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Review Article

Altered microbiome in chronic kidney disease: systemic effects of gut-derived uremic toxins

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In chronic kidney disease (CKD), influx of urea and other retained toxins exerts a change in the gut microbiome. There is decreased number of beneficial bacteria that produce short-chain fatty acids, an essential nutrient for the colonic epithelium, concurrent with an increase in bacteria that produce uremic toxins such as indoxyl sulphate, *p*-cresyl sulphate, and trimethylamine-N-oxide (TMAO). Due to intestinal wall inflammation and degradation of intercellular tight junctions, gut-derived uremic toxins translocate into the bloodstream and exert systemic effects. In this review, we discuss the evidence supporting a role for gut-derived uremic toxins in promoting multiorgan dysfunction via inflammatory, oxidative stress, and apoptosis pathways. End-organ effects include vascular calcification, kidney fibrosis, anemia, impaired immune system, adipocyte dysfunction with insulin resistance, and low turnover bone disease. Higher blood levels of gut-derived uremic toxins are associated with increased cardiovascular events and mortality in the CKD population. Clinical trials that have examined interventions to trap toxic products or reverse gut microbial dysbiosis via oral activated charcoal AST-120, prebiotics and probiotics have not shown impact on cardiovascular or survival outcomes but were limited by sample size and short trials. In summary, the gut microbiome is a major contributor to adverse cardiovascular outcomes and progression of CKD.

Introduction

A healthy adult harbors approximately 100 trillion gut bacteria – this is ten-fold greater than the number of cells in the human body [1]. The genome of the gut microbiome encompasses 3.3 million genes [2] which is 150 times larger than the human genome. The gut bacteria co-evolved in symbiosis with its host and is essential for regulation of local and systemic immunity [3,4], acting as the ‘outside-in’ modifier of T cell and natural killer cells subsets [5–7]. Over 50 bacterial phyla colonize the healthy gut, with the anaerobic Bacteroidetes and Firmicutes contributing >90% of bacterial species [2]. The abundance and diversity of bacteria increases from the stomach (10^2 – 10^4 cells/ml) to the colon ($>10^{12}$ cells/ml) concurrent with decreasing oxygen tension [3].

The intestinal microbiome is markedly altered in chronic kidney disease (CKD) and results in increased production of bacteria-derived uremic toxins such as indoxyl sulphate, *p*-cresyl sulphate, and trimethylamine-N-oxide (TMAO). These toxins induce breakdown of the gut epithelial barrier which in turn facilitates translocation of toxins into the systemic circulation. In this review, we discuss the evidence supporting a role for gut-derived uremic toxins in promoting multiorgan dysfunction (Figure 1) via inflammatory, oxidative stress, and apoptosis pathways.

Altered gut microbiome in CKD

Recent progress in genetic sequencing techniques via bacterial 16S rRNA has resulted in a more thorough characterization of the gut microbiome in health and disease. While a healthy microbiome is

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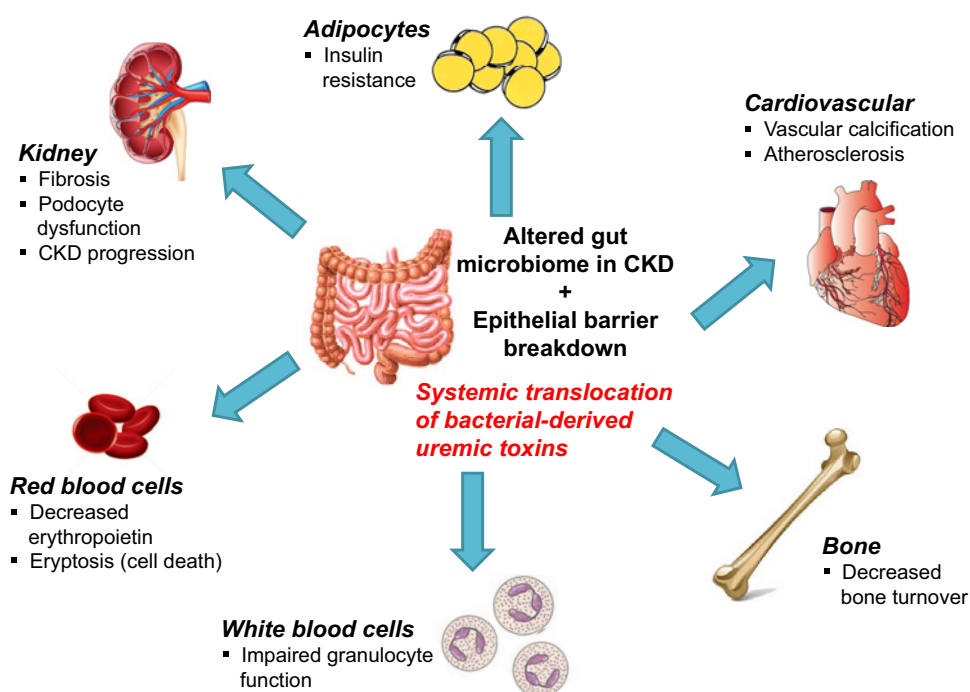


