb’s cycle. High glutathione levels correlating with high oxidative stress and high dipeptide levels were associated with aggressive renal cell carcinoma. It was also shown that α-hydroxybutyrate, a surrogate of α-ketoglutarate, was associated with tumor recurrence. α-ketoglutarate is produced when cystathionine is hydrolyzed to cysteine, a critical precursor for glutathione synthesis. The knowledge gained from metabolomics has resulted in the testing of PPAR-α inhibitors, GW6471 and NXT1120, and etomoxir, an inhibitor of carnitine phosphotransferase-1 to inhibit ω-oxidation of fatty acids in *in-vivo* renal cell carcinoma studies.

The discussion is summarized in Table 1.

**Microbiome**

Human beings harbor complex community of bacteria, archaea, viruses, and eukaryotic microbes, numbering over 100 trillion cells, 10
Increased glycolytic flux and reduced oxidative phosphorylation

High glutathione and dipeptide

Table 1. Metabolome pattern in selected kidney diseases.

| Disease                        | Metabolome alteration                                                                 |
|--------------------------------|----------------------------------------------------------------------------------------|
| Chronic kidney disease         | • Reduced serum levels of tryptophan with increase in kynurenine                        |
|                                | • Accumulation of acyl carnitines and asymmetric dimethyl arginine                     |
|                                | • Generation of indoxyl sulfate, p-cresol sulfate, trimethylamine oxide,                |
|                                |   phenylacetylglutamine, and hippuric acid                                             |
| Renal transplantation          | • Higher pretransplant kynurenine levels associated with presensitization status and    |
|                                |   longer dialysis vintage                                                              |
| Diabetic kidney disease        | • High serum levels of saturated fatty acids and low levels of high-density lipoproteins|
|                                | • Increase of γ-butyrobetaine, citrulline, symmetric dimethyl arginine                 |
|                                |   and kynurenine, and decrease in azelaic acid                                        |
|                                | • Reduced activity of peroxisome proliferator-γ co-activator 1α                      |
| IgA nephropathy                | • High fecal levels of total free amino acids, glucose, alanine, aspartate, valine,    |
|                                |   leucine, and proline and low level of ketoglutarate                                 |
| Membranous nephropathy         | • Increased urinary excretion of dicarboxylic acids, threonine, quinolinate, cholesterol, and phenolic acids |
| Acute kidney injury            | • Increase in acylcarnitines, methionine, homocysteine, phenylalanine, and             |
|                                |   asymmetric dimethyl arginine and a reduction in serum levels of arginine and several |
|                                |   lysophosphatidylcholines                                                           |
| Renal cell carcinoma           | • Increased glycolytic flux and reduced oxidative phosphorylation                      |
|                                | • High glutathione and dipeptide levels                                                |
|                                | • Increased generation of α-hydroxybutyrate                                           |

times that of host cells belonging to over 1000 species with a genome approximately 100 times that of human.16 The genes encoded by this human microbiota collectively form the microbiome referred to as the “second human genome.”17 The metabolic capacity of the human microbiome has been found to parallel that of liver. Under physiologic conditions, the microbiome performs complementary functions that have not evolved in humans such as complex carbohydrate digestion, synthesis of vitamins, maintaining gut epithelial integrity, and protection from infection by colonization resistance and immune regulation referred to as “normobiosis.”18 The human gut comprises bacteria belonging to four major phyla namely Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. In healthy state, the human gut has a predominance of saccharolytic bacteria populating the proximal colon with proteolytic bacteria in the distal colon. Firmicutes (e.g., Enterococcus, Clostridium, Ruminococcus, and Lactobacillus) contain Gram-positive bacteria that degrade indigestible polysaccharides, constituting 60% of the gut microbiota, and produce butyrate.19 Butyrate, a short-chain fatty acid, is an important nutrient for colonic epithelium.20 The Gram-negative Bacteroidetes (e.g., Bacteroides and Prevotella) comprising 15% of microbiota produce propionate, another short-chain fatty acid used in gluconeogenesis.19 The gut microbiome in health forms distinct community structures called “entero-types” encoding specific metabolic signatures with implications in health and disease.21 The variations in microbiome among individuals are driven by several factors such as age, diet, geographical location, ethnicity, antibiotic use, and genetics. Although ethnicity has been strongly correlated with microbiota diversity than other factors, it has been shown that geographical migration changes the gut microbiome, stressing upon the eternal influence of environment.22 Among Indians, Firmicutes dominate the gut microbiome followed by Bacteroidetes and Actinobacteria. Even within India, there exists variation in enterotypes between the urban and rural and also between the northern and southern regions. Among urban Indians, gut microbiome with xenobiotic metabolism signatures was found to dominate, consistent with high exposure to industrial and environmental pollutants when compared to rural residents.21
Approximately 99% of the gut microbes cannot be cultured. Recent explosion in the knowledge of human microbiome has been due to metagenomic sequencing, which, together with identification, also helps to know the information encoded in their genome.

