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Report

Validation of the Utility of the Uremic Toxin Integrated Score as a Prognostic Factor for Cardiovascular Complications in Hemodialysis Patients: A Pilot Study

Aina Sugiura,[a,1] Kaito Makise,[a,1] Yuki Narita,[a,b,c,*] Etsushi Nakata,[a] Keisuke Matushita,[a] Kazutaka Oda,[b] Junji Saruwatari,[d] Hirofumi Jono,[a,b] Kazuhiko Nishi,[e] and Hideyuki Saito[a,b]

[a]*Department of Clinical Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan;* [b]*Department of Pharmacy, Kumamoto University Hospital, Kumamoto, Japan;* [c]*Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan;* [d]*Division of Pharmacology and Therapeutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan;* [e]*Department of Urology, Kamiamakusa General Hospital, Kumamoto, Japan*

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Patients undergoing hemodialysis face a significantly elevated risk of cardiovascular disease (CVD) mortality. Uremic toxins, particularly indoxyl sulfate (IS) and p-cresyl sulfate (PCS), accumulate in patients undergoing dialysis and contribute to cardiovascular complications. Although IS and PCS concentrations are often assessed individually, this pilot study developed and validated an integrated scoring system combining these values to improve CVD mortality prediction. This prospective study included 66 patients undergoing hemodialysis at the Hitoyoshi Medical Center between 2008–2018. The IS + PCS score was calculated using Cox proportional hazards analysis. The primary endpoint was CVD death. The IS + PCS score was strongly associated with CVD mortality. IS alone exhibited high sensitivity (91.67%) but low specificity (44.44%), whereas PCS alone showed high specificity (92.59%) but low sensitivity (25.00%). The IS + PCS score achieved balanced sensitivity (83.33%) and specificity (57.41%), with a higher F1 score (0.44) than that of IS alone (0.41) and PCS alone (0.32). Leave-one-out cross-validation confirmed internal validity (mean C-index: 0.645). The IS + PCS score demonstrated potential as a prognostic predictor of CVD mortality in patients undergoing hemodialysis. While the score achieved balanced sensitivity and specificity, with an F1 score exceeding that of the individual markers, time-dependent receiver operating characteristic analysis showed no superiority over IS alone at any time point. These findings suggest that the integrated score combines the complementary strengths of both toxins, but requires validation in larger cohorts to establish clinical superiority.

Key words uremic toxin, hemodialysis, cardiovascular disease, indoxyl sulfate, p-cresyl sulfate

INTRODUCTION

Chronic kidney disease (CKD) progresses to end-stage kidney disease, necessitating renal replacement therapy, including dialysis. Despite significant advances in dialysis therapy, mortality rates remain high, representing a major clinical challenge. Cardiovascular disease (CVD) accounts for more than half of all deaths in this population.[1)] Therefore, biomarkers that enable early assessment of CVD risk in patients undergoing dialysis are essential.[1)]

The traditional CVD risk factors include advanced age, hypertension, dyslipidemia, diabetes, smoking, and physical inactivity. However, their association with survival outcomes in patients undergoing dialysis differs markedly from that in patients with CKD. For instance, renin-angiotensin system inhibitors, which effectively slow kidney function decline and reduce cardiovascular events in patients with CKD, demonstrate limited benefits in patients undergoing dialysis.[2)] Similarly, although statins reduce cardiovascular events in patients with CKD, studies targeting dialysis population have not demonstrated significant benefits.[3)] This is likely due to the unique pathophysiology of patients undergoing dialysis, which significantly affects their prognosis.

