Blood, urine and faecal metabolite profiles in the study of adult renal disease

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ABSTRACT

Chronic kidney disease (CKD) is a major public health burden and to date traditional biomarkers of renal function (such as serum creatinine and cystatin C) are unable to identify at-risk individuals before the disease process is well under way. To help preventive strategies and maximize the potential for effective interventions, it is important to characterise the molecular changes that take place in the development of renal damage.

Metabolomics is a promising tool to identify markers of renal disease since the kidneys are involved in the handling of major biochemical classes of metabolites. These metabolite levels capture a snap-shot of the metabolic profile of the individual, allowing for the potential identification of early biomarkers, and the monitoring of real-time kidney function. In this review, we describe the current status of the identification of blood/urine/faecal metabolic biomarkers in different entities of kidney diseases including: acute kidney injury, chronic kidney disease, renal transplant, diabetic nephropathy and other disorders.
1. Introduction

Chronic kidney disease (CKD) is a major public health problem affecting 10–13% of the population in Western countries and substantially increasing the risk of cardiovascular morbidity and overall mortality [1]. The metabolites creatinine and more recently cystatin C are currently used to estimate glomerular filtration rate (eGFR). However, they have been shown to be inaccurate, especially in early stages of CKD, in elderly subjects and in individuals with extreme body mass index (BMI) [2]. Moreover, their circulating levels rise after 50% of kidney function is lost, indicating the need for other early biomarkers [3].

To help preventive strategies and maximize the potential for effective interventions, it is important to characterise the molecular changes that take place in the development of kidney diseases. Metabolomics is a powerful tool to discover markers of clinical traits as it captures a snapshot of the individual metabolic profile at a particular time. Moreover, among the available omics, metabolomics is the one toward the end of the ‘omics cascade’ and it is the nearest to the renal phenotype. Metabolomics complements other ‘omics’ data and represents the downstream results in the genome, transcriptome and proteome (Fig. 1).

Different possible mechanisms have been advocated to link metabolites to kidney function: metabolites may accumulate in tubular cells, blood or urine due to an impaired renal function (e.g. creatinine); they may reflect enzyme activity primarily expressed in kidney tissue (e.g. N-acetyltransferase, N-acetyl glucosamine, aminopeptidase or components of the local renin angiotensin system) [4–6]. Moreover, renal impairment may reflect alteration in enzyme activity in various tissues influencing several metabolic processes [7]. Also, metabolites may be uremic toxins and contribute directly to the disease progression e.g. indoxyl-sulphate and asymmetric dimethylarginine (ADMA) (see European Uremic Toxin Work Group EUTox, www.uremic-toxins.org). Indeed, as the kidneys are directly involved in the handling of many biochemical classes of metabolites (by filtering through the glomeruli, transporting in tubuli or generating [8]), changes in metabolite concentrations may reflect impaired kidney function.

Previous reviews have highlighted the potential of metabolomics in the study of kidney diseases, both in humans and in animals [9–11]. They have suggested that metabolomics plays a central role in system biology as it connects molecular interactions of different kidney cells to renal pathophysiology [12–14]. Experimental animal models of CKD have identified important markers of renal function [15–17] although those are beyond the scope of this review.

Here we describe the current status of the identification of blood/urine/faecal metabolic biomarkers in adult renal function focusing on acute kidney injury (AKI), CKD, renal transplant and diabetic nephropathy among others. Studies are subdivided by the type of sample assayed and by renal disorder.

2. Metabolomic technologies

Current estimates suggest that the human metabolome comprises thousands of small molecules, both confirmed and predicted [18,19]. Metabolites vary profoundly in polarity, size and concentration (e.g. mmol/l, nmol/l, pmol/l), ranging from hydrophilic, polar metabolites with a low molecular weight (e.g. amino acids), to hydrophobic, non-polar high-molecular-weight metabolites (e.g. lipids). This diversity means that the method of unbiased detection, identification and quantification of the entire metabolome is technically challenging [19].

No single platform can measure all metabolites. Metabolomic datasets are mainly generated through proton nuclear magnetic resonance (1H NMR) spectroscopy and mass spectrometry (MS).

1H NMR spectroscopy is a quantitative technique that provides detailed information on solution-state molecular structures, based on atom-centred nuclear interactions with a high analytical reproducibility. Furthermore, it is non-perturbing (i.e. samples may...
be re-used many times) and it can be used on tissue biopsies (by magic angle spinning (MAS NMR)) and may be directly compared with histopathological findings from the same tissue sample [20,21]. 1H NMR spectroscopy has a relatively low analytical sensitivity that allows only the detection of high-abundance metabolites (concentration of 100 nmol/l to 1 mmol/l or higher). A further drawback is the relatively high amount of sample volume that is required for 1H NMR analysis [22,23].