Gut “dysbiosis,” quantitative and qualitative alterations in the microbiome, resulting in cellular and metabolic derangements, is being implicated in the causation and/or progression of disease states such as obesity, diabetes mellitus, inflammatory bowel disease, cardiovascular disease, and CKD.23

Chronic kidney disease
End-stage renal disease is characterized by the retention of solutes referred to as uremic retention solutes. The origin of these solutes could be either from endogenous metabolism, exogenous, or microbiome. Among the uremic solutes, indoxyl sulfate, p-cresol sulfate, trimethylamine oxide, phenylacetylglutamine, and hippuric acid have their origin from the gut microbiome. Indoxyl sulfate and p-cresol sulfate are protein-bound solutes derived from tryptophan and tyrosine, respectively, by the action of altered gut microbiome in CKD. Both these toxins have been associated with the progression of CKD, endothelial damage, and cardiovascular disease in in-vitro and ex-vivo studies and with increased mortality in clinical studies. Trimethylamine oxide, derived from trimethylamine oxidation in liver, is produced from choline and betaine from diet by the action of the altered gut flora. Trimethylamine oxide has been shown to be associated with cardiovascular morbidity and mortality. Normally, these solutes produced in small amounts are excreted by the kidneys. In CKD, impaired excretion due to reduced kidney function and increased production due to altered flora occur. Besides, there is also decrease in the production of short-chain fatty acids which maintain gut epithelial integrity by butyrate production and immune function by differentiation of regulatory T cells and reduction in pro-inflammatory cytokine expression initiated by toll-like receptor signalling.16,19

The major changes observed in gut microbiome of CKD include:
1. Reduced microbial diversity and number24
2. Translocation – Duodenum and jejunum which are not normally colonized are found to have an increase in their microbial content (aerobic and anaerobic)18,19
3. Predominance of proteolytic bacteria25
4. “Leaky gut,” disrupted epithelial barrier26
5. Generation of protein-bound uremic toxins. Multiple factors contribute to this altered scenario. Impaired protein assimilation in the proximal intestine generates excess protein load to the colon, resulting in the proliferation of proteolytic bacteria at the expense of normal saccharolytic bacteria.25 Urea concentration in the gut increases in uremia and hence, selection pressure causes proliferation of urease-producing microorganisms. Urea gets converted to ammonia and ammonium hydroxide which cause disruption of epithelial tight junction and barrier disruption, “leaky gut.”25

This causes translocation of microbes and microbial products including lipopolysaccharides (endotoxins) into the systemic circulation, causing immune activation, endothelial dysfunction, and systemic inflammation, which culminates in disease progression and cardiovascular disease.26 Other factors include diet, metabolic acidosis, hemodialysis, phosphate binders, and iron supplements.

Renal Transplantation
Dysbiosis in transplant patients occurs due to the use of immunosuppressants and various antibiotics used for prophylaxis and treatment, in addition to graft dysfunction. Transplant recipients have a reduction in the prevalence of Lactobacillus with a concurrent increase in Enterobacteriaceae and Enterococcus compared with healthy controls.27 Posttransplant diarrhea has been associated with reduced microbiota diversity and reduced Bacteroides, Ruminococcus, Coprococcus, and Dorea.28 Dysbiosis may also affect the bioavailability of drugs which gain importance in the transplant setting. Patients with abundance of fecal Faecalibacterium prausnitzii in the 1st week posttransplant were found to require increase in tacrolimus dosing.25 Dysbiosis may be asso-
associated with antigen cross reactivity because of molecular mimicry and precipitate rejection.28

**Glomerular diseases**

Dysregulated microbiota with a lower *Firmicutes-to-Bacteroidetes* ratio similar to that observed in other autoimmune conditions has been shown to be present in systemic lupus erythematosus. Animal studies using lpr mouse lupus nephritis model have shown that reduction in *Lactobacillus* was associated with increased intestinal permeability and that *Lactobacillus* supplementation reduced the disease severity and pathological changes in the kidney.29 Similarly, in IgA nephropathy, gut dysbiosis with increase in amino acid fermenting bacteria has been observed.