Uremic toxins are key contributors to complications in patients undergoing dialysis. While these toxins are normally excreted by individuals with healthy kidney function, they cannot be eliminated in patients with kidney failure and instead accumulate systemically. This accumulation induces reactive oxygen species (ROS) production, resulting in uremic symptoms characterized by multi-organ damage.[4)] Among

*To whom correspondence should be addressed. e-mail: y-nari@ph.sojo-u.ac.jp

[1] These authors contributed equally to the work.

these, indoxyl sulfate (IS) and p-cresyl sulfate (PCS) are particularly significant uremic toxins with potent ROS-inducing effects.[5] IS is derived from tryptophan, whereas PCS is derived from tyrosine and phenylalanine, amino acids commonly found in dietary proteins. The gut microbiota convert these amino acids into indole and p-cresol, which undergo hepatic metabolism to form IS and PCS, respectively.[6] IS and PCS are likely to increase the risk of CVD by promoting vascular endothelial inflammation and stimulating vascular smooth muscle cell proliferation and migration, leading to calcification and thrombosis.[7] Indeed, studies demonstrate that IS and PCS contribute to CKD progression, coronary artery disease, and peripheral artery disease.[8] Both are emerging mediators of the cardiorenal crosstalk.

Yamamoto *et al*. reported that IS exhibits cardiovascular toxicity and may contribute to cardiovascular events.[9] Wu *et al*. demonstrated that PCS is an independent CVD risk factor, regardless of age, sex, or diabetes status.[1] Most studies to date have focused on evaluating IS or PCS as individual markers. In contrast, Lim *et al*. suggested that uremic toxins exert synergistic effects through shared toxicity pathways.[4] They emphasized that understanding the role of uremic toxins in CVD progression requires evaluating both individual and combined pathophysiological mechanisms in patients with kidney dysfunction. Assessing IS or PCS individually may not fully capture CVD risk. Thus these toxins should be evaluated together. However, no study has comprehensively assessed the roles of these toxins in CVD development.

Therefore, this pilot study aimed to develop a combined score integrating the IS and PCS to evaluate its association with CVD prognosis and its utility as a prognostic predictor in patients undergoing hemodialysis.

METHODS

Study Design This prospective observational study enrolled patients undergoing hemodialysis at the Hitoyoshi Medical Center (renamed Hitoyoshi Hospital in 2014) in 2008 and tracked their survival until 2018. Prognostic factors (IS and PCS) were collected from a separate study conducted in 2008 using stored residual samples, which established the following exclusion criteria: a) age < 20 years, b) concurrent peritoneal dialysis, c) dialysis frequency less than three times weekly, and d) symptomatic heart failure at rest (Stage III or IV). After applying these criteria, 66 patients were included. The primary endpoint was CVD death, defined as death from cardiac or vascular diseases. Test values were collected from medical records, and baseline data were used for all analyses.

Ethical Considerations This study was approved by the Ethics Committee of Faculty of Life Sciences, Kumamoto University (Ethics No. 1457) and followed the Declaration of Helsinki and institutional guidelines. As this study utilized existing stored residual samples and medical records, informed consent was obtained through an opt-out approach with public disclosure of study information. Personal information was anonymized to protect privacy.

Measurement of Total and Free IS and PCS Serum Concentrations Total and free serum concentrations of IS and PCS were measured using API 3200™ LC-MS/MS system (AB SCIEX, Foster City, CA, USA).

The LC-MS/MS measurement conditions for IS and PCS are described below:

Column: Symmetry® C18 5 μm, 3.9 × 150 mm, Waters
Column temperature: 40 °C
Flow rate: 0.2 mL/min
Mobile phase: 10 mM ammonium acetate:acetonitrile = 73:27
Polarity/Scan type/Ion source: Negative/SRM/Turbo spray
Parameters: CUR 40.00, CAD 3.00, IS -4500.00, TEM 500.00, GSI 50.00, GS2 30.00
Injection volume: 5 μL

Statistical Analysis The cutoff values for each parameter relative to CVD mortality were determined from the receiver operating characteristic (ROC) curves, and patients were stratified into high and low groups accordingly. Cutoff values were determined using the Youden index, which maximizes the difference between the positive rate (sensitivity) and false-positive rate (1-specificity). To assess the CVD mortality risk for each parameter, univariate and multivariate Cox proportional hazards analyses were performed using the stratified groups. Variables with $p < 0.2$ in univariate analysis were included as covariates in multivariate models. Detailed results are provided in the Supplementary Data.

