Protein-Bound Uremic Toxins from Gut Microbiota and Inflammatory Markers in Chronic Kidney Disease

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Objective: Protein-bound uremic toxins from gut microbiota tend to accumulate in chronic kidney disease (CKD) patients and are poorly removed by current dialysis techniques. These toxins induce inflammation and are associated with cardiovascular disease (CVD). The aim of this study was to report the relationship between uremic toxins and inflammatory and cardiovascular markers in CKD patients.

Design: This was a cross sectional study.

Subjects: Twenty-one nondialysis patients were included (43% men, 63.0 ± 7.8 years, glomerular filtration rate: 34.4 ± 12.5 mL/min) as well as 29 hemodialysis (HD) patients [58% men, 52.7 ± 10.3 years, time on dialysis 54 (31-94.5 months)].

Main Outcome Measure: Total levels of uremic toxins (IS, p-CS, and IAA) were assessed by high-performance liquid chromatography with fluorescence detection. C-reactive protein, Interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and calprotectin plasma levels were determined by immunometric assays.

Results: HD patients presented higher inflammatory markers and uremic toxins levels than nondialysis patients. IL-6 levels were positively correlated with IS ($r = 0.49$; $P = .03$), p-CS ($r = 0.35$; $P = .04$) and IAA ($r = 0.36$; $P = .03$). A positive correlation was also observed between MCP-1 levels with IS ($r = 0.72$; $P = .001$), p-CS ($r = 0.48$; $P = .001$) and IAA ($r = 0.75$; $P = .0001$). Linear regression showed that IS was an independent predictor for IL-6 and MCP-1 levels after adjustment.

Conclusion: Plasma uremic toxins were associated with higher IL-6 and MCP-1 levels in CKD patients, potentially playing a role in the development of CVD.

Introduction

CHRONIC KIDNEY DISEASE (CKD) patients have many complications including mild-chronic inflammation and oxidative stress. There are several causes for these common features and recently, uremic toxins retained in patient's plasma could be involved in these complications. Among these toxins are indoxyl sulfate (IS), p-cresyl sulfate (p-CS) and indole-3-acetic acid (IAA) that originate from bacterial protein fermentation in the large intestine.[1]

Gut microbiota degrade tryptophan to indole, and this compound is metabolized to IAA, directly in the intestine, and to IS, in the liver.[2] Both toxins derived from tryptophan are ligands of aryl hydrocarbon receptor (AhR) whose activation is involved in atherogenesis, vascular infiammation, and oxidative stress.[2,3] In parallel, p-CS originates from bacterial fermentation of tyrosine.[4] Recent studies have reported that these toxins induce proinflammatory responses and are reliable markers of cardiovascular disease (CVD) and mortality in CKD.[1,5]

IS is one of the most extensively studied uremic toxin. Cruz et al observed that incubation of cells with uremic plasma promoted synthesis of cytokines and monocyte chemoattractant protein-1 (MCP-1).[6] The mechanism by which IS induces toxicity has recently been clarified. The intracellular accumulation of IS via organic anion transporters and the subsequent increased production of reactive oxygen species (ROS) seems to play a key role in the toxicity of IS.[7]

A recent work from our group has shown that incubation of adipose cells with IS increased ROS production mainly through activation of NADPH oxidase and exacerbated the secretion of tumor necrosis factor-α and interleukin-6.[8]

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Similarly, p-CS exhibited pro-oxidant properties in human tubular epithelial cells by enhancing NADPH oxidase activity. p-CS also upregulated mRNA levels of inflammatory cytokines and active TGF-b1 protein secretion associated with renal fibrosis.[9]

Lately, the IAA has been gaining attention. In cultured human endothelial cells, AhR/p38MAPK/NF-κB pathway was activated by IAA and induced the proinflammatory enzyme cyclooxygenase-2 synthesis.[10]

Studies in humans have confirmed the adverse effects of uremic toxins in CKD patients. A cross-sectional study in stage 3-4 CKD demonstrated that IS and p-CS were associated with elevated levels of inflammatory biomarkers as well as with increased arterial stiffness.[11] Indeed, a meta-analysis involving patients with CKD stage 3 and above concluded that elevated levels of IS and p-CS are associated with increased mortality in patients with CKD, and p-CS is associated with an increased risk of cardiovascular events.[12] Dou et al observed that IAA serum levels were a significant predictor of mortality and cardiovascular events.[10] The relationship between uremic toxins and inflammation should be further explored in the CKD population. Thus, the aim of this study was to verify the relationship between uremic toxins plasma levels and inflammatory markers in CKD patients at different CKD stages.