MS coupled with separation techniques such as gas chromatography (GC), capillary electrophoresis (CE), high/ultra performance liquid chromatography (HPLC/UPLC) or direct flow injection analysis without prior separation (FIA), offers a higher analytical sensitivity than 1H NMR and allows broader surveys of the metabolome and the potential to detect a significant number of very low-abundance metabolites. However, its preparation steps are very elaborate and can cause metabolite losses [24,25]. In MS, molecules are charged or ionized in the ionization process, and these charged molecules and their fragments are separated according to their mass-to-charge ratio. Multiple ionization techniques have been used in efforts to increase the number of detected metabolites [26]. However, their unambiguous assignment and identification still represents a significant challenge. Another general limitation of MS is its low reproducibility compared with 1H NMR. Retention times and signal intensity are sample and instrument-dependent, thus requiring the use of carefully standardized sample handling and quality controls to provide robust quality assurance [27].

Given their respective strengths and weaknesses, the complementary and simultaneous use of 1H NMR and MS would provide an excellent metabolome coverage. Both 1H NMR and MS can be used in a targeted or untargeted metabolomics.

3. Blood

In adults, the kidneys receive about 20% of the cardiac output and are the most important excretory organ for a wide variety of metabolites. Blood concentration levels of the metabolites may reflect not only a reduced renal function but may also be markers of renal damage in different renal diseases. Because blood metabolite concentrations are influenced by kidney function, some metabolites are currently used for its estimation, most importantly serum creatinine and Cystatin C are used to estimate the glomerular filtration rate. Moreover, the challenge is not only to determine new markers of renal function but also metabolites that can discriminate and detect renal damage under some specific conditions such as acute kidney injury, diabetes nephropathy, glomerulopathies, renal graft rejection etc. A list of blood metabolites consistently associated with kidney disease is presented in Table 1 and Fig. 2.

3.1. Acute kidney injury

The acute decline in kidney function is often secondary to an injury that causes functional or structural damage in the kidneys. Transient azotemia represents one-third of all causes of acute kidney injury (AKI) in hospitalized patients, and is correlated with a significantly increased risk of death [28]. AKI can be defined according to different criteria as a 2-fold increase of serum creatinine, a decrease of GFR higher than 50% or urine output lower than 0.5 mL/kg/h within 12 h. However, the validity of these definitions has been questioned and new criteria for diagnosis of AKI are needed [29]. To date, studies for early and discriminative diagnosis of AKI have focused on specific potential proteins like kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), and few studies have used a metabolomic approach. However, AKI is also a metabolic disease that is partially the result of hypoxia of renal tubular epithelial cells, such that metabolomics analysis will probably result in biomarkers that reflect the altered metabolic function of these cells. The majority of metabolomic studies of AKI have been performed in urine samples of experimental animals. However, some blood metabolite studies have analysed this complex clinical syndrome.

In a pilot study of serum metabolites, seventeen hospitalized patients with newly diagnosed AKI were compared with renal healthy voluntaries [30]. Besides an elevation of creatinine levels, an alteration of lipid profile, an activation of nitric oxide and oxidative stress pathways were also observed. Specifically in this non-targeted LC/MS study an increase in acylcarnitines and amino acids such as methionine, homocysteine, ADMA, and phenylalanine and a reduction in serum levels of arginine and several lysophosphatidylcholines were reported in patients with AKI. An increase in homocysteine is considered a markers of cardiovascular damage [31]. While ADMA and acylcarnitines that are biomarker of defective fatty acid oxidation also associate with renal disease [32–35]. The accumulations of various acylcarnitines in plasma have been also found in CKD, as we will comment on further. Results are in line with previous work on rat that provided evidence that alterations in lipid metabolism as well as oxidative stress are a consequence of kidney ischaemia/reperfusion injury [15–17,36]. These results will need to be further evaluated in larger populations, adjusting for confounding variables and comorbidities.

3.2. CKD

Metabolomics studies in blood have successfully identified metabolites associated with CKD. These include amino acids, steroids, purine, nitric oxide, tryptophan, oxidative stress and lipids, among others [7,32,34,35,37–43]. Though some of these studies are small and do not account for multiple testing corrections, the results are generally consistent across studies.

