Introduction

Cardiovascular disease (CVD) is today a major cause of death in many countries, and may be influenced by several factors. Among the modifiable factors are arterial hypertension, dyslipidemias, diabetes mellitus, the progression of chronic kidney disease (CKD), among others.

CVDs have high incidence in patients with CKD, which are up to 20 times more likely to develop them compared to other populations, which implies high mortality rates in this specific population. Traditional risk factors, such as hypertension, dyslipidemias, diabetes, smoking habits, among others, do not always explain cardiovascular events in patients with CKD. Among non-traditional factors commonly present in CKD are oxidative stress, endothelial dysfunction, aortic calcification, inflammation, and more recently, the accumulation of uremic toxins, which may cause insulin resistance, increased inflammatory cytokine production, and atherosclerosis.

In recent years, great attention has been given to uremic toxins, which are organic products usually excreted by the kidneys. They can be produced by the intestinal microflora, by the endogenous metabolism or ingested through food. In cases of renal failure, however, these toxins tend to accumulate in the plasma, and can thus accelerate the process of atherosclerosis.

There are several mechanisms that trigger CVD in CKD patients, including: systemic disorders such as arterial hypertension, anemia and alterations in calcium and phosphate homeostasis; functional changes in renal glomerulus and heart muscle; molecular changes...
involving anti- and proinflammatory nuclear factors, namely Nrf2 (nuclear factor erythroid derived 2 like-2) / NF-kB (nuclear factor kappa B) \(^2\); release of fibroblasts growth factor (FGF-23); \(^3\) activation of AhR receptor (Aryl hydrocarbon receptor) \(^11\); such mechanisms may induce the cellular senescence process and accelerate the process of atherosclerosis \(^9,11\); leukocyte activation, inflammation induced by proinflammatory cytokines and negative acute phase proteins \(^8\). Many of these may be related to the increase in uremic toxins, however, the mechanisms have not been well explained.

The purpose of this review is to discuss the role of uremic toxins, especially those formed by intestinal microbiota as indoxyl sulphate (IS), p-cresyl sulphate (p-CS) and trimethylamine-N-oxide (TMAO), in the pathogenesis of CVD in CKD patients.

**What are uremic toxins?**

They are organic solutes normally excreted and metabolized by the kidneys that tend to accumulate, causing toxicity in patients with impaired renal function. They can be classified by their size and ease of removal by dialysis techniques and according to their origin: intestinal microbiota, endogenous metabolism or ingested through food \(^6,8\).

There are approximately 152 uremic toxins listed in the European Uremic Toxin (EUTox) Work Group \(^5\), which are classified into three major groups: (1) small molecules, soluble in water, with molecular weight <500 Da, low toxicity, e.g. urea and phosphorus \(^6,8,14\); (2) intermediate molecules: molecular weight > 500 Da; insufficient removal by hemodialysis, the best option of removal is by peritoneal dialysis \(^6\) - these molecules may be toxic, e.g. parathyroid hormone (PTH) and β2-microglobulin; (3) protein-related molecules: these molecules share similarities with intermediary molecules as they bond with proteins; they have low molecular weight and may also be toxic. Hemodialysis cannot completely eliminate this kind of molecules, resulting in accumulation in the plasma and toxicity. Some examples of these toxins are indoxyl sulphate (IS), homocysteine, p-Cresol, among others \(^6,8,14\).

It is noteworthy that most of the existing toxins are bound to protein, and their weight is considered intermediate (<500 Da), and difficult to remove by dialysis. Poor removal by conventional dialysis is explained by the size of the complex (toxin + plasma protein) that cannot pass through the dialysate membrane (even when improved techniques are applied, such as in high flux hemodialysis) \(^6\). Some of these toxins are produced by the intestinal microbiota and have been associated with inflammation, oxidative stress and CVD in CKD patients \(^6\).