Figure 1. Microbiome alterations and the leaky gut epithelial barrier in CKD results in systemic translocation of bacterial-derived uremic toxins such as indoxyl sulphate and *p*-cresyl sulphate
These toxins induce oxidative stress and damage in multiple organ systems.

defined by the diversity in bacterial species [8], over 50% of healthy individuals have the same 75 bacterial species in common and only seven to nine phyla (from the 55 known bacterial phyla) are detected in humans [2]. Over 90% of the bacteria identified in the gut microbiome belong to the Bacteroidetes and Firmicutes phyla, which include the bacterial genera of *Bacteroides*, *Alistipes*, *Prevotella*, *Porphyromonas*, *Clostridium*, *Dorea*, *Faecalibacterium*, *Eu-bacterium*, *Ruminococcus*, and *Lactobacillus* [2]. Analyses of fecal metagenomes from four countries demonstrated that there are three major enterotypes of the human microbiome [9].

A symbiotic relationship with the gut microbiota is essential for health. The normal microbial flora shapes the adaptive immune system after birth [10], regulates local and systemic immunity [3–7], contributes to micronutrient homeostasis (production of short-chain fatty acids, various vitamins such as group B vitamins and vitamin K) and nitrogen balance (synthesis of amino acids, e.g. lysine and threonine) [11,12]. Disruptions in the gut microbiome have been implicated in progression of numerous illnesses including CKD, inflammatory bowel diseases, diabetes, dyslipidemia, obesity, cardiovascular diseases, cancer, allergic disorders, and IgA nephropathy amongst others [13–18].

Simenhoff and colleagues [19] performed endoscopy in CKD and non-CKD individuals in the 1970s and were the first to demonstrate markedly altered gut flora in CKD patients. They noted that antibiotic treatment altered the microbiome and favorably decreased serum levels of amine toxins, concurrent with improved mentation [20]. The duodenum and jejunum, which are only lightly colonized in a healthy individual, become intensely colonized by aerobic and anaerobic bacteria in uremic patients [19,21].

Gas chromatography studies have shown significantly altered exhaled breath gases in CKD rats [22] and end-stage kidney disease (ESKD) patients [23] compared with healthy controls, further testament to the altered gut microbe composition. It has been proposed that influx of urea and other retained toxins into the gut lumen exerts a selection pressure for microbes that express urease, uricase, and indole and *p*-cresyl-forming enzymes [24,25]. These changes in the gut flora are exacerbated by the low potassium, low phosphorus CKD diet which correlates to a diet low in fermentable plant fiber and poor in symbiont-rich cheese/yogurt [26]. In a fiber-rich diet, plant-derived resistant starches transit intact to the colon where they are degraded by *Bacteroides* and fermented to release short-chain fatty acids (acetate, butyrate, propionate, and D-lactate) which are an important nutrient source for colonic cells [3]. Fecal analysis has demonstrated that dialysis patients show decreased numbers of bacteria that are able to produce the short-chain fatty acid butyrate [25].

Table 1 Gut bacteria-derived uremic toxins that have been associated with adverse effects on the cardiovascular, kidney, and adipocyte systems in CKD

Name	Source	Class
Indoxyl sulphate	Tryptophan	Protein-bound
Indole-3 acetic acid	Tryptophan	Protein-bound
<i>p</i> -cresyl sulphate	Phenylalanine, tyrosine	Protein-bound
TMAO	Choline, L-carnitine, PPC	Water-soluble
Phenylacetylglutamine	Phenylalanine	Water-soluble

Abbreviation: PPC, phosphatidylcholine.

Therefore, the gut environment in CKD suffers from combined disruptions to the microbial framework and lack of fiber substrate, adversely impacting the local nutrient environment for enterocytes [3]. It was recently proposed that use of oral iron supplements might further contribute to gut microbiome alterations [27], extrapolating from studies in African children whereby supplemental iron led to decreased abundance of generally beneficial bacteria including *Lactobacillus* and *Bifidobacterium* species [28] as well as increased gut permeability [29].

