Protein-bound uremic toxins: a long overlooked culprit in cardiorenal syndrome

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Abstract

Protein-bound uremic toxins (PBUTs) accumulate once renal excretory function declines, and are not cleared by dialysis. There is increasing evidence that PBUTs exert toxic effects on many vital organs including the kidney, blood vessels and heart. It has been suggested that PBUTs are likely to be a potential missing link in cardiorenal syndrome, based on the high incidence of cardiovascular events and mortality in the dialysis population which is dramatically reduced in successful kidney transplant recipients. These data have led the call for more effective dialysis or additional adjunctive therapy to eradicate these toxins and their adverse biological effects. Indoxyl sulfate and p-cresyl sulfate are the two most problematic PBUTs, conferring renal and cardiovascular toxicity, and are derived from dietary amino acid metabolites by colonic microbial organisms. Therefore, targeting the colon where these toxins are initially produced appears to be a potential therapeutic alternative for the progression of chronic kidney disease. This strategy, if approved, is likely to be applicable to predialysis patients, thereby potentially preventing progression of chronic kidney disease to end-stage renal disease as well as preventing the development of cardiorenal syndrome.

Keywords: Cardiorenal syndrome, protein-bound uremic toxins, colonic microbial metabolism
Introduction

The cardiorenal syndrome has become a major concern in clinical practice. The coexistence of cardiovascular disease (CVD) and chronic kidney disease (CKD) leads to a multiplicative increase in the risk of mortality (49) as well as the complexity of clinical management.

Renal impairment has been recognized as an independent and strong cardiovascular risk factor, such that studies demonstrating even microalbuminuria and mild reductions of renal function are associated with increased cardiovascular events and mortality (8, 23). The prevalence of CVD in the elderly aged ≥ 66 years with CKD is twice that of their non-CKD counterparts (69.8% vs 34.8%), and is associated with poor survival outcomes (1). In the dialyzed CKD population, CVD is responsible for approximately half of all deaths (2). It is noteworthy that CKD itself is a progressive but silent disease. Most patients usually experience symptoms, both uremia and its complications such as heart failure and cardiovascular events, in late stages when structural pathologic remodeling is usually not reversible.

Conversely, renal dysfunction is a common complication of CVD. The degree of renal dysfunction is a powerful independent risk factor for all-cause as well as cardiovascular mortality in heart failure patients (29). In the setting of acute myocardial infarction, mild and transient renal impairment during hospitalization is associated with long-term (10-year) mortality (68).

Both CVD and CKD share common pathophysiological mechanisms (e.g. persistent neurohormonal activation, hemodynamic derangement and systemic inflammatory activation) and co-morbidities (e.g. diabetes and hypertension). However, the occurrence of adverse clinical outcomes is still unacceptably high despite the advance in therapeutic treatment of these conditions and co-morbidities. This may be for reasons that 1) the pathophysiology of cardiorenal syndrome is not well understood due to a relative lack of mechanistically orientated studies in the setting of combined cardiovascular and renal diseases, and 2) there are still missing culprits/links that play a role in crosstalk between these two organ systems (Figure 1).

Protein-bound uremic toxins (PBUTs) have recently been recognized as a potential missing link in cardiorenal syndrome. Strong evidence for the oxidative stress-inflammation-fibrosis processes in both cardiovascular and renal tissue is attributable to PBUTs. This has been well demonstrated with two of the most potent toxins: indoxyl sulfate (IS) and p-cresyl sulfate (pCS) (Table 1). Uremic toxins begin to accumulate in the circulation once renal excretory function declines, even with mild to moderate renal dysfunction (9, 42). Accumulated toxins are believed to be a cause of uremic syndrome in CKD patients and their removal generally relies on dialysis when reaching end-stage
renal disease or stage 5 CKD (54). However, removal of the key PBUTs by dialysis is problematic due to their high protein (mostly albumin) binding affinity (32).