Human intestinal microbiota has several functions, including the maintenance of the intestinal health, immune function, bioactivation of nutrients and vitamins and, more recently, complex disease phenotypes \(^6\). Researchers have shown that intestinal microbiota contributes in metabolic diseases through modulation of multiple signaling pathways in the host, such as lipid metabolism and inflammation \(^6\), insulin resistance \(^17\) and non-alcoholic hepatic steatosis \(^18\). Additionally, the intestinal flora can influence the bioavailability of dietary constituents and their metabolism in mammalian hosts \(^19\).

Below follows a description of uremic toxins from the intestinal microbiota that have been studied with respect to their impact on cardiovascular mortality: P-CS, IS, and TMAO \(^6,20\).

**p-cresyl sulphate (p-CS)**

p-Cresol belongs to the class of phenols, which are generated from the partial breakdown of phenylalanine and tyrosine by anaerobic microorganisms (facultative or obligatory) of the intestinal wall. Several phenols produced in the colon are absorbed and detoxified in the liver through conjugation processes (glucuronidation and sulfation) forming two different compounds: p-cresyl sulphate (p-CS) and p-cresyl glucuronide (p-CG) \(^6,14\).

Current studies indicate that the p-CS has greater toxic activity due to its higher plasma concentration. However, a cohort study published in 2013 by Liabeuf et al. \(^20\) evidenced that the overall levels of p-CG were also related to cardiovascular mortality, regardless of known risk factors (age, vascular calcification, anemia, inflammation) in patients with CKD \(^20\).

According to Soulage et al. \(^4\), accumulated p-CS induces adipocyte dysfunction, causing ectopic redistribution of lipids in muscle and liver, lipotoxicity and changes in insulin signaling, resembling the metabolic disorders of
CKD. Furthermore, strong evidence suggests toxicity of p-cresyl sulphate to endothelial cells in vitro.

A relevant fact is the difference between the effects of the free and conjugated form of p-Cresol. The p-Cresol measured in humans is found in its conjugated form, both in p-CS (>95%) or p-CG (<4%). The sample must undergo deproteinization to measure the p-Cresol concentration. However, once the sample is deproteinized, the p-Cresol found is below the detection limit. Thus, experimental studies and major cardiovascular events in CKD patients are related to its conjugated forms (p-CS and p-CG).

In a recent literature review, Lekawanvijit et al. mention the morphostructural changes caused by excess of p-Cresol and its metabolites in the cardiovascular and renal systems, such as: increased collagen synthesis in cardiac fibroblasts and increased protein synthesis in cardiomyocytes in vitro; increased expression of inflammatory genes in proximal tubular cell cultures; and glomerulosclerosis and interstitial renal fibrosis with activation of pro-fibrotic genes and protein expression.

The p-CS also has pro-oxidative properties in tubular cells due to the increase in NADPH oxidase activity. Administration of p-CS into mice caused oxidative stress, leading to renal tubular damage. Moreover, p-CG, its other conjugated form, has also shown production of reactive oxygen species (ROS) in endothelial cells.

**Indoxyl sulphate (IS)**

The IS is a toxin derived from indole, produced in the large intestine by intestinal bacteria, derived from dietary tryptophan. Indole, in turn, is metabolized in the liver and produces IS. The indoxyl glucuronide (IG), another conjugated form of indoxyl, is also synthesized in the liver from the indole. In healthy people, the excess of IS is excreted by renal tubules via organic anion transporters (OAT), at a speed of about 170 mmol/day. When there is reduced renal function, this toxin accumulates in the organism.

In healthy subjects, the plasma indoxyl levels usually are around zero. However, in uremic patients, this concentration is close to 40-494 mmol/L, with a mean concentration of 150-200 mmol/L. Tumlin et al. state that values between 1 to 2.9 mmol/L are acceptable concentrations in healthy subjects; however, CKD patients had concentrations of up to 500 mmol/L.

Removal of IS by conventional dialysis techniques is inefficient due to its connection to serum albumin. IG, in turn, has better debugging, being efficiently removed by hemodialysis.

