

Original Article

Serum Homocysteine (Hcy), Cystatin C (Cys-C), and Urine Microalbumin (mAlb) are critical for early diagnosis of diabetic nephropathy

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Abstract: Objectives: To evaluate the clinical value of combined serum homocysteine (Hcy), cystatin C (Cys-C), and urine microalbumin (mAlb) in early diagnosis of diabetic kidney disease (DKD). Methods: A total of 450 participants were retrospectively enrolled, including 150 DKD patients, 150 patients with type 2 diabetes mellitus (T2DM) without nephropathy, and 150 healthy controls. Serum Hcy, Cys-C, HbA1c, glucose, lipids, and urine mAlb were measured. Group differences were assessed using Kruskal-Wallis tests. Receiver operating characteristic (ROC) curves were generated to evaluate diagnostic performance. Least absolute shrinkage and selection operator (LASSO) regression and SHAP analysis were applied to identify key predictive features. Results: DKD patients showed significantly higher Hcy (22.42 ± 5.15 $\mu\text{mol/L}$), Cys-C (1.82 ± 0.41 mg/L), and urine mAlb (180.41 ± 42.81 mg/L) than T2DM patients (all $P < 0.05$). Combined indicators achieved a sensitivity of 82.0%, specificity of 86.7%, and an area under the ROC curve (AUC) of 0.928, outperforming single markers ($P < 0.001$). LASSO-SHAP analysis identified mAlb dynamics (e.g., AUC, 24-month values) as the dominant predictor. Conclusions: Combined Hcy, Cys-C, and mAlb testing improves early DKD diagnostic accuracy, enabling timely intervention. Single-center design and small sample size warrant multicenter validation.

Keywords: Homocysteine, cystatin C, microalbumin, diabetic nephropathy

Introduction

Diabetic kidney disease (DKD), one of the most devastating microvascular complications of type 2 diabetes (T2DM), affects approximately 30-40% of diabetic patients worldwide and remains the leading cause of end-stage renal disease (ESRD) [1]. Early and accurate diagnosis is therefore essential for timely intervention and delaying disease progression. Although serum homocysteine (Hcy) and cystatin C (Cys-C) reflect metabolic and filtration dysfunction, respectively, their combined diagnostic utility in a multi-dimensional DKD assessment remains under-explored [1].

Serum Hcy, Cys-C and urine microalbumin (mAlb) have each shown unique value as biochemical indicators for the early diagnosis of diabetic nephropathy, and they have attracted increasing attention in recent years [2]. Hcy, a

sulfur-containing amino acid, is closely related to the occurrence and development of diabetic nephropathy. In the diabetic state, metabolic disorders can promote elevated Hcy levels, which in turn damage vascular endothelial cells, exacerbate renal microangiopathy, and contribute to the initiation of nephropathy. Several studies have reported significantly higher serum Hcy levels in patients with early DKD compared with healthy individuals, supporting its role as a promising early warning biomarker [3].

Cys-C, an endogenous cysteine protease inhibitor, is a reliable marker of glomerular filtration rate (GFR) due to its stability and independence from confounding factors such as inflammation and muscle mass. Its levels increase in the early stages of renal dysfunction, often preceding changes in conventional indicators such as serum creatinine (Scr). Compared with Scr,

Cys-C can capture subtle fluctuations in GFR more rapidly and accurately, providing a solid basis for early detection of DKD.

Urine mAlb, which reflects glomerular filtration barrier integrity, is another key indicator of early DKD [4]. Normally, only trace amounts of albumin are present in urine. However, in the early stages of DKD, increased glomerular basement membrane permeability results in elevated urinary mAlb excretion [5]. Continuous mAlb monitoring allows the detection of early glomerular leakage and provides opportunities for timely intervention to curb DKD progression [6]. Nevertheless, the diagnostic specificity of mAlb alone is limited (~75%) due to confounding factors such as hypertension and infections. While urinary mAlb is widely used as a screening marker, its limited specificity necessitates a more comprehensive approach.

