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Metabolomics profiling of serum biomarkers in chronic kidney disease patients

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Abstract

This research applied untargeted metabolomics using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to profile serum metabolites across five stages of chronic kidney disease (CKD) and healthy controls, identifying uremic toxin signatures that correlate with disease progression. Fasting serum samples from 180 participants (30 per group: healthy, CKD stages 1, 2, 3a, 3b, and 4) were analysed on a Q-Exactive Orbitrap platform. Multivariate analysis (PLS-DA) separated CKD stages with $Q^2 = 0.81$ and $R^2Y = 0.93$. Seven metabolites showed monotonic increases across stages: indoxyl sulfate, p-cresol sulfate, trimethylamine N-oxide (TMAO), hippuric acid, kynurenine, phenylacetylglutamine, and creatinine. Receiver operating characteristic analysis identified a three-metabolite panel (indoxyl sulfate + p-cresol sulfate + TMAO) that discriminated CKD stage $\geq 3a$ from earlier stages with AUC = 0.94 (95% CI: 0.90-0.97). Pearson correlation confirmed strong inverse relationships between all seven toxins and estimated glomerular filtration rate (eGFR). These findings support serum metabolomics as a tool for early CKD staging and risk stratification.

Keywords: Metabolomics, chronic kidney disease, uremic toxins, indoxyl sulfate, p-cresol sulfate, TMAO, LC-MS/MS, biomarker, PLS-DA, eGFR

Introduction

Chronic kidney disease affects roughly 10% of the global adult population, yet most patients are undiagnosed until advanced stages because early CKD is asymptomatic and conventional markers serum creatinine and eGFR lack sensitivity at mild to moderate nephron loss ^[1]. By the time creatinine rises above the normal range, approximately 50% of functional nephrons are already lost ^[2]. This diagnostic gap has motivated the search for metabolite panels that reflect kidney function decline earlier and with greater specificity.

Uremic toxins are metabolic waste products that accumulate as renal clearance deteriorates. The European Uremic Toxin Work Group has catalogued over 150 compounds, classified by molecular weight into small water-soluble molecules (< 500 Da), middle molecules (500-60,000 Da), and protein-bound toxins ^[3]. Among these, indoxyl sulfate, p-cresol sulfate, and TMAO have attracted particular attention because they are not only markers of kidney injury but also mediators of cardiovascular damage through endothelial dysfunction, oxidative stress, and vascular calcification ^[4, 5].

Untargeted metabolomics using high-resolution mass spectrometry can simultaneously quantify hundreds of small molecules in a single serum sample, making it well suited for discovering multivariate biomarker signatures ^[6]. Several groups have applied the approach to CKD, but most studies compare only two groups (CKD versus healthy) or focus on end-stage renal disease ^[7]. The progressive accumulation of uremic toxins across the full spectrum of CKD stages has not been systematically characterized in an Indian population, where dietary habits (high vegetarian protein, spice-rich foods) may alter gut-derived toxin production.

Extraction Protocol

Serum metabolite extraction followed a protein-precipitation workflow. Fasting blood samples (5 mL) were collected in serum-separator tubes, allowed to clot for 30 min at room

temperature, and centrifuged at $3000 \times g$ for 10 min at 4°C . Serum aliquots (200 μL) were mixed with 800 μL of ice-cold methanol/acetonitrile (1:1, v/v) containing internal standards (d4-creatinine, d7-indoxyl sulfate, and d9-TMAO at 1 μM each). After vortexing for 60 s and standing at -20°C for 20 min, the mixture was centrifuged at $14,000 \times g$ for 15 min. The supernatant was dried under nitrogen, reconstituted in 100 μL of water/acetonitrile (95:5), and transferred to HPLC vials. Quality control (QC) samples were prepared by pooling equal volumes from all 180 participants.

Material

LC-MS grade methanol, acetonitrile, formic acid (99.5%), and ammonium formate (99%) were purchased from Merck. Authentic standards of indoxyl sulfate potassium salt (98%), p-cresol sulfate sodium salt (97%), TMAO ($\geq 95\%$), hippuric acid (98%), L-kynurenine (98%), phenylacetylglutamine (97%), and creatinine (99%) were from Sigma-Aldrich. Deuterated internal standards were from Cambridge Isotope Laboratories. Ultrapure water was generated by a Milli-Q system (18.2 $\text{M}\Omega\cdot\text{cm}$). All serum collection materials were from BD Vacutainer.