Importantly, many PBUTs including IS and pCS are derived from colonic microbial metabolic products using dietary amino acids tryptophan and tyrosine/phenylalanine as a substrate, respectively. Thus, the colon has become a potential novel target for treatment to reduce the toxicity caused by colon-derived PBUTs. This strategy, if applicable, could offer several benefits over conventional renal replacement therapy, in terms of easier accessibility to treatment, lower cost, reduced complexity and fewer major adverse complications. In addition, targeting the colon may be an ideal preventive strategy since it can be applied before reaching stage 5 CKD or renal failure.
<table>
<thead>
<tr>
<th><strong>Indoxyl sulfate</strong></th>
<th><strong>Cardiac effects</strong></th>
<th><strong>In Vitro studies</strong></th>
<th><strong>In Vivo studies</strong></th>
<th><strong>Clinical studies</strong></th>
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<tbody>
<tr>
<td></td>
<td>Pro-fibrotic and pro-hypertrophic effects in NCF and NCM, respectively, with OATs 1 and 3 as potential intracellular transporters of IS (44)</td>
<td>- Cardiac fibrosis in association with diastolic dysfunction via TGF-β1/NF-κB pathway in a 5/6 nephrectomy model (38)</td>
<td>- Cardiac fibrosis and hypertrophy in association with increased cardiac oxidative stress in 1/2 nephrectomy plus adriamycin and 5/6 nephrectomy models (20)</td>
<td>- Increase risk for cardiovascular and all-cause mortality (12)</td>
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<td>Pro-fibrotic, pro-hypertrophic and pro-inflammatory effects mediated via p38 &amp; p44/42 MAPKs/NF-κB pathway (37)</td>
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<td><strong>Vascular effects</strong></td>
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<td></td>
<td>Defective endothelial proliferation and wound repair without effects on cell viability and apoptosis in cultured HUVEC (15)</td>
<td>- Induce IL-6 protein expression in aortic tissue of IS-administered rats (4)</td>
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<td>Enhance oxidative stress (17, 98), inhibit cell proliferation (98) and promote cell senescence (98) in cultured HUVEC; all attenuated by OAT inhibitor probenecid and antioxidants (98)</td>
<td>- Enhance endothelial-leukocyte adhesion, leukocyte extravasation and interrupted blood flow without a vasoactive effect, fibrin deposition or capillary plugging in rats with IS-peritoneal superfusion (70)</td>
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<td>Promote rat VSMC proliferation mediated via OAT3-p44/42 MAPK pathway and increase PDGF-C and PDGF-β receptor gene expression (96)</td>
<td>- Promote aortic calcification and wall thickness in association with cell senescence in IS-administered hypertensive rats (5)</td>
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<td>Promote ROS generation and osteoblastic transformation mediated by NADPH oxidase activation in human aortic smooth muscle cell (59)</td>
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<td></td>
<td>Induce IL-6 protein expression via OAT3/AhR/NF-κB pathway in human vascular endothelial and smooth muscle cells (4)</td>
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<td><strong>Renal effects</strong></td>
<td>Increased expression of renal pro-inflammatory genes encoding major cytokines and intracellular signal mediators as well as target genes connected to TGF-β1(83)</td>
<td>- Impair renal function (56, 57, 64)</td>
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<td></td>
<td>Enhance renal oxidative stress in mesangial cells (22)</td>
<td>- Promote oxidative stress by reducing urine and renal nitric oxide in 4/5 nephrectomy rats (87) and reducing renal superoxide scavenging activity in both normal and uremic rats (67)</td>
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<td>Enhance oxidative stress, and activate NF-κB and PAI-1 promoter in human renal proximal tubular cells (58)</td>
<td>- Glomerulosclerosis (56, 57, 64), tubular atrophy (56) and renal interstitial fibrosis (56) with increased expression of pro-fibrotic genes (TGF-β1, TIMP-1 and pro-alpha 1(I) collagen) (56, 57)</td>
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<td>Promote cell senescence by down-regulation of renal klotho gene and protein expression in human proximal tubular cells which is inhibited antioxidant and NF-κB inhibitors (79)</td>
<td>- Induce renal fibrosis with renal renin-angiotensin activation and increased renal TGF-β1 protein expression in IS-administered mice (82)</td>
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<td>Increased expression of renin, angiotensinogen, and angiotensin 1 receptor genes as well as activation of Smad-associated TGF-β1 pathway in IS-treated renal tubular cells (82)</td>
<td>- Renal fibrosis (81) in association with decreased Klotho gene (a renoprotective antiaging gene) expression (79, 81) due to DNA hypermethylation in IS-administered mice (81)</td>
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<td>Induce epithelial-to-mesenchymal-like transition (82)</td>
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<td>p-cresyl sulfate</td>
<td><strong>Cardiac effects</strong></td>
<td>- Pro-fibrotic and pro-hypertrophic effects (39)</td>
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<td></td>
<td><strong>Vascular effects</strong></td>
<td>- Cardiac effects associated with cardiac fibrosis, apoptosis and oxidative stress in pCS-administered uremic rats (26)</td>
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<td></td>
<td><strong>Renal effects</strong></td>
<td>- Diastolic dysfunction in association with cardiac fibrosis, apoptosis and oxidative stress in pCS-administered uremic rats (26)</td>
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<td>p-cresol</td>
<td><strong>Cardiac effects</strong></td>
<td>- Pro-hypertrophic effects in cultured cardiac myocytes (39)</td>
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<td></td>
<td><strong>Vascular effects</strong></td>
<td>- Defective endothelial proliferation and wound repair without effects on cell viability and apoptosis in cultured HUVEC (15)</td>
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<tr>
<td></td>
<td><strong>Renal effects</strong></td>
<td>- Induce renal fibrosis with increased renin, angiotensinogen, and angiotensin 1 receptor mRNA expression and TGF-β1 protein expression in pCS-administered mice (82)</td>
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Discovery of PBUTs as a missing link in cardiorenal syndrome