As shown in Supplementary Data (Supplemental Data 4), multivariate analysis confirmed that total IS and PCS concentrations are independent risk factors for CVD mortality, distinct from established risk factors including age and NT-proBNP. Because the objective of this study was to develop a score that specifically integrates uremic toxins, we performed Cox proportional hazards analysis using only total IS and PCS concentrations as covariates to determine optimal weighting coefficients for the score.

To construct an integrated uremic toxin score (IS + PCS score), we performed a Cox proportional hazards analysis using only the total IS and total PCS concentrations as covariates. The hazard ratios served as weights reflecting each toxin's contribution to CVD mortality risk, forming the IS + PCS score as follows:

$$\begin{aligned}\text{IS + PCS score} &= \text{total IS concentration } (\mu M) \times \ln(6.3) \\ &\quad + \text{total PCS concentration } (\mu M) \times \ln(2.3) \\ &= \text{total IS concentration } (\mu M) \times 1.84 \\ &\quad + \text{total PCS concentration } (\mu M) \times 0.83\end{aligned}$$

Survival curve analysis and Cox proportional hazards regression (univariate and multivariate) were performed to assess CVD mortality risk. Survival curves were compared between the groups using the log-rank test.

The predictive performance of IS alone, PCS alone, and the IS + PCS score for CVD mortality was evaluated using time-dependent ROC curve analysis with the C-index, along with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and F1 score at multiple time points. Areas under the curve were compared using the DeLong test. Internal validity was assessed using leave-one-out cross-validation (LOOCV), with predictive performance reported as the C-index.

All analyses were performed using R (version 4.5.0, R Foundation for Statistical Computing, Vienna, Austria), with $p < 0.05$ considered statistically significant.

RESULTS

Patient Characteristics Table 1 presents the baseline characteristics of the 66 patients. Categorical variables are

Table 1. Patient Characteristics

Variables	Data are expressed as mean ± SD or n (%)
Age (year)	62 ± 12
Sex (male, %)	35 (53)
DW (kg)	52.6 ± 9.6
BW (kg)	54.7 ± 9.9
HR (bpm)	73.5 ± 5.8
SBP (mm Hg)	134.1 ± 21.5
DBP (mm Hg)	74.3 ± 9.4
Hypertension (n, %)	41 (65)
Diabetes (n, %)	10 (16)
Cardio-Thoracic Ratio (%)	51.0 ± 4.9
Ferritin (ng/mL)	550.2 ± 770
Fe (μg/dL)	77.1 ± 37.9
PTH (pg/mL)	211.3 ± 217.9
CK-MB (ng/mL)	3.7 ± 1.3
NT-proBNP (pg/mL)	6563.1 ± 13374.2
hsTnT (pg/mL)	58.4 ± 30.9
IS total(μM)	166.4 ± 72.3
IS free(μM)	5.7 ± 3.3
PCS total(μM)	261.5 ± 147.1
PCS free(μM)	9.5 ± 7.6

DW, dry weight; BW, body weight; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; Fe, serum iron; PTH, parathyroid hormone; CK-MB, creatine kinase–myocardial band; NT-proBNP, N-terminal pro-B-type natriuretic peptide; hsTnT, high-sensitivity troponin T; IS, indoxyl sulfate; PCS, p-cresyl sulfate

expressed as numbers (percentages), and continuous variables as mean ± standard deviation.

Development of IS + PCS Score and Validation of Its Predictive Capability Multivariate Cox proportional hazards analysis confirmed that total IS and PCS concentrations were independent CVD risk factors, distinct from established factors such as age and N-terminal pro-B-type natriuretic peptide (NT-proBNP; Supplementary data). Therefore, to construct the IS + PCS score, we performed multivariate analysis using only the total IS and PCS concentrations as covariates. The resulting hazard ratios served as weighting coefficients (Fig. 1A), from which the IS + PCS score was calculated.