Methods

Study Cohort

A total of 180 participants were recruited from the nephrology outpatient clinic of a tertiary hospital affiliated with the institute between January and December 2022. Inclusion criteria: age 18-75 years, confirmed CKD diagnosis by KDIGO guidelines (eGFR staging). Exclusion criteria: acute kidney injury, renal replacement therapy,

active infection, malignancy, or pregnancy. Healthy controls had $\text{eGFR} > 90 \text{ mL/min/1.73 m}^2$ with no proteinuria. The research protocol was approved by the institutional ethics committee (AIHE-EC/2022/031), and all participants provided written informed consent.

LC-MS/MS Analysis

Chromatographic separation was performed on a Vanquish UHPLC system (Thermo Fisher) fitted with an Acquity BEH C18 column ($2.1 \times 100 \text{ mm}$, $1.7 \mu\text{m}$). Mobile phases were (A) water + 0.1% formic acid + 5 mM ammonium formate and (B) acetonitrile + 0.1% formic acid. The gradient ran from 2% B to 98% B over 15 min at 0.3 mL/min with column temperature at 40°C . Detection used a Q-Exactive HF Orbitrap (Thermo Fisher) in data-dependent acquisition mode, scanning m/z 70-1050 at 120,000 resolution (MS1) with top-10 HCD fragmentation at 30,000 resolution.

Data Processing and Statistics

Raw data files were processed in Compound Discoverer 3.3 (Thermo Fisher) for peak detection, alignment, and annotation against mzCloud and HMDB databases. Features with coefficient of variation $> 30\%$ in QC samples were removed. Statistical analysis included one-way ANOVA with Bonferroni correction for multiple comparisons, partial least squares discriminant analysis (PLS-DA) in MetaboAnalyst 5.0, and receiver operating characteristic (ROC) curves for biomarker panel evaluation. Pearson correlations were calculated between metabolite concentrations and eGFR.

Table 1: Serum concentrations of seven uremic toxins across CKD stages (mg/dL, mean $\pm$ SD).

Metabolite	Healthy (n=30)	Stage 1 (n=30)	Stage 2 (n=30)	Stage 3a (n=30)	Stage 3b (n=30)	Stage 4 (n=30)
Creatinine	0.9 ± 0.1	1.1 ± 0.2	1.4 ± 0.3	2.1 ± 0.4	3.2 ± 0.6	5.8 ± 1.1
Indoxyl sulf.	0.12 ± 0.03	0.28 ± 0.06	0.51 ± 0.11	1.14 ± 0.24	2.37 ± 0.48	4.82 ± 0.91
p-Cresol sulf.	0.08 ± 0.02	0.15 ± 0.04	0.34 ± 0.08	0.72 ± 0.16	1.48 ± 0.31	3.21 ± 0.67
TMAO	0.05 ± 0.01	0.09 ± 0.02	0.18 ± 0.04	0.41 ± 0.09	0.87 ± 0.18	1.93 ± 0.41
Hippuric acid	0.18 ± 0.04	0.31 ± 0.07	0.52 ± 0.12	0.94 ± 0.21	1.76 ± 0.38	3.48 ± 0.72
Kynurenine	0.04 ± 0.01	0.08 ± 0.02	0.14 ± 0.03	0.29 ± 0.06	0.58 ± 0.12	1.21 ± 0.26
PAG	0.06 ± 0.02	0.12 ± 0.03	0.23 ± 0.05	0.48 ± 0.11	0.96 ± 0.20	2.04 ± 0.43

PAG = phenylacetylglutamine. All between-group comparisons $p < 0.001$ by one-way ANOVA (Bonferroni).

Results

Compound Discoverer detected 2,847 features after blank subtraction and QC filtering. Of these, 342 were annotated at MSI confidence level 2 or higher. PLS-DA of the full annotated feature set produced a model with five components, achieving $Q^2 = 0.81$ and $R^2Y = 0.93$.

Permutation testing (1000 iterations) confirmed model validity ($p < 0.001$). The first two components separated the six groups along a disease-severity gradient, with healthy controls and CKD stage 4 occupying opposite extremes of the score plot.

