Title: p-Cresyl Sulfate and Indoxyl Sulfate Predict Progression of Chronic Kidney Disease

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Abstract

Background: Indoxyl sulfate (IS) and p-cresyl sulfate (PCS) are similar in protein binding, dialytic clearance and proinflammtory feature. However, fewer prospective studies have evaluated the distinctive associations of these two retained solutes with the renal progression in chronic kidney disease (CKD) patients.

Methods: This prospective observational study evaluated the independent association of serum total p-Cresyl Sulfate and Indoxyl Sulfate with renal progression in a selected cohort of patients at different stage of CKD. Baseline PCS and IS were correlated with the renal progression using composite end points of decrement of eGFR > 50% of the baseline value, progression to ESRD and/or death during a follow-up period of 12 months.

Results: Of 268 patients, 25 (9.36%) patients showed renal progression after a mean follow-up of 11.7 months. Progressor patients presented higher serum PCS levels at baseline compared with non-progressors as well as serum IS. Univariate followed by multivariate Cox regression analysis showed that high serum PCS level was associated with renal progression independent of age, gender, diabetes status, albumin levels, serum IS, serum creatinine, Ca x P product, intact parathyroid hormone, hemoglobin and high sensitive C reactive protein level. The serum IS was also associated with renal progression; however, the predictive role of serum IS was weaken if serum PCS is also present in the analytical model.

Conclusions: The associations of PCS and IS with the renal progression were different Serum PCS was an independent significant risk marker for renal progression in different stage of CKD patients.

Keywords: Chronic kidney disease, indoxyl sulfate, p-cresyl sulfate, protein bound toxins, proximal tubule

Main message of the paper: Indoxyl sulfate and p-cresyl sulfate constituted novel risk factors for renal progression. The p-cresyl sulfate, especially, was associated with renal risk independently of other modifiable and non-modifiable risk factors, such as age, diabetes, calcification, anemia, malnutrition-inflammation and IS. This study provided clinical evidence of the importance of p-cresyl sulfate in the progression of CKD.

Introduction

Despite better understanding of disease mechanism and proper control of important modifiable risk factors, the decline of renal function still became imperative in substantial proportion of chronic kidney disease (CKD) patients. Traditional and uremia-related risk factors are not sufficient to explain renal outcome of CKD patients.

p-Cresyl sulfate (PCS) and indoxyl sulfate (IS) are prototypic molecules of protein bound uremic toxins. The two retained solutes are not only biomarker of renal function and also actively participate in the development of disease[1]. They share various similarities, including the production by gut bacteria[2], large albumin binding at Sudlow II site[3], significant renal metabolism, low dialytic clearance[4-5], its emerging role in cardiovascular disease and mortality of renal patients[6-7]. The overloading of IS in CKD rat results in glomerular sclerosis and interstitial fibrosis[8] via aberrant genetic expression of TGF-β1, TIMP-1 and Pro-α1 collagen[9-10], and complex redox alteration[11]. Indoxyl sulfate is also associated with endothelial and vascular dysfunction by promoting vascular smooth muscle cell proliferation[12] via activation of platelet-derived growth factor (PDGF) receptors[12] and mitogen-activated protein kinase (MAPK) pathways[13]. Clinically, IS is associated with increased aortic calcification and vascular stiffness[7]. On the other hand, the deleterious effect of PCS on renal cells is less studied. Previous studies revealed that p-cresol induce endothelial dysfunction[14] and decrease mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)[15]. However, now is well known that p-cresol represents an artifact of sample preparation from PCS[16] and both component posse different behavioral on the respiratory burst activity of leucocytes[17]. Exposure of human umbilical endothelial cell to PCS results in increased shedding of

endothelial microparticle via Rho kinase-dependent pathway[6]. High total PCS level is associated with aortic calcification and mortality in CKD[18] and hemodialysis patients[19-20]. Despite various similarities, parallel comparison in the contribution of serum PCS and IS levels to the renal progression of different stage of CKD patients, however, is unknown.

In the present study, we prospectively evaluate the association of serum PCS and IS level with renal progression (defined as defined as reduction of eGFR by 50% or end stage renal disease requiring dialysis) and all-cause mortality in CKD patients.