In an earlier study, our group isolated microbial DNA from the stool samples of a group of hemodialysis patients and a group of age-, sex- and ethnicity-matched healthy individuals; via a phylogenetic microarray technique, we demonstrated highly significant differences in the abundance of over 200 bacterial operational taxonomic units (OTUs) belonging to 23 bacterial families between the ESKD and the healthy control groups. The OTUs from the Beutenbergiaceae, Cellulomonadaceae, Dermabacteraceae, Micrococcaceae, Catabacteriaceae, Clostridiaceae, Coprobacillaceae, Polyangiaceae, Alteromonadaceae, OM60, Enterobacteriaceae, Methylococcaceae, Halomonadaceae, SUP05, Moraxellaceae, Pseudomonadaceae, Thiotrichaceae, Xanthomonadaceae, and Verrucomicrobiaceae families were markedly increased whereas those belonging to the Prevotellaceae, Lactobacillaceae and Alcaligenaceae families were markedly reduced in ESKD patients [24]. In order to isolate the effects of uremia from those of co-morbid conditions, medications, diet, and other inter-individual variations, the gut microbiome was studied in rats 8 weeks after 5/6 nephrectomy or sham operation. The CKD rats showed significant differences in the abundance of 175 bacterial OTUs, thus confirming the effect of uremia *per se* on the composition of the gut microbiome [24]. In a subsequent study, microbial genomic analyses confirmed that ESKD results in expansion of bacterial families possessing urease, uricase and *p*-cresyl- and indole-forming enzymes concurrent with depletion of the bacteria that produce short-chain fatty acids [25]. Amongst the 19 microbial families that were dominant in ESKD patients, 12 possessed urease (Alteromonadaceae, Cellulomonadaceae, Clostridiaceae, Dermabacteraceae, Enterobacteriaceae, Halomonadaceae, Methylococcaceae, Micrococcaceae, Moraxellaceae, Polyangiaceae, Pseudomonadaceae, and Xanthomonadaceae), 5 possessed uricase (Cellulomonadaceae, Dermabacteraceae, Micrococcaceae, Polyangiaceae and Xanthomonadaceae families), and 3 possessed indole and *p*-cresyl-forming enzymes (i.e. tryptophanase possessing families: Clostridiaceae, Enterobacteriaceae, and Verrucomicrobiaceae) [25]. Lactobacillaceae and Prevotellaceae, two families that possess short-chain fatty acid (butyrate) forming enzymes, were amongst the four microbial families that were depleted in ESKD patients (Figure 2).

Breakdown of the gut–epithelial barrier and systemic translocation of bacterial toxins

Uremic toxins are traditionally classified according to the physicochemical characteristics affecting their clearance during dialysis. These include small water-soluble molecules (molecular weight (MW) <500 Da), larger ‘middle molecules’ (MW >500 Da), and the protein-bound molecules [30]. Alternatively, uremic toxins may be classified according to their site of origin. In the presence of disordered intestinal colonization in CKD, there is increased production of gut-derived uremic toxins such as indoxyl sulphate, *p*-cresyl sulphate, and TMAO (Table 1) [31–33]. Primary actions of microbial flora include deamination and decarboxylation of amino acids. This results in release of hydrogen sulphide from sulphur-containing amino acids (methionine and cysteine), and production of ammonia and short-chain fatty acids or phenolic compounds (indole and *p*-cresyl) from aromatic amino acids (tyrosine, phenylalanine, and tryptophan) [34]. Decarboxylation of amino acids also results in formation of different amines which serve as precursors for nitrosamines.

There are currently five different gut-derived uremic toxins that have been associated with cardiovascular disease and mortality in CKD as well as other end-organ toxicity: indoxyl sulphate, indole-3 acetic acid, *p*-cresyl sulphate,

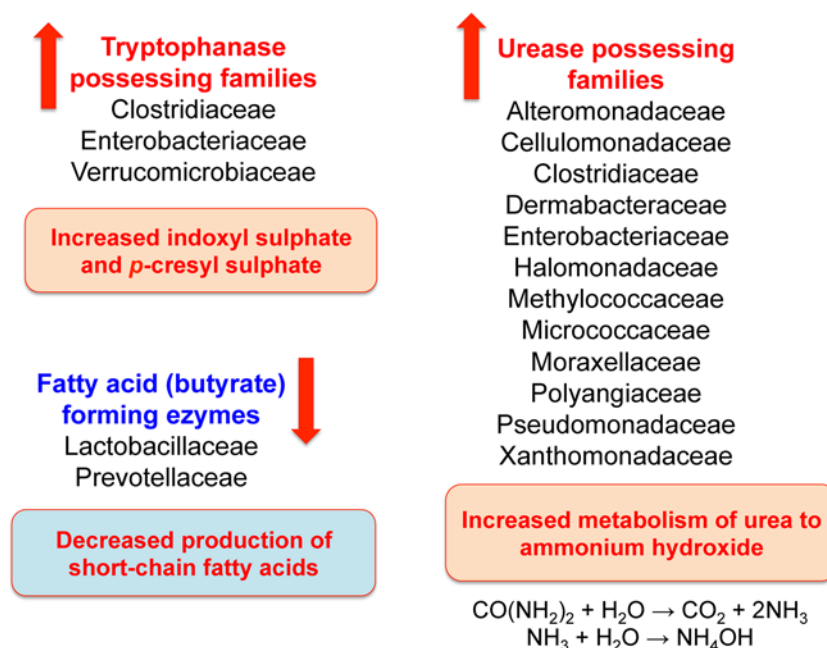


Figure 2. The gut microbiome in CKD shows expansion of bacterial families that express indole and *p*-cresyl enzymes which generate toxins from tryptophan

There is also expansion of microbial families that express urease which contributes to gut wall inflammation as follows: urea diffuses from the blood into the gut lumen and is metabolized by bacterial urease to ammonia [$\text{CO}(\text{NH}_2)_2 + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3$]; ammonia is hydrolyzed into caustic ammonium hydroxide [$\text{NH}_3 + \text{H}_2\text{O} \rightarrow \text{NH}_4\text{OH}$] which causes enterocyte damage. Finally, there is a decrease in bacterial families that produce short-chain fatty acids which are an essential nutrient source for the host enterocytes.