Differences in metabolomic profiles were observed at different stages of CKD, consistent with altered arginine metabolism, elevated coagulation/inflammation, impaired carbohydrate anion transport and decreased adrenal steroid hormone production [32,34]. Of particular interest are the differences related to arginine metabolism. Shah and collaborators reported that circulating levels of ADMA associated with increased risk of progression of loss of renal function (increase of 8.1 and 4.8 fold in subjects who reached stage 3 and 4 respectively, compared to those who remained at stage 2). Other metabolites related to arginine metabolism were also significantly different in CKD stages 3 and 4 compared with stage 2 include ornithine and citrulline. Interestingly, this negative correlation with the eGFR may reflect an impaired nitric oxide bioavailability with alteration in endothelial function and a subsequent atherosclerotic burden. More recently, Nkuipou-Kenfack and co-workers [44] examined samples from 49 patients at different stages of CKD in plasma and urine and confirmed the association with ADMA and with others acylcarnitine metabolites. They observed increases of ADMA, hydroxykynurenine, and acyl-carnitine levels in plasma and a decrease of ADMA in urine that significantly correlated with a decrease in eGFR. The accumulation of various acylcarnitines in plasma likely depicts impaired clearance due to chronic kidney dysfunction. Besides its function in fatty acid beta oxidation, c-carnitine modulates acyl-CoA levels through esterification to acylcarnitines, thus preventing the accumulation of acyl-CoAs generated in excess in renal failure [45,46]. Otherwise, an excess of acyl-CoAs may contribute to renal and cardiac lipotoxicity, hence the resulting excess acylcarnitines normally are filtered in the glomerulus and undergo only limited renal tubular reabsorption compared to free c-carnitine.

The tryptophan depletion accompanied by an increase of several
The direction of the arrows indicates metabolite concentration increased or decreased with the worsening of the different renal entities. ADMA: Asymmetric dimethylarginine. CE: Capillary electrophoresis. 1H NMR: Proton nuclear magnetic resonance spectroscopy. MS: Mass spectrometry. LC: Liquid chromatography. IDL: Intermediate-density lipoproteins. SDMA: symmetric dimethylarginine. TMAO: Trimethylamine-N-oxide.

For each metabolite, the list of publications relating previous findings does not mean to be exhaustive.
other tryptophan or tyrosine metabolite pathways have been associated with renal function decline. Those metabolites derived from bacterial protein fermentation like of indoxyl-sulphate, p-cresyl-sulphate and phenylacetylglutamine are considered uremic retention solutes. Elevated circulating levels of these metabolites may reflect changes in intestinal flora [48–50]. Moreover, their higher levels in blood might suggest the environmental changes affecting the intestinal flora could be playing a role in modifying the intestinal barrier in the onset of CKD. In this line, a markedly higher serum concentration of TMAO have been recently reported in end stage renal (ESRD) patients (n = 104) comparing to healthy subjects [51]. This metabolite derived from intestinal bacteria is associated

Fig. 2. List of blood, urine and faecal metabolites associated with renal disorders. Colours indicate metabolite concentration increased (Red) or decreased (green) with the worsening of the different renal disorders. If empty, data are not available or are inconclusive. For each metabolite, the list of publications relating previous findings does not mean to be exhaustive.

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with an increased risk of major adverse cardiovascular events [52]. Remarkably, Stubbs et al. reported that increased TMAO concentrations correlate with coronary atherosclerosis burden in a separated CKD cohort (n = 220) [51].

Metabolite analyses have also been used in the study of renal replacement. Thus, a small study on 44 hemodialysis patients and 350 metabolites reported a decrease in lower-molecular-weight triacylglycerols, and an increase in several intermediate-molecular-weight triacylglycerols in end stage renal disease (ESRD) compared to healthy controls, suggesting a disturbed triacylglyceride catabolism and/or β-oxidation [7]. Another study on hemodialysis and in peritoneal dialysis patients’ using 1H NMR showed the metabolic patterns varied depending on the dialysis modality, with hypoxanthine and inosine present only in hemodialysis patients. In line with previous studies, known uremic retention solutes, such as urea, creatinine, myo-inositol and TMAO, were higher in both dialysis groups [37,38]. This suggests that small molecules may be differentially removed depending on the dialysis technique and may provide better potential markers for monitoring patients in extracorporeal dialysis treatment than the current formulas based solely on urea clearance [53].

3.3. Renal transplant

Renal transplantation is the best therapeutic option for patients with end-stage renal disease and is associated with significant improvements in quality of life and survival. So far, clinical, analytical and histopathology parameters are used in the diagnostic of allograft function and rejection [54]. However there are still doubts about the markers that can predict a better graft function or promptly detect a kidney rejection. Blood metabolomics analyses in kidney transplantation have been used to predict acute tubular injury and necrosis as well as allograft rejection. A 1H NMR based metabolic study revealed a decrease in bacterial (TMAO) and energy (Lactate, succinate, citrate) metabolites in patients who reach normal graft function after transplantation. Circulating levels of these metabolites are also associated with eGFR indicating that they are not specific to renal transplant functionality. The same study identified time-dependent changes in the levels of serum metabolites in response to Cyclosporin A (CsA) or Tacrolimus (TAC)-based immunosuppression. The metabolites for which the levels differed between the CsA and TAC groups were glucose, hypoxanthine, lactate, succinate, and taurine. These results provide information about the monitoring of metabolic side effects associated with immunosuppressive drug therapy and graft function after renal transplant. However, its interpretation and usefulness in real clinical practice is not yet established and more studies are needed to identify a metabolite profile associated with anticalcineurinic drug toxicity [55].