Subjects and Methods

Patient Selection and Study population

Prevalent predialysis CKD patients who attended an outpatient clinic in the Nephrology Department of Chang Gung Memorial Hospital at Keelung from November 2006 to October 2007 were recruited into this study. The inclusion criteria were adults aged > 18 but < 80 year-old; no spontaneous improvement or progression of renal disease in the past 3 months. The patient was excluded from the study if any one of following condition were present: cardiovascular disease (coronary artery disease, myocardial ischemia, cerebrovascular disease or peripheral artery disease) in the past 3 months, infections requiring admission in the past 3 months, uncontrolled hypertension, serum albumin level < 2.5mg/dL or unwillingness to participate in the trial. CKD was defined as having a persistent proteinuria or a decreased eGFR < 90 ml/min per 1.73 m^2 (determined by abbreviated Modification of Diet in Renal Disease equation) in two separate measurements within an interval of 3 months. In accordance with the NKF/DOQI classification system, these patients were classified into stages I, II, III, IV, or V for descriptive purposes. A total of 268 patients were enrolled into the study and gave their informed written consent. This study was in adherence to the *Declaration of Helsinki* and approved by the ethics committee of the Institutional Review Board at Chang Gung Memorial Hospital.

Study Design

All eligible patients were interviewed carefully to identify medical disease and concomitant medications. Twelve-hour fasting blood samples were obtained for determination of serum level of PCS, IS and laboratory testing. Medical visits and renal function measurements were followed-up prospectively at 3-month, 6-month, 12-month and 24-month intervals, until commencement of dialysis therapy or death.

All eligible patients were followed-up to 15, April 2010 to note renal progression or death (Figure 1). Diabetes mellitus (DM) was defined as a fasting glucose level ≥ 126 mg/dL or use of any hypoglycemic medication. Hypertension was considered present if the patient received medical therapy for such a condition or if blood pressure was > 140/90 mm Hg.

Baseline measurements

For determination of total IS and PCS serum levels, serum samples were deproteinized by addition of 3 parts methanol to 1 part serum for determination of total IS. Total PCS was analyzed after deproteinization (acid and heat) and extraction (ethyl acetate) of serum samples. All analyses were performed on Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Milford, MA, USA), including binary solvent manager, sampler manager, column compartment and photo diode array (PDA) detector, connected with Waters Empower 2 software. IS and PCS were detected at 280 nm and 260 nm. Buffer flow was 0.4 ml/min using 10 mM $NH_4H_2PO_4$ (pH=4.0) (A) and 100% Acetonitrile (B) with a gradient from 82.5%A/17.5%B to 55%A/45%B, over 9 min. Under these conditions, IS and PCS appeared at 1.4 min and 1.7 min, respectively.[21] The limits of detection of this assay was 0.225 mg/L for IS and 1 mg/L for PCS. Calibration curves were constructed by plotting the peak areas versus the concentrations of each analysate and had average r^2 values of 0.999 ± 0.001. Quantitative results were obtained and calculated as concentrations (mg/L). Intra- and inter-assay coefficients of variation relative standard deviation were found 0.4% and 0.05% for IS and 5.50% and 7.48% for PCS, respectively. We spiked different concentrations of IS, PC, and PCS in serum of healthy individuals (n = 5). The recovery was calculated as [(final concentration - initial concentration)/added concentration]. Recoveries were 100.99% and 108.73%

for IS and PCS, respectively. Further, parallel comparison of serum total PCS and IS level obtained from UPLC and mass spectrometry in 10 random selected patients did not revealed significant difference from Bland-Altman plots (for serum IS, Pitman's Test of difference in variance showed r = -0.263, p = 0.493; and for serum PCS, r = -0.765, p = 0.124).

In addition to the demographic and clinical data, the calcium (Ca), phosphate (P), intact parathyroid hormone (iPTH), total cholesterol, hemoglobin, high sensitivity reactive-C protein (hs-CRP), uric acid and albumin were also measured at baseline. Serum creatinine (SCr) was assessed at the above-mentioned time points by spectrophotometric analysis using a modified kinetic Jaffe reaction.