TMAO, and phenylacetylglutamine (Table 1). Indoxyl sulphate and indole-3 acetic acid are protein-bound uremic toxins that result from the metabolism of dietary tryptophan [35]. Tryptophan is metabolized into indole by intestinal bacteria and after intestinal absorption is sulphated in the liver. Indoxyl sulphate cannot be efficiently removed by conventional hemodialysis because of its high binding affinity for albumin [36,37]. *p*-cresol/*p*-cresyl sulphate are products of phenylalanine and tyrosine catabolism by anaerobic gut bacteria. *p*-cresol is conjugated by intestinal microbes to *p*-cresyl sulphate (the main circulating metabolite of *p*-cresol) and *p*-cresyl glucuronide, and metabolized to *p*-cresyl glucuronide in the liver [38]. *p*-cresyl sulphate is considered the effective toxin, due to its significantly circulated concentration and biochemical impact in the body [39]. TMAO is a gut-derived toxic metabolite derived from bacterial metabolism of quaternary amines such as phosphatidylcholine, betaine, or L-carnitine to release trimethylamine. Trimethylamine is subsequently absorbed and converted into TMAO by flavin monooxygenase enzymes in the liver. Unlike the protein-bound toxic metabolites such as indoxyl sulphate and *p*-cresyl sulphate, TMAO is efficiently removed by dialysis. Phenylacetylglutamine is another colonic microbial product, produced from fermentation of the amino acid, phenylalanine. Microbes metabolize phenylalanine to phenylacetic acid, which undergoes glutamine conjugation to form phenylacetylglutamine. Only ~20% of phenylacetylglutamine is protein bound [40], thus, like TMAO it is dialyzable.

There is now a wealth of evidence demonstrating that the intestinal barrier is disrupted in CKD, which leads to translocation of bacteria-derived uremic toxins and other noxious luminal contents into the systemic circulation thus inducing inflammation and leukocyte stimulation [41,42]. Indoxyl and *p*-cresyl sulphate are associated with increased levels of inflammatory markers in stage 3–4 CKD patients, such as interleukin-6 and glutathione peroxidase [43,44]. The 10^{12} bacterial cells/ml in the colon lumen is separated from the host body by a single-layer epithelium. In the healthy gut, there is a barrier consisting of mucus, defensins, and lectins that shield the epithelium and immune system from direct contact with the microbiota [5,45]. In autopsy studies in the 1980s, Vaziri and colleagues [46] discovered chronic inflammation throughout the gastrointestinal tract of chronic hemodialysis patients, extending from esophagus to large bowel. These inflammatory changes sometimes coexisted with peptic ulcer disease and ischemic lesions [46]. Permeability of the intestinal wall in CKD rats and in CKD patients was demonstrated in the early 1990s in studies that detected appearance of orally administered PEGs of various MWs in the urine [47,48].

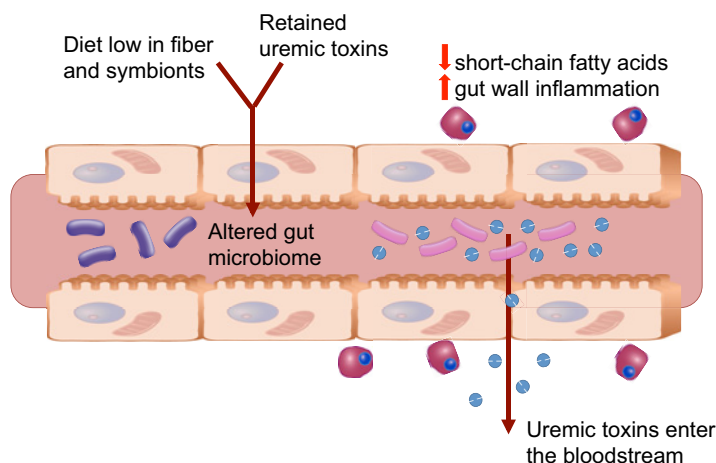


Figure 3. Influx of retained uremic toxins including urea into the gut lumen alters the microbial population

The intestinal environment is further altered due to low fiber/low symbiont intake in CKD patients who are advised to adhere to a low potassium, low phosphorus diet (thus decreased intake of fruit, vegetables, yogurt, dairy). The altered microbiome is associated with gut wall inflammation, breakdown of tight junctions, decreased production of short-chain fatty acids (an important nutrient source for enterocytes), and generation of gut-derived uremic toxins such as indoxyl sulphate and *p*-cresyl sulphate. These uremic toxins translocate into the bloodstream and induce systemic effects as outlined in Figure 1 (image credit of enterocyte, lymphocyte, and bacteria from Reactome project [133]).