Concern about the toxicity associated with PBUTs primarily originates from renal studies (Table 1). Both IS and pCS are implicated in CKD progression (94), and cardiovascular and all-cause mortality (12, 43). CKD progression induced by IS and pCS is likely to be associated with renal inflammatory activation (83), renal interstitial and glomerular fibrosis, and increased expression of pro-fibrotic genes and proteins (82). IS also promotes renal proximal tubular cell senescence via reactive oxygen species/nuclear factor kappa B (NF-κB)/p53 pathway (80). Renal fibrosis induced by IS and pCS has been demonstrated to be mediated via Smad-dependent TGF-β pathway, and is associated with epithelial-to-mesenchymal transition of renal tubular cells and intrarenal activation of renin-angiotensin-aldosterone system (82). Interestingly, administration of losartan, an angiotensin II type 1 receptor blocker, reduced renal fibrosis and its associated pathways in nephrectomized mice (82).

PBUTs are also implicated in the pathogenesis of atherosclerosis as well as non-atherosclerotic vascular diseases commonly found in the setting of CKD such as vascular stiffness, calcification and ossification (Figure 2 and Table 1). In atherosclerosis, IS and pCS promote endothelial dysfunction (15, 53), oxidative stress (17), vascular leakage by increasing endothelial permeability (70), impaired blood flow and leukocyte adhesion (70). IS can stimulate vascular smooth muscle cell proliferation, one of the hallmarks of atherogenesis (96). For non-atherosclerotic vascular diseases, IS has been implicated for contributing to aortic calcification and osteoblastic transformation of aortic smooth muscle cells (5, 59).

Direct cardiac effects of PBUTs have recently been reported (Table 1). IS has been demonstrated to have pro-fibrotic and pro-hypertrophic effects in cardiac cells as well as a pro-inflammatory effect in monocytes by increasing gene expression of key inflammatory cytokines involved in the progression of heart failure (37). The cardiac pro-fibrotic effect of IS has also been observed in renal failure and animal models of myocardial infarction with concomitant renal impairment that is likely to be mediated through the oxidative stress/NF-κB/TGF-β pathway similar to that mediating IS-induced renal fibrosis (20, 38, 40). Furthermore, p-cresol, the parent compound of pCS, has been shown to induce gap junction abnormalities in cultured cardiomyocytes, reducing myocyte conduction frequency and connexin 43 staining (69).
Therapeutic prospects

Although the issue of inadequate removal of PBUTs originates from the ESRD population receiving dialysis, PBUTs actually start accumulating at earlier stages of renal dysfunction. Hence, their biological toxicity can develop long before the commencement of renal replacement therapy which may cause irreversible damage to the target organs of PBUTs.