The activation of tryptophan catabolisation leads to the formation of kynurenine and other metabolites that counter-regulate immune activation. Recently, serum levels of kynurenine were determined in 307 renal transplant recipients. Patients with immediate functional grafts showed a lower pre-transplant kynurenine level than patients with delayed graft function. The pre-transplant elevated serum kynurenine levels, were highly associated with pre-sensitization status and longer time on hemodialysis treatments, but did not provide prognostic value [56]. Some lipids such as carnitines, choline, phosphatidylcholines, sphingomyelins and lysophosphatidylcholines were found decreased in the acute graft rejection group vs non-acute rejection group, in a small study (n = 27) performed by Zhao X et al. However, the lower serum levels of these lipids in the acute graft rejection group may also be from the reduction of phospholipase A2 activity by high-dose immunosuppressants [57].

3.4. Diabetes nephropathy and other renal disorders

Diabetic nephropathy is a microvascular complication of diabetes mellitus and is the leading cause of CKD in the Western world. Microalbuminuria is the hallmark of the onset of the disease. However, this test is not diabetic nephropathy specific and lacks sensitivity for earliest stages of the disease. Moreover, between 25 and 40% of diabetic patients will develop some grade of nephropathy, but discriminative markers for at risk patients are not well established. Thus, biomarkers of metabolomics could assist earlier diagnosis at a time when specific therapies would be more effective.

Though metabolomic biomarkers of diabetes mellitus have been successfully identified and replicated in several metabolomic studies [58–61], identifying biomarkers for progression to kidney disease in diabetic patients has proven to be challenging. Phospholipids, sphingolipids and sphingomyelins have been reported to be associated with DN in type 1 and type 2 diabetic patients [62–64]. However, most of these studies either focused on cross sectional cohorts that did not allow any causality conclusion, or on albuminuria progression rather than on the kidney failure, the ultimate outcome of DN. For example, a study in 325 type 1 diabetic patients revealed several lipid metabolites to be significantly associated with DN in type 1 diabetic patients [62–64]. Another group analysed 289 serum metabolites in 78 type 2 diabetic patients and identified 19 metabolites that could distinguish between diabetic nephropathy with macroalbuminuria and diabetic patients without albuminuria. The identified metabolites included creatinine, aspartic acid, γ-butyrobetaine, citrulline, symmetric dimethylarginine (SDMA), kynurenine, azelaic acid, and galactaric acid. Progression from microalbuminuria to macroalbuminuria was accompanied by an increase of γ-butyrobetaine and decrease in azelaic acid (β-oxidation), as well as an increase in citrulline, SDMA and kynurenine [64].

A recent interesting study looked for metabolite changes (262 metabolites) in 80 Type 2 diabetic patients with mildly impaired renal function at baseline who developed end stage renal disease (ESRD) during the follow-up. 78 uremic retention solutes were found to increase in ESRD in line with previous results. Of those, sixteen were already elevated at baseline, many years before ESRD developed. Fourteen essential amino acids and their derivatives were significantly associated with ESRD progression. Metabolites that showed the strongest association were myo-inositol, p-cresyl sulphate, phenylacetylglutamine, pseudouridine, C-glycosyl-tryptophan [65]. Notably, some of those metabolites have been previously identified as potential markers of renal function in the general population.

3.4.1. Glomerulopathies

Renal biopsy is the gold standard in the diagnostic of glomerulopathies. However, as an invasive technique it may involve complications. Metabolites have potential as non-invasive alternative diagnostic tools.

Immunoglobulin A nephropathy (lgAN) is the most common cause of CKD among primary glomerulonephritis patients. Few studies have explored biomarkers of lgAN to establish a human IgA nephropathy metabolic profile. In an interventional study on healthy controls (n = 23), low-risk (n = 23) and high-risk IgAN patients (n = 12), 1H NMR metabolomics detected different metabolic signatures in IgAN patients comparing to healthy controls, but not between the low-risk and high-risk groups [66].
Another study used a targeted LC–MS metabolomic approach to analyse oxylipin profiles of IgAN patients before and after supplementation with fish oil (ω-3 fatty acids) or placebo in young adults. Plasma total oxylipins, hydroxyoctadecadienoic and hydroxyeicosatetraenoic acids, and leukotriene B4, were found to be lower in patients whose proteinuria improved [67].

4. Urine

As the most accessible biological fluid associated with the kidney, urine is a natural choice for biomarker discovery. Also the peptides in urine are quite stable and less complex and urine metabolites could reflect changes in functions of the kidney and the urogenital tract. However some limitations should be considered. First, protein-bound metabolites may not be completely filtered through the glomeruli limiting urine detection to the free fraction. Second, an active tubular secretion and absorptions mechanism may influence urinary levels. Third, urine metabolites concentration may vary as a function of the individual’s fluid intake. Urine concentration should therefore be normalised based on creatinine or osmolality prior analysis [68]. Furthermore, certain kidney cells (e.g. medullary interstitium) or fully formed cystic, are not directly exposed to urine and this can be a limitation when studying some renal disorders such as drug nephrotoxicity or polycystic kidney disease.