Since the time of these reports, there has been significant progress toward understanding the structure of the gut–epithelial barrier. The apical junctional complex is composed of transmembrane proteins including claudins (a group of at least 20 tissue-specific proteins), occludin, E-cadherin, and cytoplasmic linker proteins such as zonula occludens [49]. Rodent studies have confirmed that CKD is associated with depletion of gut epithelial tight junction proteins including occludin and claudin-1 [50,51] and deficiency of the antioxidant transcription factor Nrf2 [52]. This leaky barrier allows translocation of gut-derived bacterial DNA and uremic toxins into the bloodstream of CKD patients [43,53–57]. Using 16S ribosomal amplification and pyrosequencing, Shi and colleagues [54] were able to detect bacterial DNA (majority of gut origin) in the plasma of 12 out of 52 chronic dialysis patients. Plasma bacterial DNA correlated with increased C-reactive protein and interleukin-6 as well as the gut permeability biomarker D-lactate [54]. As further confirmatory evidence, colon bacterial DNA is detectable in the mesenteric lymph nodes, liver, spleen, and blood of CKD rats [58]. Endotoxin, derived from the cell wall of Gram-negative bacteria, is measurable in the blood of dialysis patients and correlates with severity of systemic inflammation in the absence of overt infection [55]. Levels of circulating endotoxin increase with severity of CKD stage and are most elevated in patients on chronic hemodialysis and peritoneal dialysis [56,57].

Several mechanisms are likely to contribute to intestinal tight junction breakdown in CKD. Urea diffuses from the blood into the gut lumen and is metabolized by bacteria-derived urease, producing ammonia that is hydrolyzed into caustic ammonium hydroxide, which erodes the epithelial barrier [59,60]. This stimulates influx of leukocytes, which triggers the second mechanism whereby local inflammation and cytokine production induces retraction and endocytosis of the transcellular tight junction proteins (claudins and occludin) [61,62]. As noted above, short-chain fatty acids produced by gut bacteria are an important nutrient source for enterocytes, and a shift in the bacterial population could theoretically jeopardize the health of the epithelial barrier (Figure 3).

Although endogenous metabolism is the prime contributor to the CKD milieu, a definitive study by Aronov and colleagues [63] demonstrated that a significant fraction of uremic retention solutes originates from the intestinal tract. The investigators compared plasma solutes in three groups: healthy individuals, hemodialysis patients with intact colons, and hemodialysis patients who had undergone total colectomy [63]. Employing high-resolution MS, these investigators verified the colonic origin of a number of well-known uremic toxins such as indoxyl sulphate and *p*-cresyl sulphate as well as numerous as-yet unidentified compounds in the plasma of patients with ESKD. Over 30 mass spectroscopy-detected solutes were present in the plasma from ESKD patients with intact colons which were either absent or present in significantly lower concentration in those without colons [63]. Nearly all of these compounds were significantly more abundant in plasma from the ESKD patients than in the healthy control individuals,

and as such represented uremic solutes. These findings provided irrefutable evidence for the colonic microbes as the source of numerous uremic solutes – many of which are yet to be identified.

Systemic effects of gut-derived uremic toxins (Figure 1)

Kidney fibrosis and CKD progression

In the 1990s, Niwa and colleagues demonstrated that indoxyl sulphate stimulated monocyte infiltration in the remnant kidney of 5/6-nephrectomized rats, resulting in increased transforming growth factor- β 1 and progression of renal failure [64]. Plasma levels of gut microbial-derived TMAO have been correlated with increased 5-year mortality risk in CKD subjects [65] likely to be via cardiac and renal injury pathways. Adding TMAO to the diet in animal models has been shown to increase tubulointerstitial fibrosis with progressive loss of kidney function [65].

More recently, Ichii and colleagues [66] examined the toxic effects of indoxyl sulphate on podocytes. Sustained exposure to indoxyl sulphate in mice lead to an increase in aryl hydrocarbon receptor (AhR), a transcription regulator in podocytes [66]. Pathologic changes included wrinkled glomerular basement membrane, foot process effacement, and formation of cytoplasmic vacuoles in podocytes due to down-regulation of structural actins, integrins, and collagen [66]. Other investigators have shown that *p*-cresyl sulphate induces reactive oxygen species in HK-2 human tubular epithelial cells [67]. There was an increase in certain NADPH oxidases such as Nox4. Further, knockdown of Nox4 reduced the pro-inflammatory effects of *p*-cresyl sulphate in cell culture [67,68]. Finally, daily intraperitoneal injection of *p*-cresyl sulphate for 4 weeks in 5/6-nephrectomized rats enhanced production of reactive oxygen species in the remnant kidney and promoted interstitial fibrosis [67].