Owada et al. observed that nephrectomized mice showed increased serum levels of IS, and that the accumulation of this toxin led to decreased activity of the antioxidant enzyme superoxide dismutase (SOD).

A study with mice confirmed that the IS, in concentrations found in CKD patients (~500 mmol), contributes to the generation of cardiac fibrosis through TGF-β synthesis, a tissue inhibitor of metalloproteinase-1 (TIMP-1) and alpha collagen. Toxins bound to the protein appear to stimulate cardiac remodeling by activating p38 MAPK, cytochrome p42/44 MAPK, and through the activation of the NF-kB inflammatory marker.

In a recent study, Adesso et al. observed that IS-treated macrophages showed increased production of ROS through pro- and antioxidant mechanisms, changes in intracellular calcium homeostasis and increased production of proinflammatory cytokines, besides the induction of the proinflammatory NF-kB (nuclear factor kappa B).

The IS can generate a number of changes in renal morphology (especially in renal tubular cells), as a stimulus to tubulointerstitial fibrosis and glomerular sclerosis, thus accelerating the progression of CKD (due to accelerated loss of nephrons, progressively). The IS seems to trigger endothelial damage, inducing the production of proinflammatory molecules, the inhibition of regeneration and repair of the endothelium, and endothelial production of free radicals.

The IS is also considered an accelerator of atherosclerosis as it induces some important events related to the pathogenesis of CVD, such as endothelial release of microparticles, disruption of adherent junctions of endothelial cells, proliferation of smooth muscle cells, cardiac and kidney fibrosis, and impaired differentiation and function of osteoclasts.

The IS can also activate the Aryl hydrocarbon receptor (AhR), a nuclear transcription factor. Koizumi et al. observed an increase in oxidative stress through NADPH oxidase activation in human umbilical cells treated with IS. This group also examined the oxidative stress induced...
by decreased iNampt enzyme, which limits the production of NAD⁺ derived from nicotinamide (NAM) that reduces the activity of Sirt1 enzyme (important in the cellular senescence process)\textsuperscript{11}. The IS is also characterized as a cellular agonist of this transcription factor, since it can activate the AhR, which is shuttled from the nucleus, and starts to perform several functions in the body, e.g. it participates in the metabolism of xenobiotics; in the development of organs during the embryonic period; promotion of cancer, and more recently, it has been associated with the inflammatory vascular response and development of atherosclerosis\textsuperscript{12}.

**Trimethylamine-N-oxide (TMAO)**

TMAO is another uremic toxin produced by fermentation of choline, phosphatidylcholine and L-carnitine by the intestinal microbiota, particularly by bacteria of the families Peptostreptococcaceae and Clostridiaceae\textsuperscript{5,25}. After the ingestion of food sources of choline and phosphatidylcholine, such as eggs, red meat and milk, the intestinal microbiota transforms these precursors in trimethylamine (TMA), which is then converted into trimethylamine-N-oxide (TMAO) by the action of enzyme Flavin Monooxigenase-3 (FOM3) in the liver\textsuperscript{5,17,24}.

Studies have reported the pro-atherogenic role of this toxin, since there is evidence that TMAO can induce atherosclerosis by reducing the reverse transport of cholesterol and decreasing synthesis of bile acids\textsuperscript{5,17,24}.

Omnivorous people produce more TMAO than vegetarians and such a mechanism is dependent on the intestinal microbiota. The diet appears to modulate the intestinal microbiota, as people who ingest smaller quantities of its precursors have lower plasma concentrations of that toxin\textsuperscript{5,17,25}.

Koeth et al.\textsuperscript{17} observed that animals supplemented with L-carnitine showed changes in the composition of cecal microbiota, and increased synthesis of TMA and TMAO, which induced worsening of the atherosclerosis condition; but that did not happen with the use of oral antibiotics, capable of suppressing the intestinal microbiota. These animals also presented reduced reverse cholesterol transport, increasing the evidence of cardiovascular risk related to this toxin\textsuperscript{17}.