Though urine proteomics is considered a very promising tool to detect kidney injury, urine metabolomics offers a wide range of measurable metabolites and may offer direct insights into biochemical pathways linked to kidney dysfunction. See Table 2 and Fig. 2 for a list of urine metabolites associated with kidney disease.

### 4.1. Acute kidney injury

There are different causes of AKI and different urinary metabolites profiles can be identified, as a response to the damage to the different kidney structures. To date, no specific markers of AKI have been identified and studies have been conducted in heterogeneous samples with different etiologic backgrounds.

AKI secondary to drug toxicity has been mainly studied in experimental animal models. Similar urine metabolite profiles have been found in nephrotoxicity by aminoglycoside antibiotics, cisplatin [69,70], doxorubicin [71] or melamine [72]. These studies confirmed that these nephrotoxins cause Fanconi-like syndrome (glucosuria, hyperaminoaciduria, lactic aciduria and ketonuria). They also showed that the citric acid cycle is the pathway most affected by nephrotoxins. Thus studies in experimental animals have provided urinary metabolites panels as early markers of acute kidney injury, however their translational applications in humans are not standardized. Going beyond aminoaciduria some human clinical studies identified bacterial metabolites (TMAO), hippuric acid and others like citrate and lactate in urine samples of patients with drug induced AKI secondary to cyclosporine or statins [73,74]. Findings indicate the presence of generalized tubular and renal papillae damage, despite normal serum creatinine values, suggesting these urine metabolites may be better and earlier markers for the kidney injury.

Beger and colleagues studied 40 children undergoing cardiac surgery, prospectively collecting urine after surgery. Twenty-one of these children developed AKI. A metabolite of dopamine, homovanillic acid sulfate, was found to be a reliable maker of AKI with 90% sensitivity and 95% specificity using a cutoff value of 24 ng/ml at 12 h after surgery. The increase in homovanillic acid sulfate in

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### Table 2

<table>
<thead>
<tr>
<th>Metabolites(^4)</th>
<th>Pathway</th>
<th>Detection method</th>
<th>References</th>
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<td><strong>Acute kidney injury</strong></td>
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<tr>
<td>ADMA</td>
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<td>LC–MS, 1H NMR</td>
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<td>Krebs cycle</td>
<td>↓</td>
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<tr>
<td>Hippurate</td>
<td>Benzoate metabolism. Bacterial</td>
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<tr>
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<td>Serotonin metabolism</td>
<td>↑</td>
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<td>Lactate</td>
<td>Pyruvate metabolism. Oxidative stress</td>
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<tr>
<td>TMAO</td>
<td>Bacterial</td>
<td>↑</td>
<td>LC–MS, 1H NMR</td>
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<td>Acylcarnitines</td>
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<td>↑</td>
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<td>ADMA</td>
<td>Urea cycle. Proline metabolism. Nitric oxide</td>
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<td>Pseudouridine</td>
<td>Nucleotide</td>
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The direction of the arrows indicates metabolite concentration increased or decreased with the worsening of the different renal entities. ADMA: Asymmetric dimethylarginine. GC: Gas chromatography. 1H NMR, proton nuclear magnetic resonance spectroscopy LC: Liquid chromatography. MS: Mass spectrometry. TMAO: Trimethylamine–N-oxide.

\(^4\) For each metabolite, the list of publications relating previous findings does not mean to be exhaustive.
this ischemic-reperfusion model of AKI may be tied to homeostasis of the catecholamine system in the kidney [75–77]. These findings may help identify at risk patients and to prioritise early therapeuticst after surgery [78]. However, levels may be also affected by concomitant epinephrine or norepinephrine drugs use, which should be considered in the interpretation of the results.

More recently, a targeted metabolomic analysis was performed in urine samples of patients admitted to the intensive care unit (ICU). Thus, urinary levels of prostaglandin E2 (PGE2), PGJ2 metabolite (2,3-dinor-6-OOX-PGF1a), thromboxane A2 (TXA2) metabolite (11-dehydro-TXB2) were determined. Prostanoids urinary creatinine ratios metabolites (2,3-dinor-6-OOX-PGF1a/CRE and 11-dehy-dro-TXB2/CRE) were found to associate with the subsequent onset of AKI and poor outcomes in 93 adult heterogeneous ICU-patients [79]. Prostanoids regulate several biological functions, including hemodynamics and renal tubular transport, thus urinary levels of PGJ2 and TXA2 metabolites on admission to the ICU may serve as prognostic and diagnostic markers of AKI. However due to the different background etiologies of AKI in intensive care unit patients these results may not be generalized to all causes of AKI.