Statistical Methods

Descriptive statistics were expressed as means ± standard deviation, median, range or percentage frequency, as appropriate. All variables were tested for normal distribution by Kolmogorov-Simirnov test. The Student's *t*-test or Mann-Whitney U test was applied to compare means of continuous variables. Categorical data were tested using the Chi-square test. Pearson or Spearman correlation coefficients were appropriately used to test the correlation between PCS and IS with other variables. Data were log-transformed to approximate normal distribution. Kaplan-Meier curves were performed to assess renal and overall survival in patients with serum PCS and IS levels above and below the median. Adjusted risk estimates for endpoints were calculated using univariate, followed by, multivariate Cox proportional hazard regression analysis. The assumption of proportionality was checked graphically using the complementary log-log plot and found to be acceptable for the risk factors of interest. All statistical tests were two-tailed, and a p value of < 0.05 was considered statistically significant. Data were analyzed using the SPSS 13.0 software for

Windows XP (SPSS Inc., Chicago, IL).

Results

Baseline characteristics of study population

Table 1 shows the baseline characteristics of the study population. The mean age of patients was 67 ± 12 years, and 154 (57.5%) were male. The mean sCr was 1.9 ± 1.4 mg/dL, with a mean eGFR of 44.8 ± 32 ml/min/1.73 m^2. Serum total PCS levels were significantly higher compared with those of the healthy control (7.16 [<1.0 - 42.06] vs. 1.93 [1- 3.8] mg/L, p<0.001), as were serum total IS levels (4.63 [<0.225 - 53.58] vs. 0.88 [0.59- 1.26] mg/L, p<0.001). Of all patients, 35 (13.1%) patients had renal progression and 14 (5.2%) patients dead (7 patients from cardiovascular cause, 6 from infection and 1 from liver cirrhosis) after a mean follow-up of 21 ± 5.4 months Table 2 indicated correlation of serum level of PCS and IS with eGFR and other important risk factor of renal progression.

Serum PCS/IS and stage of CKD

The baseline serum PCS and IS levels were significantly higher in patients who had renal progression during follow-up compared with non-progressors [serum PCS levels were 10.26 (1.69-36.24) mg/L in progressor patients and 3.97 (0.35-42.06)mg/L in non-progressor patients, p < 0.001; serum IS level, 7.6 (0.19-53.58) mg/L vs. 1.94 (0.29- 39.09) mg/L, p< 0.001, respectively].

Table 3 summarizes the hazard ratios (HR) for renal progression and all-cause mortality in whole study patient and in subset group of patients according to baseline eGFR level as function of serum PCS and IS levels. Higher serum PCS levels were significantly associated with renal progression [HR, 1.092; 95% confidential interval (CI), 1.060- 1.126; p<0.001] and all-cause mortality (HR, 1.099; 95%CI, 1.053-1.148; p<0.001) in all patients. Higher serum IS level was only associated with renal progression (HR, 1.063; 95% CI, 1.041-1.085; p<0.001) but not all-cause mortality. In

subset analysis of patient with different baseline renal functions, these associations remained significant in patients with eGFR > 45 ml/min. However, we were not able to associate either serum PCS or IS with the risk of renal progression or all-cause mortality in patients with eGFR < 45ml/min.

Serum PCS/IS and progression of CKD

In crude analysis, a serum total PCS level greater to 7.16mg/L (the median) and serum total IS level greater to 4.63 mg/L (the median) were associated with renal progression, log-rank p< 0.001 (figure 2, A and 3, A). Univariate analysis (Table 4) identified that higher serum total IS (HR, 1.063; 95% CI, 1.041- 1.085; p <0.001) and PCS (HR, 1.092; 95% CI, 1.060- 1.126; p <0.001) levels were significantly associated with progression of CKD. Other significant risk factor included presence of diabetes mellitus (HR, 2.618; 95% CI, 1.282- 5.344; p =0.008), eGFR (HR, 0.96; 95% CI, 0.94- 0.981; p <0,001), calcium (HR, 0.183; 95% CI, 0.110- 0.306; p <0.001), phosphate (HR, 2.899; 95% CI, 2.136- 3.934; p <0.001), Ca/P product (HR, 1.109; 95% CI, 1.067- 1.154; p <0.001), iPTH (HR, 1.003; 95% CI, 1.001- 1.005; p <0.001), hemoglobin (HR, 0.678; 95% CI, 0.572- 0.802; p <0.001); uric acid (HR, 1.255; 95% CI, 1.094- 1.493; p <0.001) and albumin (HR, 0.236; 95% CI, 0.141- 0.392; p <0.001). Multivariate Cox regression analyses were constructed with different adjustment of important risk factors for CKD progression (Table 5). The serum PCS, analyzed as a continuous variable, was independently associated with CKD progression after adjustment of patient's demographic characteristics (age, gender and DM, model 1). The predictive role of serum PCS remained independently significant with adjustment for its binding protein (albumin, model 2), baseline renal function (eGFR, model 3), indoxyl sulfate (model 4a) and other common risk factors of CKD progression (Ca x P