Methods

Subjects

This transversal study included 21 nondialysis patients (43% of men, 63.0 ± 7.8 years, glomerular filtration rate, 34.4 ± 12.5 mL/min) and 29 hemodialysis (HD) patients [58% of men, 52.7 ± 10.3 years, time on dialysis, 54 (31-94.5 months)]. Patients aged >18 years, in CKD stage 3 and 4 and undergoing HD for at least 6 months were included. Patients with inflammatory diseases, cancer, AIDS, autoimmune disease, smokers, use of a central catheter for hemodialysis access, amputated limbs, pregnancy, and patients using catabolic drugs, antioxidant vitamin supplements pre, pro and symbiotic and antibiotics in the last 3 months before the start of this study were excluded.

Dialysis duration was 3-4.5 hours per session, three times per week, with a blood flow >250 mL/min and a dialyzate flow of 500 mL/min. The study protocol was reviewed and approved by the Ethics Committee, and all the patients were asked to sign the informed consent.

Analytic Procedures and Sample Processing

Blood samples were drawn from each subject in the morning, after overnight fasting (for HD patients before a regular HD session). Plasma was separated (15 minutes, 3000× g, 4°C) and stored in −80°C until analysis.

Total concentrations of uremic toxins IS, p-CS, and IAA were quantified by high-performance liquid chromatography (HPLC) with fluorescent detection. Briefly, plasma samples were processed as described.[13] The ultrafiltered plasma was injected into an HPLC system (Shimadzu Prominence) consisting of a Rheodyne injector (model 7125), a quaternary pump (Shimadzu LC-20AD), controlled by the LC Solution software, and a fluorescence detector (Shimadzu RF-20A). Separation was achieved with a 150 × 4.6 mm, 5 μm, C8 Luna column (Phenomenex), eluted with 50-mM ammonium formate pH 3.0 and methanol, whose proportion increased from 35% to 70% along the run, at a flow rate of 0.7 mL/min. During the run, the fluorescence wavelengths varied: λ_{exc} = 280 nm and λ_{em} = 383 nm to IS and λ_{exc} = 265 nm and λ_{em} = 290 to p-CS and IAA.[13,14]

High-sensitivity protein C reactive (CRP), interleukin-6 (IL-6), MCP-1, and Calprotectin were analyzed by immunoenzymatic assay (ELISA; R&D systems duoset kit for CRP; BosterImmunoleader kit for IL-6 and MCP-1; Bühlmann kit for Calprotectin). Routine laboratory parameters were measured by standard techniques.

Body mass index (BMI) was calculated as weight divided by height squared. Dialysis dose (Kt/V) was calculated from values of blood urea nitrogen, predialysis and postdialysis, body weight, and dialysis duration using standard formula.

Statistical Analysis

Kolmogorov–Smirnov test was used to test the distribution of variables, and results were expressed as mean ± SD (standard deviation), median (interquartile range), or percentage, as appropriate. The correlations between variables were assessed through Spearman Rho or Pearson's coefficient correlation depending on the distribution of the sample. Regression analyzes were performed to determine variables that had independent associations with cytokines. Statistical significance was accepted as $P < .05$, and analyzes were performed with SPSS 19.0 (SPSS, Inc., Chicago, IL).