4.2. CKD

Urine samples of 15 CKD patients were compared to healthy individuals to look for a metabolite signature of CKD in urine. After validation in 16 CKD patients, a panel of 7 metabolites (5-oxoproline, glutamate, guanidoacetate, a-phenylacetylglutamine, taurine, citrate, and trimethylamine N-oxide) was shown to identify and potentially monitor patients with CKD [80]. Recently, plasma and urinary amino acid of 52 individuals with different stage of CKD revealed that depending on solutes, elevated plasmalevels were associated with increased or decreased urinary excretion. Some profiles suggested an overproduction occurring at a systemic level (citrulina), urinary retention (ADMA) and urinary excretion (proline) [81]. This and others studies showed that plasma and urinary levels of ADMA could be used to determine the CKD stage [82]. A metabolomic study examining 49 patients at different stages of CKD that combines plasma and urine samples and urine proteomic analysis was performed by Nkuipou-Kenfack E et al. [44]. A combination of 13 urinary metabolites, including ADMA, acylcarnitines and amino acids, was associated with kidney function and disease progression after a mean of 2.8 years. Combining different biomarkers data (plasma or urine peptide) did not improve the prediction of disease progression and they optimistically suggested these urinary metabolites alone are an effective tool in the diagnosis and follow up of CKD. However, larger scale urine human studies are certainly needed.

4.3. Renal transplant

Biomarkers that can predict graft function and/or renal side effects of calcineurin inhibitors in kidney transplantation are badly needed. Urinary metabolomics has yielded promising preliminary findings in transplantation diagnostics. A pilot study compared urine samples from 5 transplant recipient with acute renal rejection and 8 recipients with no rejection. The analysis revealed 4 unknown small molecules in the rejection group. The authors claimed a specificity of 100% [83]. More recently, Blydt-Hansen and colleagues performed the largest study for detecting borderline and acute T cell–mediated rejection (TCMR) in kidney transplant recipients and to assess its potential as a non-invasive diagnostic tool. Urine samples were analysed using the Biocrates kit in 183 No-TCMR, 54 borderline tubulitis and 30 TCMR. A discriminate score with 10 urinary metabolites suggested an accurate urinary metabolite “signature” that is unique to TCMR. Interestingly, the metabolites that identified TCMR overlapped with those that identified borderline tubulitis, suggesting that allograft injury associated with T cell response exists on a continuum of severity [84].

Calcineurin inhibitor (CNI) related nephrotoxicity remains a major cause of long-term graft loss in kidney transplantation. Specific biomarkers could be used not only to predict the occurrence of graft injury but also to better understand the underlying CNI-related nephrotoxicity processes. Dieme B and co-workers used GC–MS to profile urine samples of 35 kidney transplant patients with Tacrolimus or Ciclosporinregimen in one year follow up. The urinary metabolic patterns varied over time. The principal metabolites that differed, regardless of the treatment used, were mainly sugars, inositol and hippuric acid, metabolites associated previously with renal function as discussed in this review. Interestingly, among tacrolimus treated patients, different metabolic signatures were found between patients with immediate or delayed graft function at day 7 after grafting [85].

Serum and urinary levels of tryptophan and kynurenic acid have been recently used as prognostic and monitoring the renal transplant function [86].

4.4. Diabetes nephropathy and others renal disorders

Non-targeted metabolomics was obtained on 52 type 1 diabetic patients from the FinnDiane study with normo-albuminuria at baseline and after 5.5 years of follow-up to identify markers that could distinguish between progressive and non-progressive albumin forms. The discriminating metabolites included acylcarnitines, acylglycines and metabolites related to tryptophan metabolism. An increase in acylcarnitines and a decrease of hippuric acid in urine are associated with early kidney damage, reflecting alterations in β-oxidation and uremic toxin elimination, respectively [33]. A more recently study was conducted to analyse 94 urinary metabolites in 85 type 1 and type 2 diabetic subjects with diabetic nephropathy, 73 diabetic without renal damage and 23 controls. 12 urinary metabolites were different in diabetic subjects with CKD compared to diabetic subjects without renal damage. Further analysis indicated those metabolites are linked to organic anion transporter-1 (OAT1) and mitochondrial metabolism and suggested a global suppression of mitochondrial activity in diabetic nephropathy [87]. These and other recent experimental studies [88] demonstrate that urine metabolomics is a reliable source for biomarkers of diabetic nephropathy and have potential to uncover new pathways involved in renal damage.

4.4.1. Glomerulopathy

Idiopathic nephrotic syndrome (INS) is caused by an increase of the permeability of the glomerular filtration barrier without evidence of a specific systemic cause. INS is the most common cause of nephrotic syndrome in children between 1 and 10 years of age. Sedic and coworkers, have recently reported a small study of INS using combined urinary proteomics and metabolomics. Label-free mass spectrometric analysis of urinary proteins and small molecule metabolites was carried out in 12 patients with INS versus 12 controls. Findings revealed hydroxyphenylacetate, uridine, glutamate and phenylalanine were involved [89]. Another important cause of nephrotic syndrome is membranous nephropathy (MN). This glomerular entity involves podocyte injury and diffuse thickening of the glomerular basement membrane with the consequent increase in the proteinuria. The metabolites that are overproduced during MN progression enter urine space and are secreted into final urine samples. For this reason, urine possibly is an ideal source to noninvasively characterize the activity of this disease.