Cardiovascular disease and mortality

The majority of outcomes research in the field of uremic toxins has been in the cardiovascular system. CKD is a state of accelerated cardiovascular disease which remains the leading cause of death in this patient population [69–71]. Non-traditional risk factors contributing to cardiovascular disease in CKD patients include chronic inflammation, oxidative stress, protein wasting, dysregulation of endogenous calcification inhibitors, and protein-energy wasting [72–74]. As mentioned above, five different gut-derived uremic toxins have been associated with cardiovascular disease and mortality in CKD: indoxyl sulphate, indole-3 acetic acid, *p*-cresyl sulphate, TMAO, and phenylacetylglutamine.

In CKD patients, serum indoxyl sulphate levels have been shown to have an inverse relationship with kidney function and a direct relationship with aortic calcification, pulse-wave velocity, first heart failure event, and all-cause and cardiovascular mortality [75–77]. The mortality association remained after adjustment for age, gender, diabetes mellitus, albumin, hemoglobin, phosphorus levels, and aortic calcification [75]. Indole-3 acetic acid is an agonist of the transcription factor AhR which regulates vascular inflammation, oxidative stress, and atherosclerosis [78,79]. In CKD patients, serum indole-3 acetic acid $>3.73 \mu\text{M/l}$ correlates with higher mortality and cardiovascular events even after adjustment for traditional cardiovascular risk factors and for other uremic toxins *p*-cresyl sulphate and indoxyl sulphate [80]. *p*-cresyl sulphate has been shown to be an independent predictor of cardiovascular events, with median levels of free (not total) *p*-cresyl sulphate $>0.051 \text{ mg/100 ml}$ being strongly associated with mortality [81]. *p*-cresyl sulphate has also correlated with increased pulse-wave velocity indicative of vascular stiffening [43]. The precursor *p*-cresol has also been associated with cardiovascular events in CKD patients [82–84].

High serum TMAO concentrations strongly associate with incident cardiovascular events in humans with intact kidney function, providing solid clinical evidence to support a link between TMAO and cardiovascular pathology [85]. Chronic dietary L-carnitine supplementation in mice significantly altered the gut microbiome, increased synthesis of TMAO, and increased atherosclerosis; these effects were ameliorated with suppression of intestinal microbiota [85]. Serum TMAO concentrations substantially increase with decline in kidney function, and this effect is reversed by renal transplantation [86]. Data from the Diabetes Genome Project demonstrated that increased TMAO concentrations correlate with coronary atherosclerosis and long-term mortality in patients with CKD undergoing coronary angiography [86]. A separate group of investigators demonstrated that plasma TMAO levels correlate with increased 5-year mortality risk in CKD subjects after multivariate adjustment [65]. Related to TMAO, polyamines are organic cations including cadaverine, spermine, spermidine, and putrescine that arise from the decarboxylation of L-arginine, L-ornithine or lysine by gut bacteria. In CKD patients, serum levels of putrescine, spermidine, and spermine are increased [87]. These molecules have been shown to interact with insulin and lipoproteins, thus promoting acceleration of atherosclerosis [88]. Another water-soluble uremic toxin, phenylacetylglutamine, has also been demonstrated to correlate independently with mortality and cardiovascular disease in a prospective study of 448 CKD patients [89].

Hypertension is a major cardiovascular risk factor arising from a complex interplay of both genetic and environmental factors [90]. Germ-free mice that lack intestinal bacteria have relatively lower blood pressure when compared with conventional mice [91] and fecal transplantation from hypertensive human donors to germ-free mice elevates blood pressure in these animals [90], suggesting that the gut flora influences blood pressure. In addition, rat studies and randomized clinical trials suggest that administration of probiotics can reduce blood pressure [92,93].

At the cellular level, uremic toxins affect the function of endothelial cells and vascular smooth muscle cells (VSMCs) and platelets [94,95]. Endothelial function is dependent on nitric oxide and nitric oxide production is decreased in patients with CKD. Both indoxyl sulphate and *p*-cresyl sulphate at concentrations typically found in uremia have been shown to reduce endothelial proliferation and repair *in vitro* [44]. One important mechanism for CKD-associated nitric oxide deficiency is increased oxidative stress. Inhibition of NADPH oxidase or use of antioxidants such as vitamin E, N-acetyl-L-cysteine, and vitamin C suppressed indoxyl sulphate-induced production of reactive oxygen species and preserved endothelial cell viability [96]. Further, indoxyl sulphate in higher concentrations has been shown to negatively influence protective properties of endothelial cells, such as migration and tube formation, by depleting nitric oxide bioavailability [97,98]. Of note, indoxyl sulphate at normal physiologic levels in non-CKD individuals appears to have an antioxidant effect via scavenging free radicals [99]. Finally, indoxyl sulphate influences cellular senescence and has been shown to aggravate aortic calcification in tandem with asymmetric dimethylarginine in hypertensive rats [100,101].