Survival curve analysis showed significantly lower survival in the high IS + PCS score group (log-rank $p = 0.02$) (Fig. 1B). Univariate Cox regression identified the IS + PCS score as a significant risk factor (hazard ratio [HR]: 5.05, 95% confidence interval [CI]: 1.11–23.07, $p = 0.037$). In multivariate analysis adjusting for age and NT-proBNP, all three variables remained independent predictors: IS + PCS score (HR: 34.13, 95% CI: 4.93–236.36, $p < 0.001$), NT-proBNP (HR: 21.06, 95% CI: 4.56–97.28, $p < 0.001$), and age (HR: 12.46, 95% CI: 2.74–56.64, $p = 0.001$) (Fig. 1C).

Evaluation of the Predictive Performance of the IS + PCS Score ROC curve analysis compared the discriminatory performance of IS alone, PCS alone, and the IS + PCS score for predicting CVD mortality (Fig. 2). Time-dependent ROC analysis revealed that the IS + PCS score had C-index values of 0.857 at 3 years and 0.630 at 5 years, which were lower than those of IS alone (0.984 at 3 years and 0.729 at 5 years). However, at longer follow-up periods of 7 and 10 years, the discriminatory performance of the IS + PCS score (0.638 and 0.627) and IS alone (0.646 and 0.634) became comparable, with minimal differences observed.

Table 2. Predictive Performance of IS, PCS, and IS + PCS Scores

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	F1 score
IS	91.67	44.44	26.83	96.00	0.41
PCS	25.00	92.59	42.86	84.75	0.32
IS+PCS score	83.33	57.41	30.30	93.94	0.44

IS, indoxyl sulfate; PCS, p-cresyl sulfate; PPV, positive predictive value; NPV, negative predictive value

To evaluate the predictive performance in detail, we calculated the sensitivity, specificity, PPV, NPV, and F1 score (Table 2). IS alone demonstrated high sensitivity (91.67%) but low specificity (44.44%), whereas PCS alone showed high specificity (92.59%) but low sensitivity (25.00%), revealing complementary characteristics. The IS + PCS score integrates the advantages of both toxins, achieving balanced predictive performance. Notably, the IS + PCS F1 score (0.44) exceeded both IS alone (0.41) and PCS alone (0.32), suggesting the value of the integrated approach. Furthermore, the IS + PCS score maintained a sensitivity of 83.33% and a negative predictive value of 93.94%, while improving specificity to 57.41% compared with IS alone (44.44%), thereby reducing false positives. These results demonstrate that integrating multiple uremic toxins improves the predictive accuracy beyond what single indicators can achieve.

Validity Assessment of IS + PCS Score We performed LOOCV to evaluate internal validity (Fig. 3). The mean C-index was 0.645 (standard error = 0.001), confirming high stability and validating the IS + PCS score.

DISCUSSION

In this pilot study, we evaluated the utility of the IS + PCS score as a prognostic predictor of CVD in patients undergoing hemodialysis. The IS + PCS score demonstrated balanced predictive performance compared with IS or PCS alone, with the F1 score slightly exceed those of the individual markers. Furthermore, IS and PCS may be risk factors for CVD mortality independent of age and traditional cardiac biomarkers, which have long been recognized as CVD risk factors. However, since the IS + PCS score did not exceed IS alone at any time point in the time-dependent ROC analysis, validation in larger cohorts is essential.