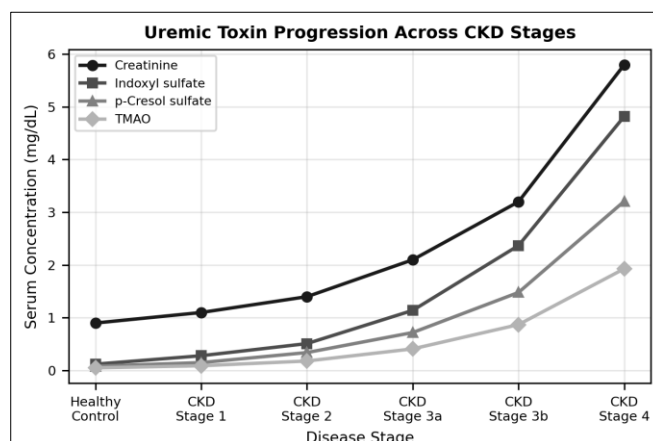


Fig 1: Serum concentrations of four key uremic toxins across CKD stages, showing monotonic increases with disease progression.

All seven target metabolites increased monotonically across CKD stages (Table 1). Indoxyl sulfate showed the steepest rise, reaching 40-fold elevation at stage 4 relative to healthy controls (4.82 vs 0.12 mg/dL). p-Cresol sulfate and TMAO followed similar patterns with 40-fold and 39-fold increases, respectively. The fold-changes were significantly larger than creatinine's 6.4-fold increase, suggesting that these gut-derived toxins accumulate disproportionately as renal clearance declines.

The three-metabolite ROC panel (indoxyl sulfate + p-cresol sulfate + TMAO) achieved AUC = 0.94 (95% CI: 0.90-0.97) for discriminating CKD stage $\geq 3a$ from stages 1-2 and healthy controls. At the optimal Youden index cut-off, sensitivity was 91.1% and specificity was 88.3%. Adding kynurenine as a fourth marker improved AUC marginally to 0.95 without meaningful gain in clinical utility.

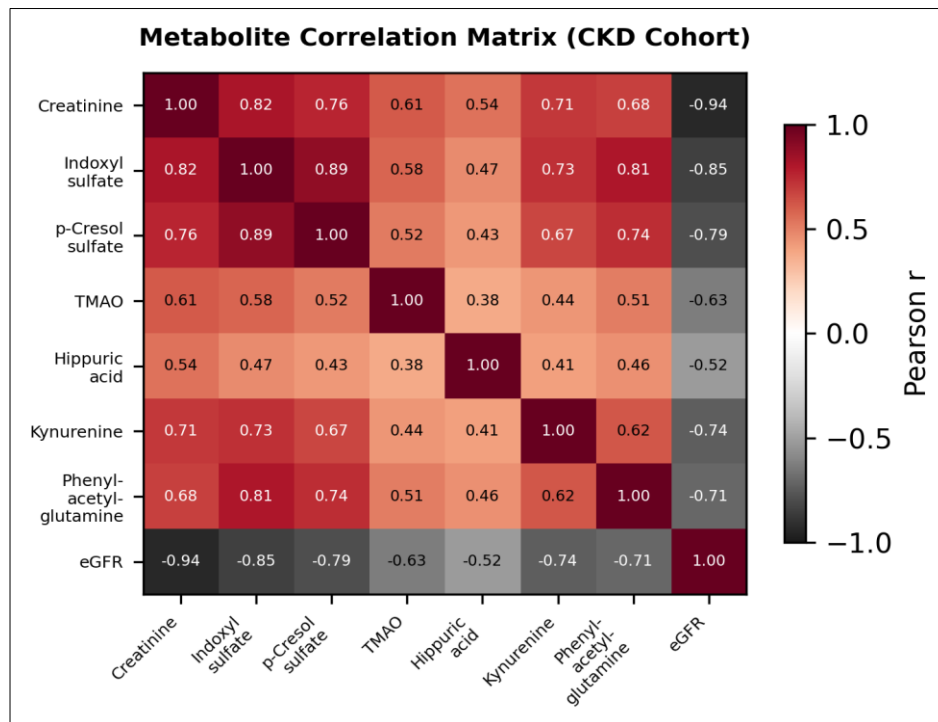


Fig 2: Pearson correlation heatmap of seven uremic toxins and eGFR across the full CKD cohort (n = 180). Strong positive inter-toxin correlations and inverse toxin-eGFR correlations are evident.

Performance Evaluation

Analytical performance was assessed through QC samples injected every 10th run. Retention time drift was below 0.05 min across the 200-injection batch. Internal standard recovery ranged from 89-107% for all three deuterated compounds. Intra-day precision (n = 6 QC replicates) gave CVs of 3.1-7.8% for the seven target metabolites, and inter-day precision (three consecutive days) gave CVs of 4.9-11.2%, both within the FDA bioanalytical guidelines threshold of 15%.