Ideally, any additional therapeutic strategies to conventional dialysis that could improve clinical outcomes closer to successful kidney transplantation has the potential to be included as part of the standard treatment for cardiorenal syndrome, especially in patients at high risk for progression to ESRD. Targeting PBUTs, which play an important role in the pathophysiology of CVD and renal disease, are believed to be an important missing link of cardio renal syndrome that could offer better outcomes (Table 2). As the two most damaging PBUTs with regard to their renal and cardiovascular toxicity, IS and pCS which originate from gut microbial metabolism (7, 92), addressing their production in the colon could prevent the progression of cardiorenal syndrome (Figure 2). In addition, information regarding signaling pathways involved in PBUTs-associated cardiorenal toxicity may provide selective targets for treatment (Figure 2), however this still requires further well-designed experimental studies and randomized controlled trials.

Preventing toxin production

Targeting colonic production of gut-derived PBUTs appears to be the most feasible approach to prevent their biological toxicity. A previous study demonstrated using liquid chromatography/mass spectrometry an absence or reduced levels of more than 30 uremic solutes in dialysis patients with a colectomy compared to those with an intact colon (7). Several of these suspected gut-derived PBUTs were inadequately removed by dialysis as indicated by a substantially lower reduction ratio of pre- and post-dialysis concentrations compared to the reduction ratio of urea (7).

Prevention of gut-derived PBUT production can be divided into three main strategies: dietary protein restriction, maintenance of gut homeostasis and oral sorbents (Figure 2 and Table 2).

Firstly, protein restriction diets limit the amount of substrate for PBUT production. A very low protein diet (0.3 g/kg body weight/day) has been demonstrated to significantly reduce serum IS levels in predialysis CKD patients after only one week (47). Of note, supplementary ketoanalogues and essential amino acids are recommended together with protein restriction to maintain the nitrogen balance.

Secondly, maintenance of normal gut homeostasis has been shown to decrease circulating levels of PBUTs in both CKD animal models and patients. In the setting of CKD, changes in colonic microbial
fermentation from a saccharolytic to proteolytic pattern (11) as well as prolonged colonic transit time (95) enhance the production of PBUTs from dietary amino acids. There are three major ways to modify gut homeostasis: probiotic, prebiotic and synbiotic treatments. Administering microorganism or ‘probiotics’ such as lactic acid bacilli (28) and *Bifidobacterium longum* (84) in dialysis patients can reduce circulating IS levels. A randomized controlled trial reported an improvement of renal function in CKD patients (n=46) receiving 6 months of probiotic treatment, however this study did not investigate serum levels of PBUTs. On the other hand, ‘prebiotics’ which use non-digestible dietary fiber to modify the gut microbial milieu have been demonstrated to lower serum IS levels and improve renal injury in an animal CKD model after administration of galacto-oligosaccharides for two weeks (21). A clinical study using oligofructose-enriched inulin for four weeks in dialysis patients showed a significant reduction of serum pCS levels but not IS levels (52). Synbiotics, a combination of probiotic and prebiotic treatment, significantly decreases circulating levels of p-cresol in both predialysis and dialysis CKD patients (25, 60). In addition, decreasing colonic transit time of amino acid substrates by simply using laxatives in conjunction with colonic microbiome modification strategies will help decrease colonic production of PBUTs.