The improvement in the F1 score by the IS + PCS score represents a significant finding from the perspective of clinical practicality. Previous studies have reported that IS and PCS individually correlate with CVD risk but have noted limitations in their predictive performance.[1,9] In this study, IS alone demonstrated high sensitivity (91.67%) but low specificity (44.44%), indicating a high false-positive rate that may misclassify low-risk patients as high-risk, and lead to unnecessary testing or treatment. Conversely, PCS alone showed high specificity (92.59%) but low sensitivity (25.00%), risking oversight in many high-risk patients. Notably, the IS + PCS score demonstrated balanced performance with sensitivity (83.33%) and specificity (57.41%), while maintaining a particularly high NPV (93.94%). The novelty of this integrated score is its demonstrated potential to overcome single marker limitations by combining complementary predictive characteristics, supporting the utility of a new combined approach to evaluat-

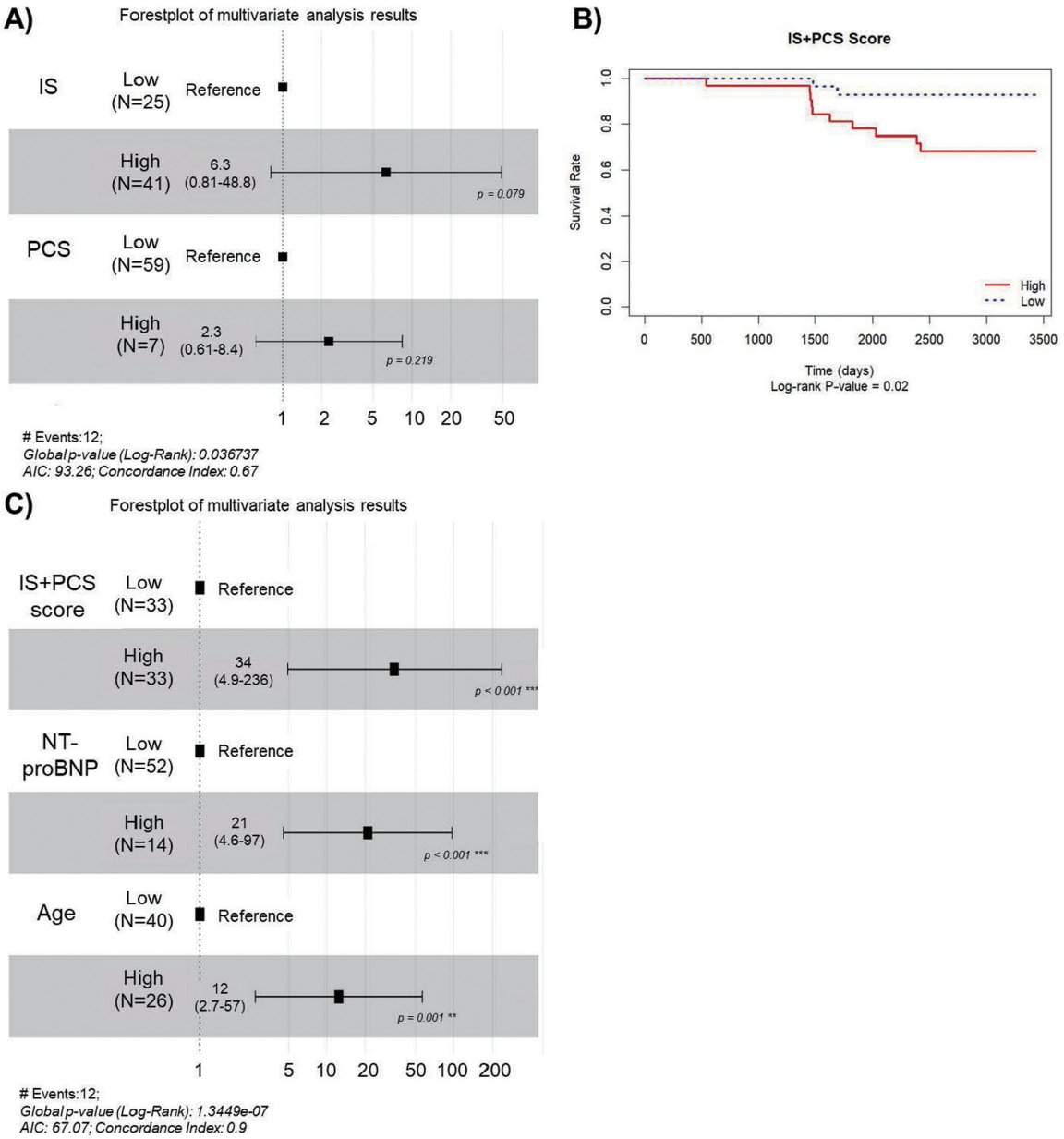

Fig. 1. Development and Validation of IS + PCS Score for Risk Prediction

A) Multivariate analysis of indoxyl sulfate (IS) and p-cresyl sulfate (PCS), B) Survival curve analysis based on IS + PCS score, C) Multivariate analysis of IS + PCS score, NT-proBNP, N-terminal pro-B-type natriuretic peptide (NT-proBNP), and age