Gao and colleagues studied serum and urine metabolites
profiles in subjects with histological diagnostic of MN. A non-target metabolomic approach was performed by GC/MS in 29 patients with urine protein lower than 3.5 g in 24 h collected urine and those with protein higher than 3.5 g in 24 h collected urine. They identified a panel of 26 differential metabolites between the groups. In contrast to lower urine protein subjects the majority of metabolites were increasingly excreted in the urines of higher urine protein excretion individuals. The significantly increased excretion of dicarboxylic acids, threonic acid, quinolinic acid, cholesterol, and phenolic acids in urines with higher protein indicate greater oxidative stress associated with higher kidney damage in MN patients. Those findings are non-specific, but may help in the understanding the pathological pathways and may help in the detection of more severe forms of the disease beyond proteinuria [90].

Urinary metabolites have also been used in the study of other glomerulopathies as IgA. De Angelis and collaborators demonstrated different urinary metabolite profile between patients with non-progressive and progressive IgA glomerulopathy [91]. This study combined microbiota and faecal metabolites analysis and will be discussed in the next section.

5. Faecal

Study of faeces (particularly the gut microbe portion) is proving to be highly effective in health. So far, metabolomic analyses of faecal samples is in its infancy and have been mostly experimental studies in animals but recently, some studies have been conducted on humans and identified promising association with dietary product [92,93] and colo-rectal cancer [94].

It is well recognized that impaired renal function directly affects the intestinal milieu and the uremic environment affects the intestinal barrier leading to bacterial dysbiosis [95,96]. This activates inflammatory pathways and immune processes and leads to systemic inflammation. In this regard, the study of faecal metabolites may help to identify different microbes patterns related to renal function decline.

In animal models of renal tubulointerstitial fibrosis, nine endogenous metabolites relating to bile acids and phospholipids were found to be associated with CKD [97] while seven markers, including Phytosphingosine, Palmitic acid, Cholic acid, Chenodeoxycholic acid, were found to associate with a better therapeutic response after ergone administration [98].

A very recent study in humans (n = 48 patients) looked for faecal microbiota, urine and faecal metabolomein-non-progressive (NP) and progressive IgAN patients and healthy controls (HC). After a six-month-follow-up, patients were classified as NP and progressive according to their proteinuria and renal function. Faecal samples of progressive patients showed the highest level of total free amino acids (FAA) Glucose, Alanine, Aspartate, Valine, Leucine and Proline and the lowest level of Ketoglutaric acid, comparing to NP or HC patients. Faecal samples of HC contained the highest concentration of Cystatin. Faecal 4-Methyl-phenol (or p-cresol) seemed to be over-synthesized in IgAN patients in line with previous studies, highlighting the potential use of faecal metabolites in the study of renal damage [91].

To date, there are no faecal metabolomics renal function studies published in the general population.

In a preliminary yet unpublished study on TwinsUK data, we have looked for association between faecal metabolites and renal function. 57 healthy individuals from the TwinsUK cohort, a National register of adult twins [99], were included in the analysis of 297 known faecal metabolites measured using the Metabolon platform [100]. 96% of the individuals were females, aged 46–88 years and with estimated glomerular filtration rate (eGFR) 81.83 mL/min/1.73 m² [62.01–102.46 mL/min/1.73 m²].

Among the 297 known metabolites we identified eleven faecal metabolites nominally associated to eGFR after adjusting for age, sex, BMI and family relatedness. Of these, two metabolites 3-hydroxybutyrate (BHBA) Beta(SE) = (−4.99 ± 1.30), P = 6.82 × 10⁻⁴, and serotonin Beta (SE) = (−5.59 ± 1.56), P = 1.30 × 10⁻⁵, were insignificant after adjusting for multiple testing using False Discovery Rate (FDR) (Table 3).

These metabolites are very interesting in the study of renal function. Butyrate is a preferred energy source for colonic epithelial cells and is thought to play an important role in maintaining colonic health in humans [101,102]. On the other hand, BHBA, is a precursor of butyrate belonging to the glutarate pathway and has been shown to have a protective effect against oxidative stress [103,104]. In our data, high BHBA correlates with worse renal function suggesting a compensatory response. Serotonin is particularly interesting as it belongs to the tryptophan pathway. Circulating levels of tryptophan have been associated with CKD. It is interesting that, faecal levels of serotonin showed an inverse association with eGFR, which suggest an increase in faecal excretion of tryptophan metabolites with worsening renal function. Among the 11 metabolites associated with eGFR in faeces, only one show a good correlation with circulating blood levels. However due to the small sample size we cannot yet draw firm conclusions (Table 3).