Results

Biochemical, anthropometric, and inflammatory parameters are shown in Table 1. As expected HD patients presented higher creatinine and urea as well as inflammatory markers levels when compared to CKD nondialysis patients. HD patients also presented higher uremic toxins levels than nondialysis patients (Table 2). In HD patients, the average levels of p-CS were above the high range for uremic patients presented by Uremic Solutes Database (Eutox). IL-6 levels were positively correlated with IS (r = 0.49; P = .03; Fig. 1), p-CS (r = 0.35; P = .04), and IAA (r = 0.36; P = .03). A positive correlation was also observed between MCP-1 levels and IS (r = 0.73; P = .001; Fig. 2), p-CS (r = 0.48; P = .001), and IAA (r = 0.75; P = .0001). Linear regression showed that IS was an independent predictor for IL-6 levels after adjustment on age, gender, BMI, IAA, and p-CS levels. IS was also predictors for MCP-1 levels after adjustment (Table 3). No association was observed between uremic toxins and CRP or calprotectin. In addition, MCP-1 levels

Table 1. Biochemical, Anthropometric, and Inflammatory Parameters of Nondialysis and Hemodialysis Patients

Parameters	Nondialysis	Hemodialysis	*P* value
Urea (mg/dL)	68.1 ± 23.5	148.8 ± 36.9	.000
Creatinine (mg/dL)	1.94 ± 0.8	10.1 ± 3.7	.004
CrCl (mL/min)	34.4 ± 12.5	—	—
Albumin (g/dL)	4.3 ± 0.3	4.2 ± 0.2	.47
Hemoglobin (g/dL)	11.7 ± 1.4	11.5 ± 1.3	.65
Hematocrit (%)	35.4 ± 4.5	34.9 ± 3.7	.74
BMI (kg/m^2)	27.0 ± 4.4	25.5 ± 4.9	.17
CRP (mg/dL)	1.8 (0.7-5.4)	3.6 (1.2-9.0)	.054
IL-6 (pg/mL)	26.0 ± 13.0	40.0 ± 22.0	.013
MCP-1 (pg/mL)	144.2 ± 28.4	263.9 ± 53.5	.0001
Calprotectin (ng/mL)	13.4 ± 4.9	11.2 ± 3.1	.13

CrCl, creatinine clearance; BMI, body mass index; CRP, protein C reactive; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1.

Data expressed as mean ± SD or median (interquartile range).

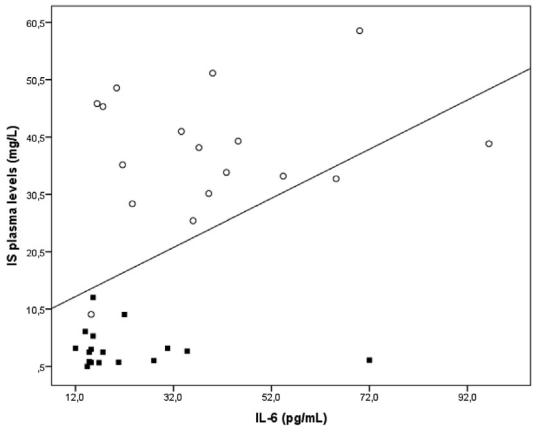

Figure 1. Correlation between IL-6 and IS plasma levels in chronic kidney disease patients (r = 0.49, P < .03; circle-HD patients; full square–nondialysis patients).

were positively correlated with IL-6 plasma levels ($r = 0.63$; $P = .0001$).

Discussion

Inflammation is common features of CKD patients and is related with CVD, the major cause of death in CKD. Accumulating evidence reveals that gastrointestinal tract can be an important source of chronic inflammation in CKD patients. The present study showed that patients at different CKD stages presented high levels of uremic toxins produced by gut microbiota (IS, p-CS, and IAA), which were positively associated with IL-6 and MCP-1 plasma levels. These findings have clinical relevance to suggest a partial contribution of these uremic toxins in inflammation, and consequently CVD, in CKD patients.