As a leading cause of end-stage renal disease (ESRD), DKD imposes a significant global health burden, making early diagnosis imperative for timely intervention. By integrating mechanistic insights, this study elucidated the scientific rationale for combined testing, providing a theoretical foundation for its clinical application. Additionally, we validated a low-cost detection protocol based on routine biochemical equipment, rendering the approach suitable for large-scale screening in primary healthcare settings. The clinical significance of this study lies in providing an efficient tool for assessing early renal injury in diabetic patients, enabling timely clinical interventions to slow disease progression, reduce the risk of ESRD, and alleviate the associated medical burden.

Materials and methods

Case selection

This retrospective study included 150 patients with diabetic nephropathy (DKD group), 150 patients with T2DM (Non-DKD group) admitted to the 965th Hospital of the PLA from January 2023 to December 2024. Additionally, 150 healthy controls (Control group) undergoing physical examination during the same period were also enrolled and the control group, these patients only provided baseline (t0) data and did not undergo subsequent follow-up. Data were collected at multiple time points: t0 (baseline); t1 (6 months after enrollment,

with a ± 3 -day window); t2 (12 months after enrollment, ± 7 days); t3 (18 months after enrollment, ± 7 days); and t4 (24 months after enrollment, ± 7 days). The research was approved by the Ethics Committee of the 965th Hospital of the PLA. All study procedures were in accordance with the Declaration of Helsinki (2013).

Inclusion criteria

T2DM: Patients with a definite diagnosis of T2DM based on WHO or ADA criteria, defined as glycosylated hemoglobin (HbA1c) $\geq 6.5\%$, fasting blood glucose (FPG) ≥ 7.0 mmol/L, or a 2-hour blood glucose ≥ 11.1 mmol/L during an oral glucose tolerance test (OGTT).

Age: 30-70 years.

Urinary Microalbumin Excretion Rate (UAER): For the DKD/T2DM group: UAER 20-200 $\mu\text{g}/\text{min}$ (microalbumin) or ≥ 200 $\mu\text{g}/\text{min}$ (macroalbuminuria) was used for DKD confirmation (per KDIGO 2022 guidelines). Healthy control group: UAER < 20 $\mu\text{g}/\text{min}$ and no history of diabetes or renal disease.

Data availability: Availability of complete medical records, including baseline demographics, laboratory parameters, and clinical history.

Definition of early DKD: Persistent moderately increased albuminuria (UAER 20-200 $\mu\text{g}/\text{min}$ or UACR 30-300 mg/g), as per KDIGO 2022 criteria.

Exclusion criteria

Primary renal diseases: History or presence of chronic glomerulonephritis, lupus nephritis, polycystic kidney disease, or other non-diabetic renal disorders (confirmed by urinalysis, imaging, or biopsy).

Acute diabetic complications: Occurrence of diabetic ketoacidosis (DKA) or hyperosmolar hyperglycemic state (HHS) within the past 3 months.

Malignancy: Active cancer (except non-melanoma skin cancer) within the past 5 years.

Severe cardiovascular/cerebrovascular diseases: Recent myocardial infarction (within 6 months), stroke (within 1 year), New York Heart

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Association (NYHA) class III/IV heart failure, or severe peripheral artery disease (Fontaine stage III/IV).

Pregnancy/Lactation: Pregnant or lactating women, or those planning pregnancy (confirmed by urine pregnancy test if applicable).

Medication interference: Use of angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), or mineralocorticoid receptor antagonists (MRAs) within the past 4 weeks, unless on stable regimen (≥ 3 months, unchanged dose in the past 2 months). Use of nephrotoxic drugs (e.g., aminoglycosides, non-steroidal anti-inflammatory drugs [NSAIDs] ≥ 150 mg/day ibuprofen equivalent) within the past month. Supplementation with folic acid (> 1 mg/day), vitamin B12 (> 500 μ g/day), or betaine within the past 2 weeks (due to potential influence on Hcy levels).

Other conditions: Cognitive impairment or inability to adhere to study requirements.