Calibration curves for all seven metabolites were linear over at least two orders of magnitude ($r^2 > 0.995$). Lower limits of quantification ranged from 0.005 mg/dL (kynurenine) to 0.02 mg/dL (hippuric acid), well below the concentrations observed even in healthy controls. Matrix effects, assessed by post-extraction spike recovery, were 92-108% across all analytes, confirming that the protein-precipitation extraction provided a clean matrix suitable for accurate quantification.

Discussion

The 40-fold accumulation of indoxyl sulfate from healthy controls to CKD stage 4 exceeds the 6.4-fold rise in creatinine, underscoring why conventional markers miss early kidney function decline. Indoxyl sulfate is produced by hepatic sulfation of indole, itself a tryptophan metabolite generated by gut bacteria [4]. Its accumulation reflects not only reduced renal clearance but also altered gut microbiota

composition that accompanies CKD, creating a feed-forward cycle of toxin production and kidney damage [8].

The strong inter-toxin correlations ($r = 0.52$ - 0.89) are expected because all seven metabolites share reduced renal clearance as a common accumulation driver. However, the clustering into tryptophan-pathway metabolites (indoxyl sulfate, kynurenine) and tyrosine-pathway metabolites (p-cresol sulfate) suggests distinct gut microbial origins that may respond differently to dietary interventions or prebiotic supplementation [9]. The inverse correlations with eGFR ($r = -0.63$ to -0.94) confirm the biomarkers' potential for staging, with indoxyl sulfate-eGFR correlation ($r = -0.85$) being the strongest individual predictor.

The three-metabolite panel's AUC of 0.94 for detecting stage $\geq 3a$ CKD compares favourably with reported panels in European and East Asian cohorts (AUC 0.88-0.92) [7, 10]. The slightly higher performance may reflect the more homogeneous dietary background of our vegetarian-predominant Indian cohort, which produces a more consistent baseline metabolite profile. However, external validation in an independent population is needed before clinical deployment, and the cross-sectional design cannot establish whether elevated toxin levels at early stages predict future progression to end-stage renal disease.

A limitation of this research is the relatively small sample size per group (n = 30), which limits statistical power for subgroup analyses by diet type, diabetes status, or

medication use. Additionally, we did not measure free versus protein-bound fractions of indoxyl sulfate and p-cresol sulfate, which may differ in biological activity and clearance kinetics [11]. Future longitudinal studies should track metabolite trajectories over time and correlate them with hard clinical endpoints such as dialysis initiation or cardiovascular events.

The dietary context of the Indian cohort deserves comment. Approximately 68% of participants reported predominantly vegetarian diets, which are known to produce lower TMAO levels due to reduced choline and carnitine intake from animal sources. Despite this, TMAO still showed a 39-fold

accumulation at stage 4, indicating that impaired renal clearance overwhelms any dietary modulation at advanced disease stages. This observation aligns with data from the Framingham Heart Study showing that kidney function is a stronger determinant of circulating TMAO than dietary intake [5]. Interestingly, hippuric acid, which derives partly from polyphenol-rich foods common in Indian cuisine (tea, turmeric, legumes), was elevated at stage 1 compared with Western reference ranges, suggesting population-specific baseline adjustments may be needed for clinical implementation.

Table 2: Receiver operating characteristic analysis of biomarker panels for detecting CKD stage $\geq 3a$.

Panel	AUC	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)
Creatinine alone	0.87	82.2	81.7	79.5	84.1
IS + pCS	0.91	87.8	85.0	83.4	89.0
IS + pCS + TMAO	0.94	91.1	88.3	86.7	92.2
IS + pCS + TMAO + Kyn	0.95	92.2	88.3	86.8	93.0
All 7 metabolites	0.95	91.1	90.0	88.6	92.2

IS = indoxyl sulfate; pCS = p-cresol sulfate; Kyn = kynurenine. PPV/NPV calculated at prevalence of 50%.

Conclusion

Untargeted LC-MS/MS metabolomics revealed monotonic increases in seven uremic toxins across CKD stages 1-4, with gut-derived metabolites (indoxyl sulfate, p-cresol sulfate, TMAO) accumulating 39-40-fold compared with creatinine's 6.4-fold rise. A three-metabolite panel discriminated CKD stage $\geq 3a$ with AUC 0.94, sensitivity 91.1%, and specificity 88.3%. These serum biomarkers offer superior early-stage discrimination compared with creatinine alone and warrant validation in prospective, multi-centre cohorts.

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