The intestine and its microbial flora have been explored as a potential source of inflammation in CKD. Studies have reveled significant changes in the composition and function of colonic bacterial flora in humans and animals with advanced CKD.[15-17] These patients often are advised to adhere to low potassium and low phosphorus diets that leads to inadequate intake of fermentable plant fiber and symbiotic bacteria. Alterations in fermentable substrates lead to low production of short-chain fatty acids that are important nutrients for colonocyte and regulatory T lymphocytes, thus influencing the colonocyte integrity and impairing the protective mucosal barrier.[18]

Additionally, there is an influx of urea from the blood circulation into the gut lumen. These factors promote dysbiosis, characterized by expansion of bacterial that possess urease and indole and p-cresol-forming enzymes.[16] The disordered bacterial colonization promotes increased permeability of the intestinal barrier once urea in the gut lumen is metabolized by gut bacterial urease to ammonia leading to the breakdown of gut epithelial barrier structure and function.[17,19-21]

In recent elegant review, Vaziri et al. showed that CKD patients present deep alterations in the gut microbiota profile and impairment of the intestinal epithelial barrier, which has been recognized as the important cause of systemic inflammation in uremia.[17]

The colonic bacteria are the main source of well-known pro-inflammatory uremic toxins such as indoxyl sulfate,

Table 2. Uremic Toxins Plasma Levels in Nondialysis and Hemodialysis Patients

Uremic Toxins Levels	Nondialysis	Hemodialysis	*P* value
IS (mg/L)	3.4 ± 2.9	35.0 ± 12.9	.001
p-CS (mg/L)	20.9 (10.2-28.0)	52.6 (32.7-69.5)	.001
IAA (ug/L)	149.8 ± 79.5	553.0 ± 342	.001

IS, indoxyl sulfate; p-CS, p-cresyl sulfate; IAA, indole-3-acetic acid.

Data expressed as mean ± SD or median (interquartile range).

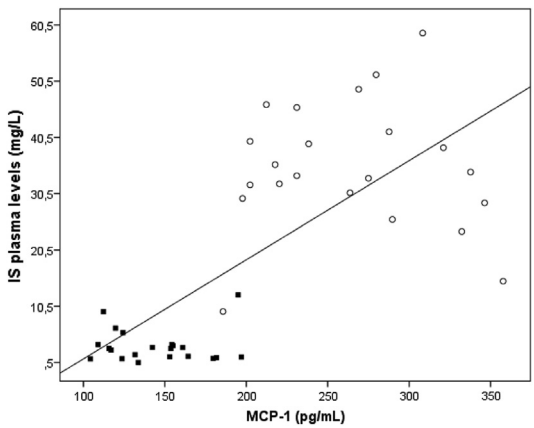

Figure 2. Correlation between MCP-1 and IS plasma levels in chronic kidney disease patients (r = 0.73; P = .001; circle-HD patients; full square–nondialysis patients).

Table 3. Multiple Regression Models of Determinants for Plasma IL-6 and MCP-1 in Total CKD Patients

Variable	IL-6 Model (β, P value)	MCP-1 Model (β, P value)
Age	0.18, .32	0.009, .94
BMI	0.22, .21	0.033, .79
Gender	0.12, .42	0.03, .76
p-CS	−0.09, .66	0.037, .8
IAA	−0.40, .08	0.32, .07
IS	0.97, .001	0.49, .008

BMI, body mass index; p-CS, p-Cresyl sulfate; IAA, Indole-3-Acetic Acid; IS, Indoxyl Sulfate (IS).

p-cresol sulfate, indole-3-acetic acid, and others retained compounds in CKD patients.[17] Studies have suggested that uremic toxins from gut microbiota are associated with increased levels of inflammatory markers in CKD patients, such as TNFα and interleukin-6 and with vascular damage by inducing vasoactive substances related to atherogenesis such as chemokines, cytokines, or cell-adhesion molecules.[9,11,20,21] Conventional dialysis is not effective in controlling the levels of these solutes contributing to uremic toxicity and inflammation in these patients.[15]

Given the critical role that chronic inflammation plays in the development and progression of CVD, the increased levels of circulating proinflammatory substances may lead to drastic consequences for CKD patients.[22] Barreto et al. observed that high IS levels in nondialysis CKD patients were significantly associated with cardiovascular and overall mortality.[23] Meijer et al. also reported that p-CS was predictive of cardiovascular risk in nondialysis CKD patients.[24] A cross-sectional observational study by Rossi et al. showed that IS and p-CS were independently associated with the presence of cardiovascular disease in nondialysis and HD patients.[25]