Serum biochemical markers

Fasting venous blood (5 ml) was collected from the elbow vein within 24 hours of admission, and on the day of physical examination for healthy controls. Samples were centrifuged at 3000 r/min for 5 min, and the serum was separated for biochemical analysis. HbA1c: Measured using a Bio-Rad (USA) HbA1c analyzer (models: D-100™) with high-performance liquid chromatography (HPLC). Glu, TC, TG, HDL, LDL, BUN, and Scr: Measured using an automatic biochemical analyzer (Roche Cobas® c501/c502). CysC and CRP: Detected using immunoturbidimetry (reagents: Roche Cobas® C3/C503). Hcy, TNF- α , and IL-6: Quantified using enzyme-linked immunosorbent assay (ELISA) with commercial kits (Quantikine®, R&D Systems). All procedures followed the manufacturer's protocols.

Urine biochemical tests

An early morning urine sample (5 mL) was collected, left to stand, and centrifuged at 1500 rpm for 5 minutes. The supernatant was analyzed for the following parameters: Urinary N-acetyl- β -D-glucosaminidase (UNAG): Measured using an automatic urine analyzer (Siemens CLINITEK® Novus). UAER: Assessed

by immunoturbidimetry (Tina-quant Albumin Gen.2, Roche Cobas®). Urine Creatinine (Ucr): Determined via the creatinine oxidase method (Creatinine Plus, Roche Cobas®).

Primary and secondary outcomes

Primary outcomes: sensitivity, specificity, and area under the receiver operating characteristic curve (AUC) for serum Hcy, Cys-C, and urinary mAlb in the early diagnosis of diabetic nephropathy.

Secondary indicators included group differences in baseline clinical data and diabetes-related biochemical parameters, and correlation analyses between each indicator and the UAER.

Feature selection and model explanation

LASSO regression for feature selection: To identify the most informative biomarkers and reduce model complexity, we employed the Least Absolute Shrinkage and Selection Operator (LASSO) regression. This technique is particularly suitable for high-dimensional data with potential collinearity among predictors, as it performs feature selection by shrinking less relevant coefficients to zero.

SHAP analysis for model interpretation: To enhance interpretability of the predictive model, we used SHapley Additive exPlanations (SHAP). SHAP values provide a game-theoretic approach to attribute each model prediction to individual features, thereby quantifying their contribution to overall model performance.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 29.0 (SPSS Inc., Chicago, IL, USA). The normality of continuous variables was assessed using the Shapiro-Wilk test. Continuous variables conforming to a normal distribution were presented as mean $\pm$ standard deviation and were analyzed using Student's t-test or one-way ANOVA followed by Tukey's post-hoc test. For overall comparisons among the three groups, statistical significance was set at $P < 0.05$. For pairwise post-hoc comparisons, the significance level was adjusted to $P < 0.017$ using the Bonferroni correction (three comparisons).

Table 1. Comparison of baseline values between the three groups

Indicator	Control group (n = 150)	Non-DKD group (n = 150)	DKD group (n = 150)	F	P
Age (years)	56.46±7.86	55.89±7.89	58.98±8.02	0.282	0.754
Gender				1.000	0.465
Male [n, (%)]	79 (52.7)	84 (56.0)	80 (53.3)		
Female [n, (%)]	71 (47.3)	66 (44.0)	70 (46.7)		
BMI (kg/m ²)	23.55±3.12	24.12±3.06	24.34±3.01	2.657	0.071
PT (s)	12.56±3.21	12.97±3.10	12.89±2.89	0.752	0.472
aPTT (s)	26.02±6.35	26.75±6.32	26.89±6.19	0.828	0.437
TT (s)	16.42±5.56	17.57±5.24	17.25±5.66	0.437	0.174
D_D (μg/L)	1.02±0.23	1.04±0.28	1.06±0.27	0.881	0.414
IL_6 (pg/ml)	7.46±0.21	7.48±0.19	7.45±0.20	0.873	0.418
CRP (mg/L)	4.59±2.35	4.87±2.98	5.12±2.75	1.441	0.238

Note: BMI, Body Mass Index; PT, Prothrombin Time; aPTT, Activated Partial Thromboplastin Time; TT, Thrombin Time; D_D, D-dimer; IL_6, Interleukin-6; CRP, C-reactive Protein; DKD, Diabetic Kidney Disease.