Lastly, administration of oral sorbents can inhibit gastrointestinal absorption of PBUT precursors by adsorbing and enhancing their excretion into the feces. AST-120 is a synthesized carbon adsorbent, selectively adsorbing low molecular weight compounds in the lower gastrointestinal tract where PBUT precursors are metabolized by colonic microbiota. AST-120 has been demonstrated in pre-clinical and non-randomized clinical studies to reduce serum levels of PBUTs, mostly focusing on IS, improve clinical outcomes and inhibit their biological toxicity on the kidney, blood vessels and heart (Table 2). Two large scale randomized controlled trials of AST-120 have been conducted in moderate to severe CKD patients. The first study (n=460) did not observe a substantial delay in CKD progression within one year of follow-up, although the decline in estimated creatinine clearance was greater in placebo than AST-120 treated groups (6). The recent randomized placebo-controlled EPPIC trial (n=2,035) also reported that AST-120 did not show renal benefits using the composite endpoint of dialysis initiation, kidney transplantation and serum creatinine doubling (77). Major limitations of both trials were infrequent primary endpoint events due mainly to a longer actual than estimated median time to primary endpoint events. The actual time to primary endpoint was reported up to 170-190 weeks for the EPPIC trial and the authors also selectively recruited high risk patients with a urine protein/urine creatinine ratio of ≥ 1.0 and positive hematuria (77). In addition, simultaneous assessment of cardiovascular endpoints could provide useful additional information for the management of cardiorenal syndrome as CKD patients generally are at higher risk for cardiovascular events than progression to ESRD (33).
These colon-targeting strategies can be applied from pre-ESRD stages, unlike renal replacement therapy. However there has been a shortage of appropriately designed large scale trials to prove their beneficial therapeutic effects on the kidney and/or cardiovascular system. This is likely because the cardiorenal toxicity of gut-derived PBUTs is still a novel area of research requiring further mechanistic evaluation.

**Targeting toxin-induced signaling pathways or related mechanisms (Figure 2)**

**Inhibition of intracellular uptake:** Organic anion transporters (OATs) are involved in the intracellular uptake of IS in the kidney (18), blood vessels (96) and cardiac cells (44). OATs inhibitors, including probenecid and cilastatin, have been demonstrated *in vitro* to suppress IS-induced cardiomyocyte hypertrophy and cardiac fibroblast collagen synthesis (44). Probenecid also inhibits free radical production in renal proximal tubular cells stimulated with IS (58). Use of OAT inhibitors in cardiorenal syndrome with preserved renal function needs to be further clarified since normal function of renal OATs in the proximal tubular cells is to excrete PBUTs into the urine.

**Inhibition of intracellular signal mediators:** PBUTs-induced renal and cardiovascular toxicity is most likely mediated through the oxidative stress-inflammation-fibrosis pathway. Antioxidant and NF-κB inhibitors have been shown to dose dependently inhibit the IS-induced activation of plasminogen activator inhibitor-1, a factor known to promote tubulointerstitial fibrosis, in cultured renal proximal tubular cells (58). The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, may be another possible target of treatment for IS-induced renal and vascular toxicity. AhR activation by IS in endothelial cells promotes oxidative stress (48) and cellular senescence (34), as well as expression of monocyte chemoattractant protein-1, a chemokine involved in atherosclerosis which is abolished by AhR inhibitors in a dose-dependent manner (91). IS has recently been demonstrated to be involved in renal glomerular podocyte injury by increasing activity of the AhR (30). This suggests that podocyte injury may be a cellular mechanism of IS-induced glomerulosclerosis which may be prevented by AhR inhibitors.

One of the greatest difficulties regarding cell signaling inhibition is to selectively target the specific site of activation in the affected organs. Data on targeting signal transduction pathways activated by PBUTs are mainly based on experimental *in vitro* studies. Further *in vivo* investigations are required for clinical translation.
Table 2 Summary of studies on targeting production of protein-bound uremic toxins in cardiovascular and renal disease