Dou et al. revealed that the IAA levels were higher in more advanced CKD stages and all-cause mortality, and cardiovascular events were significantly higher in patients with high IAA levels. The authors also demonstrated that IAA increased the expression of endothelial inflammatory genes such as IL-6, IL-8, ICAM-1, and MCP-1 in endothelial cells.[10]

A number of studies support the hypothesis that AhR activation by tryptophan-derived uremic toxins (IS and IAA) is related with inflammation, and consequently CVD during CKD. This receptor is a ligand-dependent transcription factor that generate biological responses to environmental pollutants; however, recently, studies have shown that this receptor is also involved in activating diseases by modulating the biological responses of critical cell types at the barrier and mucosal interfaces.[2]

Watanabe et al. showed that IS induced the expression of MCP-1, whereas AhR inhibitors abolished the IS-induced increase in MCP-1 expression.[9] Additionally, MCP-1 induction by IS involves a ROS/MAPK/NF-κB pathway.[20,26] MCP-1 is a chemokine that is involved in early stage of atherosclerosis through the recruitment of monocytes from the blood stream to the sub-endothelial space.[27,28]

In this study, plasma levels of the three toxins analyzed, IS, p-CS, and IAA, showed positive association with MCP-1 and IL-6 plasma levels and, notably, IS was independently associated with IL-6 levels, whereas IS and p-CS were predictors of MCP-1 levels. In agreement with our results, Rossi et al. showed that IS and p-CS were associated with IL-6 plasma levels.[11] In a study in stage 2-5 CKD patients, Barreto et al. showed that IL-6 levels significantly predicted CVD and all-cause mortality. Furthermore, IL-6 was superior to CRP, albumin, or TNF-α in predicting mortality in this patient cohort.[29]

This is the first study in CKD that demonstrates a relationship between uremic toxins and MCP-1. Despite a known relationship between CKD and atherosclerosis, the causative role of uremic toxins in leukocyte–endothelial interactions has not been clearly elucidated. Ito et al. showed that IS increased leukocyte–endothelial interactions, in human umbilical vein endothelial cells, through upregulation of E-selectin.[21] Other studies also showed that IS induces endothelial dysfunction by releasing endothelial microparticles[30] and producing ROS.[31] Our findings suggest that uremic toxins, mainly IS and p-CS, mediate MCP-1 over expression, and this could be one possible mechanism for the induction of vascular proinflammatory processes observed in CKD.

In vitro studies suggest that uremic toxins induce generation of ROS, which activate the NF-kB pathway, resulting in both oxidative stress and proinflammatory factors production.[32,33] Our group observed in a previous study that IS and IAA plasma levels were positively correlated with malondialdehyde, and IS, p-CS, and IAA plasma levels were positively correlated with NF-κB expression in CKD patients in HD (unpublished). Besides, Rossi et al. observed in CKD patients an inverse association between IS, p-CS, and glutathione peroxidase activity, an important antioxidant enzyme.[11] These data confirm the stimulating impact of these toxins on oxidative stress and inflammation.

This study has limitations. Food intake was not evaluated to assess the intake of tryptophan, tyrosine, and fibers. Because of the cross-sectional nature of the study, we cannot infer any causality, rather only establish associations. Moreover, the results are vastly exploratory in nature, and the sample size may have limited the appearance of other associations.

In conclusion, the results of the present study suggest that IS, p-CS, and IAA may contribute to the chronic and vascular inflammation in CKD and provide insights into the mechanisms that lead to higher cardiovascular mortality

and death in CKD patients. Strategies to reduce the generation of uremic toxins could contribute to reduce inflammation and thereby help to prevent CVD in CKD patients.

Practical Application

Uremic toxins may contribute with inflammation and cardiovascular morbidity in CKD patients. Thus, these toxins as well as the intestinal epithelial barrier could be considered important targets for treatment of these patients.

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