These data should encourage larger studies to investigate the relationships between faecal, urine and blood metabolites. Our pilot highlights the potential of faecal metabolomics as a promising tool to discover early biomarkers and the effect of the diet in the context of complex diseases and renal function.

### Table 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Super pathway</th>
<th>Sub pathway</th>
<th>eGFR</th>
<th>Corr. with blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-hydroxybutyrate (BHBA)</td>
<td>Lipid</td>
<td>Ketone bodies</td>
<td>−4.99(1.3)</td>
<td>0.023</td>
</tr>
<tr>
<td>Serotonin (5HT)</td>
<td>Amino acid</td>
<td>Tryptophan metabolism</td>
<td>-5.59(1.56)</td>
<td>0.03522</td>
</tr>
<tr>
<td>Stearidionate (18:4n3)</td>
<td>Lipid</td>
<td>Long chain fatty acid</td>
<td>3.74(1.26)</td>
<td>0.064</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>Lipid</td>
<td>Bile acid metabolism</td>
<td>4.12(1.53)</td>
<td>0.089</td>
</tr>
<tr>
<td>1-oleylglycerol (1-monooleoin)</td>
<td>Lipid</td>
<td>Monoaoylglycerol</td>
<td>4.38(1.67)</td>
<td>0.097</td>
</tr>
<tr>
<td>acetylcarnitine</td>
<td>Lipid</td>
<td>Carnitine metabolism</td>
<td>3.02(1.19)</td>
<td>0.105</td>
</tr>
<tr>
<td>10-heptadecanoate (17:1n7)</td>
<td>Lipid</td>
<td>Long chain fatty acid</td>
<td>4.35(1.89)</td>
<td>0.136</td>
</tr>
<tr>
<td>1-linoleoylglycerol (1-monolinolein)</td>
<td>Lipid</td>
<td>Monoaoylglycerol</td>
<td>4.07(1.78)</td>
<td>0.137</td>
</tr>
<tr>
<td>docosapentaenoate (n3 DPA; 22:5n3)</td>
<td>Lipid</td>
<td>Essential fatty acid</td>
<td>3.35(1.59)</td>
<td>0.167</td>
</tr>
<tr>
<td>Hyodeoxycholate</td>
<td>Lipid</td>
<td>Bile acid metabolism</td>
<td>3.55(1.7)</td>
<td>0.175</td>
</tr>
<tr>
<td>3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPP)</td>
<td>Lipid</td>
<td>Fatty acid, dicarboxylate</td>
<td>4.03(1.55)</td>
<td>0.175</td>
</tr>
</tbody>
</table>

Table shows results of a pilot study of 57 individuals from the TwinsUK cohort. Right column depicts correlation coefficient between faecal and blood metabolite. *q* value = false discovery rate; is the significance threshold after applying false discovery rate (FDR <5%) adjustment.
6. Future perspectives

There is a real need for novel and reliable biomarkers for early diagnosis and prediction of kidney disease, detection, progression and treatment decisions. More studies in populations with early renal function decline, when therapeutic strategies may be more efficient, are needed.

Metabolomics emerges as a promising tool for biomarker discovery. As we have shown throughout this review, metabolic profiling does not only focus on a single marker but rather on a panel of metabolites. As shown in Fig. 2, some metabolites are associated with multiple kidney phenotypes indicating that some pathways of kidney damage are shared regardless of the etiology.

In this review we have highlighted some examples of clinical relevance. However, any renal disorder may have its specific biomarkers and the challenge remains to uncover those metabolites capable to properly identify and predict the disease outcome. For example diabetic nephropathy is the leading cause of CKD in the Western world, however to date we lack a specific marker to diagnose early renal damage rather than albuminuria which may be present in other several renal pathologies. Another example is the lack of markers in renal transplant that can help discern between drug toxicity and allograft rejection.

Integrating the results of more than one biosample may provide better clues to biological and pathological pathways involved in the renal damage. A systematic integration of the multidimensional ‘omics’ data and a translation into biologically meaningful functions are the next steps. Pesce and co-workers [105] reviewed a holistic approach by integrating experimental data from ‘omics’ technologies with mathematical models in nephrology. However, methods to integrate and analyse metabolites correlation across different biological samples are still needed. Faecal metabolites are a promising tool to further understand pathophysiological pathways of renal damage. Indeed, they may aid the integration between microbiota and systemic metabolism.

Unfortunately we are still some way from translating the current findings into real clinical practice. We need to perform more quantitative analyses that allow us to extrapolate the findings to the clinic with proper reference values. Multicentre studies with larger populations and joint collaborative efforts should help overcome some of the current road blocks and form the basis for precision medicine approach in managing the renal diseases (Fig. 1).

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