High levels of plasma TMAO were correlated with increased risk of adverse cardiovascular events (myocardial infarction, cerebral vascular accident and death), even after adjustments for compliance with supposedly traditional factors\textsuperscript{26}. The risk decreased in individuals whose intestinal microbiota was suppressed by broad spectrum antibiotics, which gives evidence of the importance of intestinal microbiota for the metabolism of this toxin\textsuperscript{17,26}.

From the summary of information found in different articles, it is possible to establish mechanisms that generate morphostructural changes and changes at cellular and molecular levels. These changes related to the accumulation of uremic toxins generate toxicity to both the cardiovascular and renal systems, and are associated with several toxins. Such information is presented in Chart 1.

**Cardiovascular mortality in patients with CKD: link to the uremic toxins**

There are several evidences of the relationship between increased uremic toxins and cardiovascular mortality. Although the molecular mechanisms that lead to CVD are not well explained yet, many studies now indicate that cardiovascular mortality is correlated with increased uremia and its toxic effects.

Studies carried out in animals predicted adverse cardiovascular effects. Koeth et al.\textsuperscript{17} observed that animals supplemented with L-carnitine had increased production of TMAO, which induced the process of atherosclerosis; Furuse et al.\textsuperscript{27} observed that the IS causes tubulointerstitial injury through oxidative stress and endoplasmic reticulum stress, which is mitigated by the use of galacto-oligosaccharide prebiotic (GOS)\textsuperscript{27}.

In vitro studies were also able to show that the IS stimulates macrophage function, inducing inflammatory response\textsuperscript{23}. This toxin also appears to activate the AhR receptor, which induces oxidative stress\textsuperscript{11,12} and increases the expression of MCP-1 (monocyte chemoattractant protein-1) adhesion molecule\textsuperscript{12}.

High levels of IS are associated with increased levels of inflammatory marker interleukin 6 (IL-6), coronary artery disease, vascular injury, the progression of CKD and cardiovascular mortality, a fact that has been confirmed in several clinical studies\textsuperscript{14}.
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<th>Toxins</th>
<th>Cellular and molecular changes</th>
<th>Morphostructural changes</th>
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| **p-cresyl sulphate (p-CS)** | • Adipocyte dysfunction  
• Induction of expression of genes involved in inflammation and fibrosis  
• Insulin resistance  
• Increased expression of inflammatory genes in proximal tubular cell cultures  
• Increased protein synthesis in cardiomyocytes in vitro  
• Increased expression of inflammatory genes in proximal tubular cell cultures  
• Oxidative properties in tubular cells due to the increase in NADPH oxidase activity | • Changes in cardiomyocyte structure and function of gap junctions  
• Increased collagen synthesis in cardiac fibroblasts, and increased protein synthesis in cardiomyocytes in vitro  
• Glomerulosclerosis and interstitial kidney fibrosis with activation of pro-fibrotic genes and protein expression  
• Inducer of renal tubular adenoma |
| **Indoxyl sulphate (IS)**   | • Activation of leukocytes and macrophages  
• Release of proinflammatory cytokines  
• Activation of AhR  
• Activation of NF-kB  
• Oxidative properties in tubular cells due to the increase in NADPH oxidase activity  
• Release of adhesion molecules | • Increased collagen synthesis in cardiac fibroblasts, and increased protein synthesis in cardiomyocytes in vitro  
• Aortic calcification due to increased smooth muscle cells, coronary calcification, reduced aortic compliance  
• Glomerulosclerosis and interstitial kidney fibrosis with activation of pro-fibrotic genes and protein expression  
• Increased atheromatous plaque  
• Cardiac remodeling |
| **Trimethylamine N-oxide (TMAO)** | • Reduced reverse cholesterol transport  
• Reduced synthesis of bile acids | • Increased atheromatous plaque |

**Chart 1**  
Molecular and structural changes related to the increase in uremic toxins in CKD patients

In a cohort of patients on hemodialysis, the total levels of free p-Cresol and p-CG were correlated with cardiovascular mortality, regardless of the risk factors already known (age, vascular calcification, inflammation and anemia); the levels of IS were also correlated with the mortality of these patients.