Uremic toxins have been shown to promote VSMCs proliferation and transformation into osteoblast-like cells capable of producing a matrix of bone collagen and non-collagenous proteins. This matrix in the setting of dysregulated mineral metabolism leads to vascular wall thickening and calcification [102,103]. Yamamoto and colleagues [104] demonstrated that indoxyl sulphate causes VSMCs proliferation via activation of the p44/42 mitogen-activated protein kinase pathway *in vitro*. Treatment with a MAP kinase inhibitor or blockade of the organic anion transporter ameliorated indoxyl sulphate's effects and decreased VSMCs proliferation [104].

Leukocyte dysfunction

In the 1990s, Vanholder and colleagues [105] demonstrated that *p*-cresol exerted dose-dependent inhibition of granulocyte respiratory burst reactivity and phagocytosis. Longer incubation times to simulate *in vivo* conditions further depressed granulocyte function and was confirmed by three different methods (carbon dioxide production, chemiluminescence, flow cytometry) [105]. Aside from cardiovascular events, *p*-cresyl sulphate levels were associated with increased infection-related hospitalization in dialysis patients [81].

Anemia

Indoxyl sulphate interferes with the production of erythropoietin partly by impairing cellular oxygen-sensing mechanisms [106,107]. Treatment of HepG2 human hepatoma cells with indoxyl sulphate impaired nuclear accumulation of hypoxia-inducible transcription factors and decreased erythropoietin mRNA [106]. Further, indoxyl sulphate has been shown to induce eryptosis (programmed death of red blood cells), partly by increasing cytosolic calcium [108].

Adipocyte dysfunction and insulin resistance

The mechanisms underlying the insulin resistance that frequently accompanies CKD are poorly understood, but there is evidence that gut-derived uremic toxins play a role. Uremic toxins have been associated with impaired insulin response in CKD models. In rats with subtotal nephrectomy, oxidative stress and suppressed insulin signaling in adipose tissues correlates with increased circulating indoxyl sulphate [109]. Adipose tissue expression of the AhR is increased in rats with CKD. *In vitro* treatment of adipocytes with indoxyl sulphate induces production of reactive oxygen species and this effect is prevented by the NADPH oxidase inhibitor apocynin [110]. Koppe and colleagues [111] demonstrated that *p*-cresyl sulphate induced insulin resistance in cultured muscle and fat cells, and intraperitoneal injections of the toxin for 4 weeks in healthy mice similarly triggered insulin resistance concurrent with ectopic redistribution of lipid in skeletal muscle and liver. Treatment of CKD mice with the prebiotic arabino-xylo-oligosaccharide to decrease intestinal production of *p*-cresol ameliorated these metabolic derangements [111].

Bone disease

Indoxyl sulphate has been shown *in vitro* to promote dose-dependent oxidative stress in osteoblasts and resistance to parathyroid hormone [112]. This may predispose clinically to adynamic bone disease, as suggested by studies in rats after parathyroidectomy where dietary supplementation with indole (a precursor of indoxyl sulphate) raised blood

levels of indoxyl sulphate and decreased bone remodeling [113]. In CKD patients, there is a positive correlation between serum fibroblast growth factor-23 with indoxyl sulphate levels, supporting an association between gut-derived uremic toxins and metabolic bone disease [114].

Interventions to attenuate gut microbiome disturbances in CKD

CKD patients are placed on a low potassium, low phosphorus diet as high serum levels of these electrolytes correlate strongly with increased mortality risk [115]. Unfortunately, these dietary restrictions alter gut microbial composition, and the potassium restriction in particular goes against the ‘heart healthy’ vegetable-rich diet that is associated with less cardiovascular events in the general population. Theoretically, judicious intake of vegetable fiber and symbiont-rich yogurt/cheeses (perhaps with concomitant use of potassium-binding resins and phosphate binders) may result in a more balanced gut microbiome and thus improve inflammatory parameters, but randomized controlled trials are lacking in this area.

Various approaches have been explored in an attempt to attenuate gut microbial alterations and hence systemic inflammation and uremic toxicity in the CKD population. While some therapies appear promising in terms of decreasing circulating levels of gut-derived uremic toxins and slowing eGFR loss, none have been shown at this time to impact cardiovascular or mortality outcomes in randomized controlled trials.

Activated charcoal has been widely used as a decontaminant in cases of acute poisoning and as an effective degassing agent to reduce abdominal bloating. AST-120, a highly potent activated charcoal preparation has been shown in animal models to partially restore expression of colonic tight junction proteins, reduce monocyte activation, and lower inflammatory markers such as endotoxin, IL-6, and TNF- α [116,117]. In small patient cohorts, AST-120 therapy was reported to lower plasma levels of indoxyl sulphate and *p*-cresyl sulphate and retard progression of CKD [118–120]. However, randomized controlled trials in Japan and the U.S.A. failed to show beneficial effects in CKD patients in terms of slowing CKD progression [121,122].