ing uremic toxins, in contrast to existing biomarker research that has largely assessed them individually. This aligns with prior research showing that integrating multiple biomarkers improves predictive accuracy. Indeed, Zethelius *et al*. reported that combining four biomarkers (troponin I, NT-proBNP, cystatin C, and C-reactive protein) in elderly men improved the C-statistic from 0.664 to 0.766.[10] Wong *et al*. demonstrated that using multiple biomarkers could overcome individual marker limitations and improve prognostic accuracy.[11] The practicality of this contribution to clinical decision-making is highlighted by the score's balanced performance in achieving the highest F1 score (0.44). In counterpoint to its high sensitivity, IS alone yields "a high false-positive rate" owing to its extremely low specificity (44.44%). Excessive false positives complicate clinical decisions, potentially leading to "unnecessary testing or treatment" as mentioned previously. The IS+PCS score provides a practical solution by significantly improving specificity to 57.41%, thereby reducing the false-positive burden. This superior balance, which avoids the unacceptably low sensitivity of PCS alone (25.00%), enables reliable risk stratification. Clinicians can thus use this integrated score as a more dependable basis for clinical decisions, including identifying high-risk individuals, that is more trustworthy (less diluted by false positives) for targeted interventions or monitoring. However, as this was a pilot study, further validation is required to confirm clinical applicability.

In this study, the higher weighting coefficient for IS (1.84) compared with PCS (0.83) in calculating the IS + PCS score represented an interesting biological finding. Both IS and PCS act as aryl hydrocarbon receptor (AhR) ligands; howev-