product, iPTH, hemoglobin and hs-CRP, model 5). The analysis of serum IS (as continuous variable) resulted in significant association with CKD progression in abovementioned models (model 1, 2, 3 and 5), except for adjustment for serum PCS (model 4b, table 5).

Serum PCS/IS and all-cause mortality

The baseline serum PCS and IS levels were also significantly increased in deceased patients [serum PCS levels were 12.07 (0.9-42.06) mg/L in deaths and 4.1 (0.35-36.24) mg/L in survivors, p=0.002; serum IS levels, 4.78 (0.7-12.54) mg/L vs. 2.07 (0.19-53.58), p=0.05, respectively]. Univariate analysis showed that higher serum total PCS (HR, 1.099; 95% CI, 1.053- 1.148; p < 0.001), age (HR, 1.102; 95% CI, 1.036- 1.173; p =0.002), hemoglobin (HR, 0.7; 95% CI, 0.538- 0.910; p = 0.008) and albumin (HR, 0.277; 95% CI, 0.118- 0.665; p =0.003) were significantly associated with all-cause mortality in CKD patients. The serum total IS level was not associated with all-cause mortality. The serum PCS, analyzed as a continuous variable, remained independently associated with all-cause mortality in multivariate Cox regression analysis with different adjustment (Table 5, model 1 to 5). The figure 2 and 3 showed Kaplan–Meier estimates of all-cause mortality as a function of total PCS and IS levels relative to the median.

Serum PCS/IS and collinearity

Despite certain correlation between log-transformed serum total PCS and IS, the model 4 indicated a significant competitive effect of serum PCS and IS for the study endpoints. Therefore, there was no significant effect of co-linearity phenomena impacted on the instability of regression model.

Discussion

In the study, we evaluate the association between total PCS and IS with renal progression and all-cause mortality in different stage of CKD patients. We found that serum total PCS was associated with renal progression independently of baseline renal function and other modifiable and non-modifiable risk factors, such as age, diabetes, calcification, anemia, malnutrition-inflammation and IS. The serum total IS was associated with renal progression; however, this associated was lost if serum PCS is present in the analytical model.

Renal progression constitutes troublesome dilemma of clinical practice. Despite proper control of "classical" and uremia related risk factors, the deterioration of renal is still inevitable in a substantial proportion of patients. The impact of known risk factors is not enough to predict renal progression. Our study has demonstrated for the first time that both PCS and IS may not be only marker of renal function and also could predict its progression. The baseline renal function and proteinuria are important predictors of subsequent renal progression in both diabetic and non-diabetic CKD patients[22-23]. The present study had prospectively followed-up different stage CKD patients and had included a diversity of common measurable risk factors. Our finding suggested that serum IS and PCS levels are novel predictors of renal progression, and could provide additional information beyond the baseline renal function, other traditional and uremia-related predictors.

Despite significant association of high serum PCS and renal progression, the exact mechanism to the disease remains to be elucidated. In vitro, PCS significantly increased the percentage of leucocytes displaying oxidative burst activity at baseline. [17]. *p*-Cresyl sulfate also induces a dose-dependent shedding of endothelial

microparticles in the absence of overt endothelial damage[6]. For these reasons, PCS has a proinflammatory effect and can alter endothelial function. Although the relationship of PCS with cardiovascular disease and mortality has been evaluated in previous investigations[18,20,24], there were no clinical evidence indicating the association of PCS and renal progression. Further In-vivo or in-vitro investigations demonstrating the active role of PCS in stimulate renal progression remain awaited.