<table>
<thead>
<tr>
<th>Animal studies</th>
<th>Clinical studies</th>
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<tbody>
<tr>
<td><strong>Protein restriction diet</strong></td>
<td><strong>Protein restriction diet</strong></td>
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<tr>
<td>- Improved renal function and delayed progression of glomerulosclerosis as</td>
<td>- Reduced serum IS levels with a low protein diet (consisting a mixture of ketoanalog</td>
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<td>well as reduced serum and urine IS levels in a model of uremia (64)</td>
<td>and amino acid supplements) in predialysis CKD patients (47)</td>
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<td><strong>Maintain gut homeostasis</strong></td>
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<td><strong>Prebiotics</strong></td>
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<tr>
<td>- Reduce serum IS levels, cecal indole, endoplasmic reticulum stress and</td>
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<td>apoptosis, and improved renal injury in association with gut microbiota</td>
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<td>modification after 2 weeks administration of galacto-oligosaccharides in</td>
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<td>5/6 nephrectomized rats (21)</td>
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<td><strong>Laxatives</strong></td>
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<td>- Accelerate intestinal transit with alterations of gut microbiota in</td>
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<td>association with a decrease in IS levels, improved renal function, attenuated</td>
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<td>renal fibrosis, and a reduction in the expression of renal fibrotic and</td>
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<td>inflammatory cytokine genes by using lubiprostone in mice with renal failure</td>
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<td>mice (55)</td>
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<td><strong>Oral sorbents</strong></td>
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<td><strong>CV Endpoints</strong></td>
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<tr>
<td>- Prevent atherosclerotic plaque extension, inflammation and necrosis in</td>
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<td>apolipoprotein E-deficient mice with CKD (97)</td>
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<tr>
<td>- Reduce cardiac fibrosis, cardiac TGF-β protein expression, cardiac NF-xB</td>
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<td>phosphorylation and cardiac oxidative stress in association with reduced serum</td>
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<tr>
<td>IS levels in 5/6 nephrectomized rats (75)</td>
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<tr>
<td>- Decrease serum IS levels in association with improved renal function and</td>
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<td>glomerulosclerosis (56, 64), decreased oxidative stress by restoring urine and</td>
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<td>renal nitric oxide (87), reduced interstitial fibrosis and tubular injury with</td>
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<td>decreased expression of pro-fibrotic genes (TGF-β1, TIMP-1 and pro-alpha 1(1)</td>
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<td>collagen) (56), attenuated renal fibrosis with concomitant inhibition of TGF-β1</td>
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<td>gene expression and renal cortical NF-xB-DNA binding activity in a model of</td>
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<td>uremia (85)</td>
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<td><strong>Renal Endpoints</strong></td>
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<tr>
<td>- Improve renal function after 1 year duration of AST-120 treatment in</td>
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<td>predialysis CKD patients (45)</td>
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<tr>
<td>- Reduce risk for initiation of dialysis or diagnosis with renal failure after</td>
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<td>2 years of</td>
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- Reduce IS-induced renal and cardiac fibrosis, as well as the expression of both renal and cardiac pro-fibrosis markers including normalizing cardiac microRNA-21, -29b and angiotensin-converting enzyme and angiotensin receptor 1a gene expression in a myocardial infarction model with coexisting renal impairment (42, 71)

<table>
<thead>
<tr>
<th>AST-120 administration in predialysis patients (89)</th>
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<tbody>
<tr>
<td>- Improve renal function and delay CKD progression in association with decreased levels of serum and urinary IS (65) and plasma TGF-β1 (31)</td>
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<tr>
<td>- Preserve renal function (73), improve survival and reduce cost in diabetic patients with CKD (27)</td>
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<td>- Provide synergistic benefits on preserving renal function in CKD patients who are on a low-protein diet (66)</td>
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<tr>
<td>- Preservation of renal function without benefits on delaying CKD progression or survival after a 1-year administration of AST-120 in a phase III multicenter, randomized, controlled trial in moderate to severe predialysis CKD patients (n=460) (6)</td>
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<tr>
<td>- No observed benefit by adding AST-120 to standard therapy on a composite endpoint of dialysis initiation, kidney transplantation and serum creatinine doubling in a multicenter randomized trials of AST-120 (EPPIC) in 2,035 moderate to severe CKD patients (77)</td>
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**Cardiorenal Endpoints**

- Improve renal function and heart failure symptoms after 2 years of AST-120 administration in heart failure patients with moderate CKD (non-randomized trial, n=20) (78)

**Other Endpoints**

- Improve 5-year survival in AST-120-treated hemodialysis patients with an average treatment duration of 15 months starting from the predialysis stage (88)

- Improve nutritional status by increasing albumin and transferrin levels, and decreasing the free fraction of tryptophan, a substrate for serotonin (an appetite suppressor) production (86)

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Value and roadblocks in cardiorenal research focusing on PBUTs

Most reports investigating the biological effects of PBUTs on renal and/or cardiovascular systems have been derived from preclinical research that has demonstrated a potential causative role of these nondialyzable toxins in the development and progression of cardiorenal syndrome. Such cumulative evidence has been initiated a research direction to further find therapeutic strategies to eradicate accumulated PBUTs and reduce their toxicity in CKD patients. Ideally, successful kidney transplantation is the most effective way but donor kidneys are in extremely short supply. In parallel with ongoing studies to improve dialysis performance, targeting colonic production of PBUTs has been increasingly investigated. However there have been many clinical concerns and major research hurdles in this area that need a sophisticated approach.