An area of potential therapeutics that is being actively studied is the use of prebiotics (non-digestible food ingredients that can stimulate growth and/or activity of beneficial gut bacteria) and probiotics (living organisms ingested via food or supplements that can improve the health of the host). Our group previously reported that feeding uremic rats amylose maize-resistant starch (a prebiotic) improved creatinine clearance and reduced kidney inflammation and fibrosis [123]. A follow-up study revealed marked improvements in serum, urine, and intestinal fluid metabolomics in conjunction with decreased gut microbial dysbiosis [124]. Resistant starches transit to the colon undigested and are metabolized by bacteria to short-chain fatty acids which are important nutrients to enterocytes. Small trials in hemodialysis patients have demonstrated that oligofructose-inulin or resistant starch supplementation significantly reduced circulating indoxyl sulphate and *p*-cresyl sulphate levels [125,126]. A meta-analysis of controlled feeding trials found that fiber supplementation significantly decreased serum urea levels in a pooled cohort of 143 CKD patients but there was significant interstudy heterogeneity and urea lowering was not dose dependent; end points such as cardiovascular events were not addressed [127].

Marques and colleagues [128] examined the effect of control diet compared with high-fiber or acetate-supplemented diet in mineralocorticoid excess-treated mice. Both fiber and acetate decreased gut dysbiosis as measured by the ratio of Firmicutes to Bacteroidetes, and increased the prevalence of *Bacteroides acidifaciens*. Compared with mineralocorticoid-excess mice fed a control diet, both high-fiber diet and acetate supplementation significantly reduced systolic and diastolic blood pressures, cardiac fibrosis, and left ventricular hypertrophy [128]. These data suggest that the gut microbiome contributes to the association between diet high in fruits/vegetables and lower incidence of hypertension.

Another strategy that can be used to increase delivery of undigested carbohydrates to the colonic bacteria is administration of an inhibitor of small intestinal α -glycosidase such as acarbose. Evenepoel and colleagues [129] demonstrated that administration of acarbose significantly reduced *p*-cresol levels in the urine, plasma, and feces of a group of individuals with normal kidney function. Acarbose has not been tested in CKD patients and its use may be limited by side effects of bloating and diarrhea.

Probiotics have been tested in CKD with the goal to produce a less pathogenic microflora and thus reduce generation of uremic toxins but results have been mixed. A pilot multinational trial in patients with CKD stages 3 and 4 noted significant decrease in blood urea levels and improved quality of life scores after treatment with the Renadyl formulation of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum* over 6 months [130]. However, the follow-up randomized controlled trial (which had only 22 patients) failed to reduce plasma concentrations of uremic toxins and did not improve quality of life parameters [131]. The lack of benefit with

probiotics may be explained by persistent uremia-induced changes in the gut biochemical environment and dietary and medicinal regimens which create an unfavorable milieu for the symbiotic microbiota [34]. Consequently, attempts to restore the desired microbiome without simultaneously improving the gut's biochemical milieu will be futile [34]. To address this deficit, the SYNERGY trial examined the combination of pro- and prebiotic therapy over 6 weeks in predialysis CKD patients and noted decreased serum *p*-cresyl sulphate and gut microbiome alterations [132]. Studies of longer duration are needed to assess hard clinical outcomes of pre- and probiotics in the CKD population. We also caution that choice of probiotic microbe is important; inclusion of bacteria that express urease with the intention to metabolize gut urea would be misguided since the downstream products ammonia and ammonium hydroxide would promote intestinal wall inflammation [26,34].

Conclusion

The gut environment is altered in CKD due to the restrictive diet and influx of urea from the circulation, favoring a microbial population that generates uremic toxins including indoxyl sulphate, *p*-cresyl sulphate, TMAO, indole-3 acetic acid, and phenylacetylglutamine. Intestinal inflammation and breakdown of the epithelial barrier promote systemic translocation of these bacterial by-products with widespread oxidative stress damage to the cardiovascular, kidney, erythropoiesis, bone-mineral, and endocrine systems (Figure 1). Blood levels of indoxyl sulphate, *p*-cresyl sulphate, and TMAO independently correlate with increased mortality risk. Studies are needed to determine whether etiology of CKD (e.g. diabetes mellitus compared with hypertension compared with primary glomerulonephritis) affects the gut microbiome differentially. Strategies to reverse microbiome dysbiosis are a novel therapeutic target. Data are lacking on optimal dietary guidelines in CKD that would favor the growth of a more symbiotic microbiome while avoiding potassium and phosphorus overload. Small randomized clinical trials using pre- and probiotics have shown some benefit in terms of decreasing circulating levels of uremic toxins and improving quality of life, but larger trials are needed to examine impact on cardiovascular or kidney morbidity.

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Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

AhR, aryl hydrocarbon receptor; CKD, chronic kidney disease; ESKD, end-stage kidney disease; IL-6, interleukin 6; MW, molecular weight; Nrf2, nuclear factor erythroid 2-related factor 2; OTU, operational taxonomic unit; TMAO, trimethylamine-N-oxide; TNF- α , Tumor necrosis factor- α ; VSMC, vascular smooth muscle cell.

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