The detrimental effect of IS on the renal progression has been extensively evaluated in various experimental and in-vivo studies[8,25]. The present longitudinal study confirmed the association of serum IS with renal progression in CKD patients. However, the power of IS was reduced when the serum PCS increased. Serum PCS and IS are competitive binding inhibitor for the same albumin binding site (Sudlow site II)[3]. It is unknown if the high serum levels of PCS and IS could also behaved as competitive inhibitor at cellular level. Our finding offered new insight in the different pathogenic mechanism of PCS and IS in the genesis of renal progression. Further experimental model capable of clarifying the biological role of PCS (in conjunction with IS) should be constructed to confirm our finding.

Our significant association of serum PCS and all-cause mortality was similar to various previous studies[18-19,26]. Barreto et al demonstrated that high serum IS was associated with vascular disease and mortality in CKD patients[7]. However, this association was not observed in our patients. We speculate that the number of death in our study was not sufficient to preclude firm conclusion on the mortality.

From the temporal relationship between serum IS/PCS and renal progression in this prospective study, we suggested that the significant association is valuable. However, these data cannot be interpreted in causal terms. Our small-scale study revealed the importance of serum PCS and IS in CKD progression; however,

limitations of generalizability were found, including different ethnic groups, observation time, single-center experience, and unavailability of free form of toxins. Association of free solute concentration with mortality[19] and cardiovascular disease[20] has been well established in hemodialysis patients but less unclear in CKD patients not yet on dialysis. Recently, Liebeuf et al[18] demonstrated that free p-cresylsulphate is a predictor of mortality in patients at different stages of chronic kidney disease. However, the 65.5% of their patients were on stage 4, 5 or 5D. One third of all study population was patients on dialysis. Previous investigations showed that unconjugated p-cresol is not detectable in normal and predialysis CKD human plasma and almost the 99% of circulating toxins are in its sulfated form[16], the main culprit of tissue damage[17,18]. Our collages revealed that the free forms of indoxyl sulfate and *p*-cresol represent small amounts (approximately 10%) of the total forms in their blood concentrations in peritoneal dialysis patients. The presence of residual kidney function affects significantly the levels of free and total indoxyl sulfate[21]. Since all of our participants are predialysis CKD patients with a mean eGFR of 44.8 ± 32 ml/min, the free form of IS and PCS was not detected in large proportion of patients.

Several small interventional studies demonstrated that AST-120, an orally ingested charcoal adsorbent, could reduce IS levels[27], slow renal progression[28-29] and delay the initiation of dialysis[30]. However, a multicentric randomized control trial with a follow-up time of 1 year found that administration of AST-120 slowed the decrease in estimated CCr, but did not delay the occurrence of the serious clinical events, such as doubling of sCr level, increase in sCr level > 6.0 mg/dL , need for dialysis or transplantation, or death[31]. The effect of AST-120 on retard of renal progression remains to be proven.

In conclusion, serum IS and PCS levels may help to predict risk of renal

progression in different stage of CKD patients beyond traditional and uremia related risk factors including renal function. Additional studies may be needed to elucidating the mechanistic pathway of this finding and to directing further therapeutic strategies for protein bound toxin lowering.

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Transparency declarations

Conflicts of interest: None to declare

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Table 1: Baseline characteristics of all patients

	All patients (n= 268)
Age, y	66.9 ± 12
Male, n, %	154 (57.5%)
BMI, kg/m2	25.8 ± 3.5
Diabetes, n, %	126 (47%)
SBP, mmHg	135 ± 15
DBP, mmHg	71 ± 8
eGFR, ml/min/1.73 m2	44.8 ± 32
Initial CKD stage	
I, n,%	25 (9.3%)
II, n,%	42 (15.7%)
IIIa, n,%	37 (13.8%)
IIIb, n,%	54 (20.1%)
IV, n,%	76 (28.4%)
V, n,%	34 (12.7%)
sCr, mg/dL	1.9 ± 1.4
Ca, mg/dL	9.2 ± 0.5
P, mg/dL	3.8 ± 0.9
Ca x P, mg2/dL2	35.5 ± 7.1
iPTH, pmol/L	89.9 (1 - 692)
Cholesterol, mg/dL	193 ± 60
hemoglobin, g/dL	12.6 ± 2.1
hs-CRP, mg/L	3.2 (0.2 - 48.4)

Uric acid, mg/dL	6.9 ± 1.8
Albumin, g/dL	3.9 ± 0.4
Microalbumin, mg/day	61.4 (2-16900)
Total PCS, mg/L	7.16 (<1- 42.06)
Total IS, mg/L	4.63 (< 0.225- 53.58)

Abbreviation: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease; sCr; serum creatinine; Ca, calcium; P, phosphate; iPTH, intact parathyroid hormone; hs-CRP, high sensitive- C reactive protein; PCS, p-cresyl sulfate; IS, indoxyl sulfate.