**Strategies to mitigate the effects of uremic toxins**

Inhibition of uremic toxins has been effective in reducing inflammatory processes related to uremia, endothelial dysfunction, and aortic calcification. Today the mechanisms used to reduce uremia include advances in hemodialysis process, peritoneal dialysis and the use of...
drugs that interfere with the techniques. The preservation of the residual renal function and the improvement of toxin removal techniques appear to increase the survival of patients with CKD.

Other studies also highlight the use of new drugs that can reduce the production of IS, such as meclofenamate, administered to animals as a potent inhibitor of the production of this toxin. Saigo et al. suggest that meclofenamate inhibits hepatic sulfonation required for the production of IS, thus reducing its accumulation. This drug also reduces proinflammatory prostaglandins (E2 series), which attenuates the inflammatory process and generates a nephroprotective effect on ischemic acute kidney injury.

The use of intestinal adsorbent AST-120 and Sevelamer (a polyamine hydrochloride polymer) were effective in reducing uremic symptoms. Rastogi found that the use of Sevelamer caused the decrease in vascular calcification; decrease in total cholesterol and LDL cholesterol; reduction of the fibroblast growth factor FGF-23 (which increase is associated with left ventricular hypertrophy) and reduction of circulating inflammatory molecules and endotoxins absorbed in the intestine. The use of intestinal adsorbent AST-120 decreased creatinine and urea levels, and also proved effective in reducing atherosclerosis and kidney damage typical of CKD progression in mice.

The use of these drugs associated with the modulation of the intestinal microbiota using prebiotics and probiotics seems to benefit patients with CKD due to evidence in the reduction of uremic toxins. Koeth et al. found that the percentage of bacteria belonging to the families Clostridiaceae and Peptostreptococcaceae was positively associated with omnivorous diet and the production of TMAO in humans, suggesting that the ability of these bacteria to metabolize L-carnitine is unique to this group of microorganisms. Therefore, diet modulation is a strategy that can be used to reduce the levels of these toxins, combined with the use of probiotics with the highest percentage of these specific families of bacteria.

Hemodialysis efficiency can also mitigate the effects of the accumulation of uremic toxins. A study published by Riccio et al. found the efficiency of removal of IL-6 and p-Cresol in new dialysis techniques, such as hemodiafiltration (HFR), with two stages: an absorption cartridge and another diffusion stage. Circulating levels of p-Cresol actually decreased, while IL-6 concentrations were not significantly affected after the treatment.

Another study has compared the effectiveness of three different types of high-flux and low-flux membranes in the removal of uremic toxins. The removal of protein-bound toxins or lipophilic solutes was inefficient regardless of the type of membrane used. In turn, the peritoneal dialysis improved the removal of average-sized molecular toxins and protein-bound molecules, which reduced the mortality and preserved residual renal function; nevertheless, all dialysis techniques only partially remove the uremic toxins.

In addition, the administration of galacto-oligosaccharide (GOS) for two weeks, a prebiotic recently discovered, derived from lactose and serving as food for the intestinal bacteria that possess the β-galactosidase enzyme to consume it, caused significant reduction of cecal indole concentrations and current IS levels, and the injury was reduced due to less infiltration of macrophages in mice treated with GOS. This prebiotic also improved the composition of the intestinal microbiota of animals, reducing uremia and kidney injury.

The molecular mechanisms that generate endothelial dysfunction and structural changes that lead to CVD should be further studied, due to the great importance of these factors for the development of CVD, particularly in patients with CKD whose mechanisms seem to be exacerbated. However, with this review, it can be concluded that previously published studies indicate that patients with chronic renal failure present high concentrations of uremic toxins on the intestinal microbiota, and that such condition is related to increased cardiovascular mortality.

Potential Conflicts of Interest
No relevant potential conflicts of interest.
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**References**


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