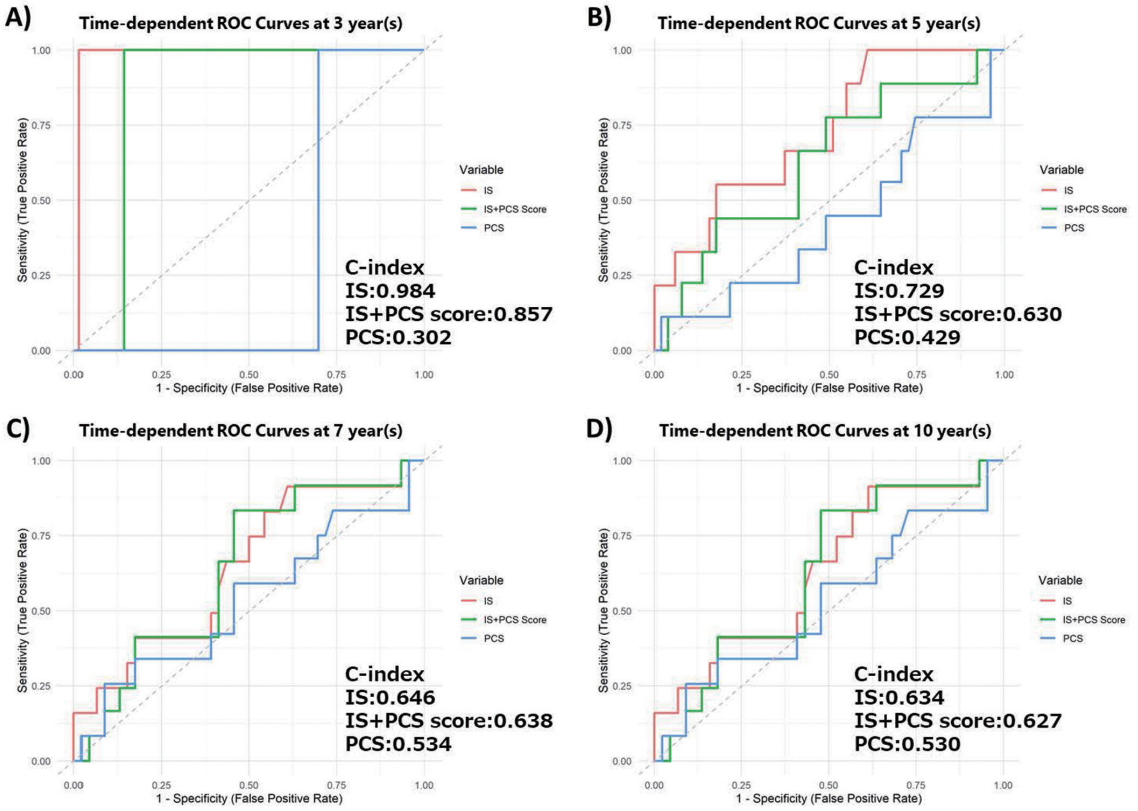

Fig. 2. Time-dependent ROC Curves for Prognostic Comparison of IS, PCS, and IS + PCS Score

Time-dependent receiver operating characteristic (ROC) curves at A) 3 years, B) 5 years, C) 7 years, and D) 10 years. C-index values are displayed for each model

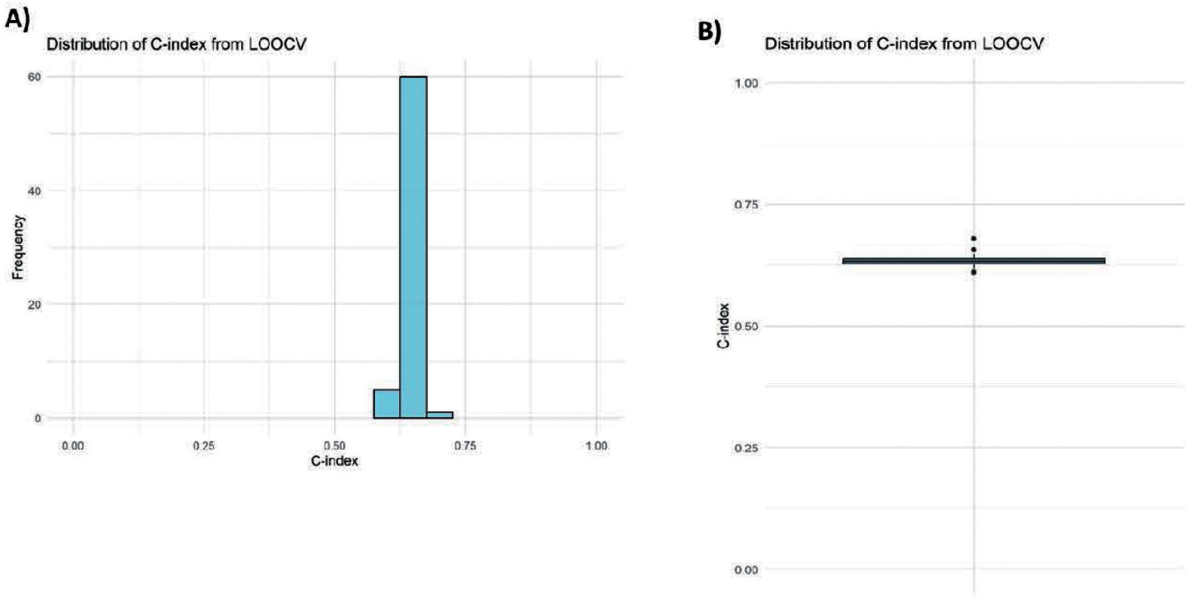

Fig. 3. Distribution of C-index from LOOCV

Leave-one-out cross-validation (LOOCV) results shown as A) histogram and B) box plot