Table 2: Correlation between log-transformed serum total PCS, IS and selected risk factors

	log-PCS		log-IS	
	r	p value	r	p value
—log-eGFR	0.642	< 0.001	0.720	< 0.001
Potassium	0.269	< 0.001	0.194	< 0.001
Ca x P	0.233	< 0.001	0.184	< 0.001
Hemoglobin	-0.513	< 0.001	-0.546	< 0.001
Albumin	-0.317	< 0.001	-0.394	< 0.001
Log-IS	0.655	< 0.001	—	—

Abbreviation: eGFR, estimated glomerular filtration rate; Ca, calcium; P, phosphate; PCS, p-cresyl sulfate; IS, indoxyl sulfate

Table 3: Univariate Cox proportional Hazard regression analysis in subset group of patient according to eGFR level

Variables	All patients		eGFR < 45 ml/min		eGFR > 45 ml/min	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
For renal progression	n=35/268		n=31/164		n=4/104	
Serum total IS, mg/L	1.063 (1.041-1.085)	<0.001	1.051 (1.027- 1.075)	<0.001	0.553 (0.09-3.405)	0.523
Serum total PCS, mg/L	1.092 (1.060-1.126)	<0.001	1.074 (1.038- 1.111)	<0.001	1.049 (0.821-1.340)	0.702
For all-cause mortality	n=14/268		n=11/268		n=3/104	
Serum total IS, mg/L	1.014 (0.956-1.075)	0.647	1 (0.934- 1.071)	0.993	0.248 (0.015-4.2)	0.334
Serum total PCS, mg/L	1.099 (1.053-1.148)	<0.001	1.104 (1.049-1.160)	<0.001	0.883 (0.468-1.668)	0.702

Abbreviation: PCS, p-cresylsulfate; IS, indoxyl sulfate

Table 4: Unadjusted HR for different endpoints

Baseline Variable	Units of Increase	Renal progression (event/total= 35/268)		All- cause mortality (event/total= 14/268)		Composite enpoints (event/total= 46/268)	
		Unadjusted HR (95% CI)	p	Unadjusted HR (95% CI)	p	Unadjusted HR (95% CI)	p
Age, y	1 year	1.003 (0.976-1.031)	0.828	1.102 (1.036-1.173)	0.002	1.018 (0.992-1.044)	0.184
Male (vs female)	—	0.439 (0.221-0.872)	0.019	1.369 (0.459-4.086)	0.573	0.560 (0.312-1.003)	0.051
Diabetes (yes vs. no)	—	2.618 (1.282-5.344)	0.008	1.128 (0.396-3.216)	0.822	1.792 (0.991-3.240)	0.054
eGFR, ml/min/1.73 m2	1 ml/min/1.73 m2	0.96 (0.940-0.981)	<0.001	0.98 (0.956-1.004)	0.101	0.971 (0.955-0.987)	<0.001
Ca, mg/dL	1 mg/dL	0.183 (0.110-0.306)	<0.001	0.564 (0.256-1.240)	0.154	0.239 (0.154-0.373)	<0.001
P, mg/dL	1 mg/dL	2.899 (2.136-3.934)	<0.001	1.132 (0.661-1.939)	0.651	2.211 (1.704-2.870)	<0.001
Ca x P, mg2/dL2	1 mg2/dL2	1.109 (1.067-1.154)	<0.001	1.002 (0.931-1.078)	0.96	1.076 (1.037-1.116)	<0.001
iPTH, pmol/L	1 pmol/L	1.003 (1.001-1.005)	0.001	1.001 (0.997-1.006)	0.527	1.003 (1.001-1.005)	0.008