**In vitro studies using PBUTs in cardiovascular research,** should use concentrations of toxin within their clinical uremic range. Regarding their protein binding capacity, ideally culture conditions should replicate the conditions observed in CKD patients by using human serum albumin at the average uremic concentration of 35 g/L in any cell-based system not containing protein as recommended by the European Uremic Toxin Work Group (13). To test effects of phenyl derivatives in vitro, p-cresol should be avoided because p-cresol is not present in the human circulation but it is an artifact caused by the acid hydrolysis of its conjugate during the measurement (14). Although in vitro analysis is of importance to explore the mechanistic pathway of PBUT toxicity, it has no relevance for the investigation of interventions preventing colonic production of these toxins.

**The in vivo investigation** of PBUT administration to animals with healthy kidneys presents difficulties in obtaining sufficient levels of toxin accumulation in the circulation. A surgery-induced 5/6 nephrectomy CKD model with or without PBUTs administration is the most commonly used; this model development requires a skillfully performed surgery utilizing a renal artery ligation technique or a renal parenchymal ablation technique to achieve an actual 5/6 nephrectomy. Moreover, a cardiorenal model, such as combined nephrectomy and coronary artery ligation model, adds more substantial difficulty and provides a challenge to interpret the differences. At present, there is no model reproducing the pathophysiology of cardiorenal syndrome observed in humans. In addition, studies of PBUTs with simultaneous assessment of renal and cardiovascular endpoints is very rare.

**In clinical studies and trials on patients with CKD,** heterogeneity of the complex CKD population despite strict inclusion criteria and a proper sample size appears to be problematic especially in large-scale trials. CKD patients commonly have concomitant comorbidities, such as diabetes, hypertension and cardiovascular disease, which can vary in number, subtype and degree of severity. This may indirectly but substantially contribute to an inadequate incidence rate of study endpoints within a defined study period (6) or to incorrectly underestimate the time to events (77). This was reflected in
the EPPIC trials with an increase of an actual average time to event of 170-190 weeks from an
estimated time to event of 124 weeks (77). Adjusting the inclusion criteria to recruit participants at
high risk for progression of CKD or for adverse CV outcomes, when it comes to evaluating CV
endpoints, should reduce the heterogeneity of the study population.

In addition, clinical studies with renal outcomes commonly use serum creatinine concentration to
monitor renal function. Serum creatinine concentration has been considered suboptimal or not
sensitive enough to detect minimal renal parenchymal damage due to the functional reserve capacity
of the kidney, although creatinine levels are not disturbed by AST-120, and has been proved to be
valid for renal function monitoring in AST-120 treated patients (46). There have been studies
reporting potential novel biomarkers which directly represent renal parenchymal damage such as
kidney injury molecule-1 (90) and neutrophil gelatinase-associated lipocalin (35) or reflect renal
filtration function with less bias than serum creatinine such as cystatin C (19). Use of such novel
biomarkers in addition to conventional serum creatinine may be useful in refining renal outcome
assessment.

Importantly from the cardiorenal viewpoint, adding cardiovascular outcomes/endpoints could provide
additional clinical-useful information to clinical trials on CKD population. For instance,
hospitalization rate for CVD has been demonstrated to be approximately twice as common in CKD
patients with concomitant CVD compared with those without, that is independent of the stage of the
disease (3). Noting that all three large randomized trials with AST-120 in CKD patients (6, 77) did
not virtually exclude all types of CVD, but only severe heart failure, arrhythmias, and recent
cardiovascular events, this suggests a percentage of the participants had underlying CVD at baseline,
however subgroup analysis (groups with the presence vs the absence of CVD at baseline) was not
demonstrated in the study results.

