First Prize Essay

METABOLOME AND MICROBIOME IN KIDNEY DISEASE

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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. Also 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 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 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 to study its various perturbations. The gut microbiome, characterised 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 since inception of nephrology. Salvageable renal function is lost in most situations prior to diagnosis with currently available diagnostic tools. There is an unmet need for markers for prediction, risk stratification, early diagnosis, treatment response and prognostication. Metabolomics, though 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 are available at present.

METABOLOME

Metabolomics is the study of all small molecules (exogenous and endogenous with molecular weight < 1000Da) in biological fluids and tissue of an organism.[1] 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.[1]

WHY METABOLOMICS

Genomics has the problem of post transcriptional modification and that not all genes are transcribed. Post translational modification of proteins and lack of correlation between level and activity of enzymes pose hurdles in proteomics.[2] In contrast, metabolomics represents the actual real time events happening in an organism and it also captures the exogenous influences such as diet, gut flora which the upstream ‘omics’ lack.[3]

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 abnormal pattern of metabolite peaks specific to a disease, a metabolic ‘fingerprint’ could be generated which could 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.[3] For example, if the metabolites altered in a specific disease in question clusters in a single metabolic pathway, then the specific enzyme upstream to the metabolites could be identified and modulators of the enzyme could be tested.[2]

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.[1] Nuclear magnetic resonance spectroscopy and mass spectroscopy with their latest improvisations are the techniques used in metabolomics. Nuclear magnetic resonance spectroscopy utilises the atomic properties to identify metabolites. Its principal advantages being high reproducibility, better structure elucidation, no prior sample processing, ability to use on tissue biopsies while high sample volume, relative insensitivity (detects only metabolites with high abundance) and ability to detect fewer metabolites are its disadvantages.[3] Mass spectroscopy utilises mass to charge ratio to characterise metabolites. It requires prior sample processing with chromatographic techniques such as gas chromatography, liquid chromatography or capillary electrophoresis which may result in metabolite loss.[4] Also, 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.[3]

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 characterised, but the entire metabolome has not yet been elucidated in contrast to genomics where the entire genome has been studied.[5]
2. Non-overlapping – available techniques even when applied to same sample results in differential metabolite identification due to their inherent limitations.[5,6]

3. No standardisation in sample collection, processing and normalisation – difficulty in comparison across studies.[5]
4. Validity – metabolite alterations currently identified have not gone beyond discovery stage except for a few. Large multicentre studies are required to validate before they could be applied in clinical practice.

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

METABOLOMICS AND KIDNEY

Metabolomics is not a new concept in nephrology as it has been used from ancient times to detect diseases based on urine odour and colour and also in our day-to-day practice when we use urine dipsticks.[1] Kidneys, next to heart in mitochondrial content are 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 relies on structural or functional decline in kidney function. But there are no markers for risk prediction, early diagnosis and identify progression. Metabolomics could potentially cater to these needs. Metabolic alterations related to chronic kidney disease 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 chronic kidney disease resulting in reduced serum levels of tryptophan with increase in kynurenine.[8] Acyl carnitines generated from esterification of acyl coA with L-carnitine accumulates due to impaired clearance. Cardiovascular disease remains the leading cause of death in chronic kidney disease patients. In addition to traditional risk factors, asymmetric dimethyl arginine (ADMA) derived from L-arginine, an inhibitor of eNOS (endothelial nitric oxide synthase) is found to accumulate in patients with chronic kidney disease.[9] Asymmetric dimethyl arginine accumulation occurs due to increased proteolysis of arginine residues in proteins and reduced activity of

dimethylarginine dimethyl aminohydrolase, an enzyme in the kidney which normally degrades asymmetric dimethyl arginine to citrulline. Apart from causing endothelial dysfunction, asymmetric dimethyl arginine has been shown to cause tubulointerstitial fibrosis by upregulating collagen deposition and Transforming Growth Factor- β expression causing chronic kidney disease progression.[9] Metabolites accumulating due to altered gut flora as uremic retention solutes are discussed later. A study on urinary metabolic profile has revealed 5- oxoproline, glutamate, guanidoacetate, phenylacetylglutamine, taurine, citrate, and trimethylamine N-oxide to identify chronic kidney disease.[7] Adequacy of dialysis techniques are now being determined with traditional urea clearance calculation with inherent limitations. Metabolomics dealing with small molecules could provide better markers for such purpose in the 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 chronic kidney disease are also associated with allograft dysfunction. A study demonstrated higher pre transplant kynurenine levels to be associated with pre sensitisation 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 chronic kidney disease 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.[11] 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 localisation) occurs in diabetes.[12] They also validated their findings by demonstrating reduced expression of OAT1 & 3 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 patients. Going further to identify the cause for

mitochondrial dysfunction, they demonstrated reduced activity of Peroxisome proliferator-γ Coactivator 1α in biopsy specimens thereby identifying a potential target to modulate for this otherwise unabated disease.[12]

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 distinct metabolic signature to differentiate IgA patients from controls but not low risk from high risk patients. In another study using faecal microbiomics, high levels of total free amino acids, Glucose, Alanine, Aspartate, Valine, Leucine and Proline and low level of ketoglutarate were associated with disease progression.[7] Urine metabolomics in membranous nephropathy patients identified significantly increased excretion of dicarboxylic acids, threonine, quinolinate, cholesterol, and phenolic acids to be associated with higher protein excretion indicating greater oxidative stress.[7]