hemoglobin, g/dL	1 g/dL	0.678 (0.572-0.802)	<0.001	0.70 (0.538-0.910)	0.008	0.687 (0.593-0.797)	<0.001
Uric acid, mg/dL	1 mg/dL	1.255 (1.094-1.439)	0.001	1.194 (0.942-1.514)	0.143	1.188 (1.042-1.354)	0.01
Albumin, g/dL	1 g/dL	0.236 (0.141-0.392)	<0.001	0.277 (0.118-0.665)	0.003	0.270 (0.173-0.420)	<0.001
Total IS, mg/L	1 mg/L	1.063 (1.041-1.085)	<0.001	1.014 (0.956-1.075)	0.647	1.050 (1.028-1.071)	<0.001
Total PCS, mg/L	1 mg/L	1.092 (1.060-1.126)	<0.001	1.099 (1.053-1.148)	<0.001	1.090 (1.062-1.118)	<0.001

Abbreviation: CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; Ca, calcium;

P, phosphate; iPTH, intact parathyroid hormone; PCS, p-cresyl sulfate; IS, indoxyl sulfate.

Table 5: Multivariate Cox regression analysis for primary and composite endpoints

Models	Renal progression			All-cause mortality			Composite endpoints		
	HR	95%CI	p	HR	95%CI	p	HR	95%CI	p
Serum p-cresyl sulfate- continuous variable									
Unadjusted	1.092	1.060-1.126	<0.001	1.099	1.053-1.148	<0.001	1.09	1.062-1.118	<0.001
Model 1	1.086	1.52-1.121	<0.001	1.134	1.076-1.196	<0.001	1.089	1.059-1.119	<0.001
Model 2	1.076	1.042-1.110	<0.001	1.083	1.033-1.136	0.001	1.075	1.047-1.104	<0.001
Model 3	1.061	1.020-1.103	0.003	1.101	1.044-1.162	<0.001	1.074	1.040-1.109	<0.001
Model 4a	1.066	1.016-1.119	0.009	1.162	1.099-1.229	<0.001	1.094	1.053-1.137	<0.001
Model 5	1.057	1.019-1.098	0.003	1.119	1.058-1.184	<0.001	1.075	1.040-1.111	<0.001
Serum indoxyl sulfate- continuous variable									
Unadjusted	1.063	1.041-1.085	<0.001	1.014	0.956-1.075	0.647	1.05	1.028-1.071	<0.001
Model 1	1.058	1.035-1.081	<0.001	1.022	0.954-1.094	0.536	1.048	1.025-1.071	<0.001
Model 2	1.06	1.037-1.085	<0.001	0.997	0.929-1.070	0.932	1.045	1.022-1.069	<0.001
Model 3	1.04	1.012-1.068	0.004	0.981	0.904-1.065	0.651	1.03	1.004-1.057	0.026

Model 4b	1.025	0.988-1.062	0.188	0.903	0.812-1.004	0.059	0.995	0.964-1.028	0.769
Model 5	1.034	1.004-1.064	0.028	0.97	0.876-1.074	0.558	1.025	0.995-1.056	0.104

Model 1 was adjusted for age (1-year increment), male gender and diabetes status.

Model 2 was adjusted for serum albumin (1 g/L increments)

Model 3 was adjusted for eGFR (1 ml/min increments)

Model 4a was adjusted for indoxyl sulfate (1mg/L increments)

Model 4b was adjusted for p-cresyl sulfate (1mg/L increments)

Model 5 was adjusted for Ca x P product(1 mg2/dL2 increments), intact parathyroid hormone (log 1 pmol/L increments), hemoglobin (1g/dL increments) and hs-CRP (log 1 mg/L increments)

Figure legends

Figure 1: Flow chart indicates patient enrolment.

Figure 2: Kaplan-Meier survival curves in all patients according to serum PCS level above and below the median of 7.16 mg/L; A, cumulative renal survival (censored for death), log-Rank p< 0.001; B, cumulative survival, log-Rank p= 0.002; C, cumulative proportion of patients who did not reach composite endpoints, log-Rank, p< 0.001.

Figure 3: Kaplan-Meier survival curves in all patients according to serum IS level above and below the median of 4,63 mg/L; A, cumulative renal survival (censored for death), log-Rank p< 0.001; B, cumulative survival, log-Rank p= 0.062; C, cumulative proportion of patients who did not reach composite endpoints, log-Rank, p< 0.001.

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