er, several factors may explain IS's higher weighting. First, there may be differences in AhR activation intensity or downstream signaling. Schroeder *et al*. reported that IS has potent AhR-activating ability,[12] and Adelibieke *et al*. reported that IS strongly induces inflammatory cytokines via AhR.[13] Conversely, PCS toxicity involves multifaceted pathways, including AhR activation and altered microbiota expression.[14] Therefore, although AhR-mediated pathways play a central role in IS toxicity, multiple non-AhR pathways are also important in PCS toxicity. This difference in pathway dependency likely explains why IS is weighted more heavily. Second, pharmacokinetic differences between the two substances may also contribute. PCS has a high protein-binding rate (~95%)[15], making it difficult to remove via dialysis, whereas IS has a slightly lower rate (~90%)[15], resulting in a higher proportion of biologically active free form. This difference in the free form may influence tissue migration and cytotoxicity, potentially leading to higher cardiovascular toxicity from IS. Such differences in weighting coefficients are not merely statistical findings; they suggest that IS and PCS, although both are AhR ligands, may contribute to CVD pathophysiology with differing intensities through distinct pathways. This finding demonstrates the importance of individualized uremic toxin assessment in patients undergoing dialysis and may provide useful information for elucidating mechanisms and selecting therapeutic targets.

This study has several limitations. First, as this was a pilot study, the sample size was limited, particularly since the number of CVD deaths was low (n = 12), potentially resulting in insufficient statistical power. Second, in the time-dependent ROC analysis, the IS + PCS score showed comparable discriminatory performance to IS alone at long-term follow-up (7 years and beyond) but did not exceed IS at any time point. This suggests that the limited number of events may have been insufficient to detect the superiority of the integrated score. Third, because this was a single-center pilot study, the generalizability of the results is limited. Furthermore, while internal validity was confirmed using LOOCV, external validation is necessary to confirm the validity and reproducibility. Fourth, potential confounding factors such as dialysis conditions, residual renal function, and medication adherence were unadjusted and may have influenced the IS/PCS concentrations and CVD mortality risk. Based on these results, validation in a larger multicenter study is essential to establish the clinical utility. Specifically, it will be necessary to reassess the prognostic ability of the integrated score after ensuring a sufficient number of events and adjusting for important confounders. Fifth, this study analyzed only IS and PCS as uremic toxins, although numerous other uremic toxins and inflammatory and nutritional markers have been reported to be associated with CVD.[16] Therefore, the results did not comprehensively evaluate the impact of the full spectrum of biomarkers on CVD mortality. Further improvements in the predictive performance may be achieved by developing integrated models that incorporate a greater number of uremic toxins and other relevant markers.

This pilot study highlights the significance of a novel integrated approach for evaluation of uremic toxins. The combined IS + PCS score demonstrated a balanced predictive performance, distinct from that of IS or PCS alone, in forecasting CVD mortality among patients undergoing hemodialysis. The slight improvement in F1 score underscores the value of this combined approach from the perspective of balancing sensitivity and specificity. Furthermore, the different weighting coefficients of IS and PCS are intriguing findings, suggesting that these uremic toxins may contribute to the pathophysiology of CVD with differing intensities or mechanisms. Given this study's limitations, these preliminary findings provide a strong rationale and foundation for future large-scale validation studies to establish the clinical utility of the score.

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Conflict of interest The authors declare no conflict of interest.

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