**Nephrolithiasis**

Patients with calcium oxalate crystal formation due to genetic or medical causes have been found to be deficient in *Oxalobacter formigenes* in their gut. This bacterium utilizes oxalate as a source for carbon and also for adenosine triphosphate, besides increasing oxalate secretion by the colon.30 In the Chinese epidemic of melamine stones due to adulteration of milk with melamine, it was observed that cyanuric acid derived from melamine by gut microbe *Klebsiella* was required for melamine crystallization.31

**Acute kidney injury**

Altered entero-renal cross talk is a less ventured avenue with regard to AKI. In an animal model of ischemia/reperfusion injury AKI model, a reduction in *Bifidobacterium* with concomitant increase in *Lactobacillus, Clostridium*, and *Ruminococcus* was observed.32 With regard to the metabolome alteration, D-amino acid oxidase activity was found to be reduced with increase in serine racemase activity, resulting in increased D-serine concentration, which was shown to promote tubular cell proliferation and to mitigate hypoxia-induced tubular damage.32 The short-chain fatty acids, especially acetate, produced by commensal gut bacteria have been shown to mitigate renal damage in AKI. In humans, a retrospective study on cirrhotic patients on rifaximin observed a reduced AKI incidence, which was presumed to be due to rifaximin-induced microbiome modulation, though formal study on gut flora changes was not carried out.33

**Measures to Restore Normobiosis**

Several approaches have been made to restore the normal microbiome, but the strength and quality of evidence is debatable as contradictory results have been observed among studies:

1. Diet: CKD diet is classically a low-phosphorus and low-potassium diet which translates into deficient prebiotics (from dairy products) and dietary fiber (from fruits and vegetables), respectively. Interventions with high-fiber diet have been shown to reduce the level of uremic toxins in few studies19

2. Probiotics, prebiotics, and symbiotics: Probiotics are living microorganisms with potential health benefits. Various studies have shown reduced levels of uremic solutes, indoxyl sulfate, and p-cresol sulfate, with probiotic supplementation in CKD and hemodialysis patients. However, the major concern is the duration of survival of these supplements in the gut. Prebiotics are indigestible ingredients favoring the growth of endogenous probiotics. The major classes of prebiotics include galacto-oligosaccharides and the inulin-type fructans. Symbiotics are a combination of prebiotics and probiotics supplemented together19,23

3. Adsorbents: AST-120 adsorbs indoxyl sulfate and has been shown in small studies to reduce the rate of kidney function decline and postpone the initiation of dialysis, but failed to replicate similar results in a larger study23

4. Acarbose: α-glucosidase inhibitor prevents the degradation of complex polysaccharides and hence, increases the carbohydrate load of distal colon, favoring proliferation of saccharolytic bacteria.18
5. Novel therapies:
   a. “Smart” bacteria: They are genetically modified bacteria tailored to produce a continuous supply of required therapeutic molecules for treatment or scavengers to remove toxic molecules.25
   b. Fecal microbiota transplant (FMT): It is originally used to treat refractory Clostridium difficile diarrhea, and it can also be potentially utilized to restore gut normobiosis.25,27

Conclusion

Unraveling the renal–metabolome–microbiome axis link could potentially alter the way kidney diseases are being diagnosed and treated.28 Although metagenomics, the direct study of genetic material in a natural sample, has furthered our knowledge on gut microbiome, knowing beyond the microbial DNA contents is essential to understand the dynamic functional role of the microbiome and its host interactions, which is best complemented by metabolomics. Although gut–microbiome interactions with the host are reflected by the changes in metabolomic signature of all body fluids, the fecal metabolome provides a direct insight into their interactions. Metabolomics along with other “omics” technologies multiplexed with currently available tools and clinical data, could lead to personalized medicine in the near future, by the way of identifying an individual’s metabolic signature and to individualize treatment accordingly.34 However, although the task is humongous, the future is bright.

Conflict of interest: None declared.

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