ACUTE KIDNEY INJURY

Metabolomics studies in acute kidney injury are sparse. A pilot study in acute kidney injury identified increase in acylcarnitines, methionine, homocysteine, phenylalanine and asymmetric dimethyl arginine 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 acute kidney injury due to aminoglycosides, cisplatin and doxorubicin yielded similar urinary metabolic profile indicating proximal tubular dysfunction including glucose, aminoacids, lactate and ketones.[7,13]

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.[14,15] 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-hydroxy butyrate, a surrogate of α-ketoglutarate was associated with tumour

recurrence. α-ketoglutarate is produced when cystathionine is hydrolyzed to cysteine, a critical precursor for glutathione synthesis.[14] The knowledge gained from metabolomics have resulted in the testing of PPAR-α inhibitors - GW6471 and NXT1120, and etomoxir, an inhibitor of carnitine phosphotransferase-1 to inhibit β-oxidation of fatty acids in *in vivo* renal cell carcinoma studies.[1]

MICROBIOME

Human beings harbour complex community of bacteria, archaea, viruses and eukaryotic microbes numbering over 100 trillion cells, 10 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, degrade indigestible polysaccharides, constitute 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] 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 alteration in the microbiome results 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 chronic kidney disease.[21]

CHRONIC KIDNEY DISEASE

End Stage Renal Disease is characterised 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 sulphate, p-cresol sulphate, trimethylamine oxide, phenylacetylglutamine and hippuric acid have their origin from the gut microbiome. Indoxyl sulphate and p-cresol sulphate are protein bound solutes derived from tryptophan and tyrosine respectively by the action of altered gut microbiome in chronic kidney disease. Both these toxins have been associated with chronic kidney disease progression, 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 theses solutes produced in small amounts are excreted by the kidneys. In chronic kidney disease, impaired excretion due to reduced kidney function and increased production due to altered flora occurs. 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, 21]

The major changes observed in gut microbiome of chronic kidney disease include

1. Reduced microbial diversity and number[22]
2. Translocation - duodenum and jejunum which are not normally colonised are found to have an increase in their microbial content (aerobic and anaerobic).[18,19]
3. Predominance of proteolytic bacteria[24]
4. “Leaky gut” -disrupted epithelial barrier[23]
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 proliferation of proteolytic bacteria at the expense of normal saccharolytic bacteria.[24] Urea concentration in the gut increases in uremia and hence, selection pressure cause 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”.[23] This causes translocation of microbes and microbial products including lipopolysaccharide (endotoxin) into the systemic circulation causing immune activation, endothelial dysfunction

and systemic inflammation which culminates in disease progression and cardiovascular disease.[23] Other factors include diet, metabolic acidosis, hemodialysis, phosphate binders and iron supplements.

RENAL TRANSPLANTATION

Dysbiosis in transplant patients occurs due to immunosuppressant use, 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.[25] Post transplant diarrhoea has been associated with reduced microbiota diversity and reduced Bacteroides, Ruminococcus, Coprococcus, and Dorea.[26] Dysbiosis also may affect the bioavailability of drugs which gain importance in the transplant setting. Patients with abundance of fecal *Faecalibacterium prausnitzii* in the first week post transplant were found to require increase in tacrolimus dosing.[26] Dysbiosis may be associated with antigen cross reactivity because of molecular mimicry and precipitate rejection.[26]

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 Lactobacillales was associated with increased intestinal permeability and that Lactobacillales supplementation reduced the disease severity and kidney pathological changes.[27] 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 utilises oxalate as a source for carbon and also for ATP, besides increasing oxalate secretion by the colon.[28] In the Chinese epidemic of melamine stones due to melamine adulteration of milk, it was observed that cyanuric acid derived from melamine by gut microbe *Klebsiella* was required for melamine crystallisation.[29]

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 – chronic kidney disease diet is classically a low phosphorus and low potassium diet which translates into deficient prebiotics (from dairy products) and dietary fibre (from fruits and vegetables) respectively. Interventions with high fibre diet have been shown to reduce the level of uremic toxins in few studies.[19]
2. PROBIOTICS, PREBIOTICS AND SYNBIOTICS – Probiotics are living microorganisms with potential health benefits. Various studies have shown reduced levels of uremic solutes – indoxyl sulphate and p-cresol sulphate with probiotic supplementation in chronic kidney disease and hemodialysis patients. But the major concern is the duration of survival of these supplements in the gut. Prebiotics are indigestible ingredients favouring the growth of endogenous probiotics. The major classes of prebiotics include galacto-oligosaccharides and the inulin-type fructans. Synbiotics are a combination of prebiotics and probiotics supplemented together.[19,21]
3. ADSORBENTS – AST-120 adsorbs indoxyl sulphate 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 study.[21]
4. ACARBOSE – α-glucosidase inhibitor, preventing the degradation of complex polysaccharides and hence increases the carbohydrate load of distal colon favouring proliferation of saccharolytic bacteria.[18]
5. NOVEL THERAPIES
    a. “Smart” bacteria – genetically modified bacteria tailored to produce a continuous supply of required therapeutic molecules for treatment or scavengers to remove toxic molecules.[24]
    b. FECAL MICROBIOTA TRANSPLANT (FMT) - originally used to treat refractory *Clostridium difficile* diarrhoea, it can also be potentially utilised to restore gut normobiosis.[21,25]

CONCLUSION

Unravelling the renal-metabolome-microbiome axis link could potentially alter the way kidney diseases are being diagnosed and treated.[20] Metabolomics along with other

'omics' technologies multiplexed with currently available tools and clinical data could lead to personalised medicine in the near future, by way of identifying an individual's metabolic signature and to individualise treatment accordingly.[30] Though the task is humongous, the future is bright.

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Respected Sir,

I've applied for the ISN and southern chapter membership.

Forwarded through:

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