Lastly, measurement of PBUTs levels is usually performed in clinical studies on PBUTs effects
without interventions/treatment which rarely occurs in clinical trials. However, PBUT levels in
clinical trials provide supportive evidence of the causative role of PBUTs in humans and can be used
to monitor compliance of the participants.
Conclusions

With emerging evidence of the toxicity associated with PBUTs, monitoring them as a marker for evaluating dialysis adequacy in addition to conventional biomarkers such as urea and creatinine should be considered. More mechanistic insights into cardiorenal toxicity induced by individual PBUTs are needed, including the toxins newly discovered by high throughput mass spectrometry, for which the physicochemical characteristics and function are not yet identified. Finally, potential therapeutic strategies for cardiorenal syndrome still require supportive *in vivo* studies and translation by appropriately designed randomized controlled trials.
Acknowledgements

This work was supported by National Health and Medical Research Council of Australia (Program Grants 334008 and 546272), Thailand Research Fund (MRG568079) and The Anandamahidol Foundation.

The authors would like to thank Dr. Bing Wang and Dr. Ingrid Hopper for the valuable comments and suggestions.
Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.


Figure Legends

Figure 1 The pathophysiology and characteristics of the cardiorenal syndrome. Protein-bound uremic toxins are a potential missing link in cardiorenal syndrome.

Figure 2 Therapeutic strategies in targeting protein-bound uremic toxins. In addition to conventional toxin removal by renal replacement therapy, two major adjunctive strategies have been proposed: 1) preventing toxin production and 2) targeting toxin-induced cell signaling pathway. Used with permission from Lekawanvijit et al. (41) and (36)
Cardiovascular disease

Kidney disease

Missing links: PBUTs?

- Renal blood flow
- Venous congestion
- Neurohormonal activation

↓ Renal blood flow
↑ venous congestion
Neurohormonal activation

Neurohormonal activation
Electrolyte imbalance
Anemia
Calcium -Phosphate dysregulation

Cardiorenal syndrome

- Persistent neurohormonal activation
- Resistance to natriuretic peptides
- Sodium and water retention
- Systemic arteriolar resistance
- Systemic inflammatory activation
- Systemic endothelial dysfunction
- Increased oxidative stress
- Progressive cardiorenal fibrosis

↓ Quality of life
↑ Health care cost
High morbidity and mortality

- Progression of heart and kidney failure
- Therapeutic difficulties e.g.
  • Diuretic resistance
  • Progression of left ventricular hypertrophy during the course of dialysis treatment

Figure 1
Dietary amino acids
Colonic microbes
Microbiotic metabolites e.g. indoles, phenols

Folate

Hepatic conjugation
Circulating toxins e.g. IS, pCS

Impaired renal function
Renal fibrosis
Cardiac fibrosis
Atherosclerosis
Vascular stiffness, calcification
Cardiorenal syndrome

Preventing toxin production*
(Predialysis CKD, renal failure)
• Protein restriction diet
• Probiotic, prebiotic and synbiotic treatment
• Oral sorbents e.g. AST-120

Toxin removal
(Renal failure)
• Dialysis
• Kidney transplantation

Expression of pro-inflammatory and pro-fibrotic genes (e.g. IL-1β, IL-6, TNF-α, TGF-β, PAI-1)
Expression of osteoblast-specific proteins
Inhibition of renal Klotho gene expression

Expression of senescence-related proteins in endothelial cells (e.g. p16[INK4a], p21[WAF1/CIP1], p53 and retinoblastoma)

OATs 1 and 3 OAT inhibitors e.g. probenecid and cilastatin

Oxidative stress
Increased ROS production
Increased NADPH oxidase activity
Reduced glutathione level

Antioxidants

RAAS blockade
Intrarenal renin and angiotensin activation

MAPKs
NF-κB
NF-κB inhibitors

Inhibition of renal Klotho gene expression
Glomerular podocyte injury

Expression of osteoblast-specific proteins
Vascular calcification
Vascular ossification
Cellular senescence

Renal fibrosis
Cardiac fibrosis
Atherosclerosis

Targeting toxin(IS)-induced cell signaling pathways*

Figure 2