Results

Baseline characteristics of the three groups

A total of 450 participants were enrolled, including 150 patients with DKD (DKD group), 150 patients with T2DM without nephropathy (non-DKD group), and 150 healthy controls (Control group). Baseline characteristics, such as age, gender distribution, body mass index (BMI), coagulation parameters (prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT)), inflammatory factors (interleukin-6 (IL-6)), and C-reactive protein (CRP), were comparable across the three groups (all $P > 0.05$), indicating balanced baseline profiles (**Table 1**).

Group differences in serum biochemical indicators

Significant group differences were observed in several diabetes-related biochemical parameters (all $P < 0.01$). Glu levels were significantly elevated in both DKD and Non-DKD groups compared to controls at baseline. Baseline HbA1c, Scr, and BUN levels were highest in the DKD group, followed by the non-DKD group. β_2 -microglobulin (β_2 -MG) levels were high in the non-DKD group. These findings indicate distinct hierarchical differences across metabolic (Glu and HbA1c), renal (Scr and BUN), and coagulation-related (Fbg and FDP) indicators, reflecting progressive deterioration in metabolic and renal function from healthy individu-

als to non-DKD patients, and further to DKD patients (**Table 2**).

Intergroup differences in Hcy, Cys-C, and mAlb levels

Baseline serum Hcy, Cys-C, and mAlb levels differed significantly among the three groups (all $P < 0.001$). Specifically, the DKD group exhibited the highest levels of all three biomarkers, significantly exceeding those in the non-DKD group at non-baseline (**Table 3**). Pairwise comparisons at T4 confirmed these differences, with the DKD group showing a 43.5% increase in Hcy, a 43.3% increase in Cys-C, and nearly a 4-fold elevation in mAlb compared to the non-DKD group (all $P < 0.001$) (**Figure 1**).

Longitudinal biomarker trends over 24 months

Longitudinal analysis revealed distinct biomarker trajectories in the DKD group over 24 months compared to the non-DKD group. Hcy and Cys-C levels in DKD patients gradually declined from baseline to 24 months but remained significantly elevated relative to the non-DKD group, which remained stable at lower levels (**Figure 2A, 2B**). In contrast, mAlb in the DKD group increased progressively throughout the 24-month period, whereas the non-DKD group maintained low and stable levels (**Figure 2C**). Scr in the DKD group decreased over time but remained higher than in the non-DKD group (**Figure 2D**). Individual trajectory analyses further confirmed these

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Table 2. Comparison of serum biochemical indexes among the three groups

Indicator	Time Point	Control group (n = 150)	Non-DKD group (n = 150)	DKD group (n = 150)	F	P
BUN	Baseline	4.56±1.45	5.83±1.56*	7.03±1.63* [#]	95.021	< 0.01
	t1	N/A	5.60±1.55	6.84±1.37 [#]	70.469	< 0.01
	t2	N/A	5.28±1.60	6.14±1.31 [#]	34.314	< 0.01
	t3	N/A	5.09±1.43	5.85±1.24 [#]	34.891	< 0.01
	t4	N/A	5.24±1.50	5.75±1.20 [#]	23.548	< 0.01
FDP	Baseline	3.53±1.99	4.98±2.23*	7.74±3.16* [#]	108.752	< 0.01
	t1	N/A	5.03±2.33	7.10±3.16 [#]	90.335	< 0.01
	t2	N/A	4.35±2.31	5.80±2.25 [#]	48.303	< 0.01
	t3	N/A	4.48±2.06	5.64±2.27 [#]	40.701	< 0.01
	t4	N/A	4.27±2.03	5.41±2.33 [#]	37.251	< 0.01
Fbg	Baseline	3.03±0.07	4.15±1.76*	5.97±2.53* [#]	104.593	< 0.01
	t1	N/A	3.79±1.98	5.59±2.58 [#]	74.056	< 0.01
	t2	N/A	3.37±1.75	4.87±2.21 [#]	55.154	< 0.01
	t3	N/A	3.12±1.66	4.30±2.12 [#]	31.751	< 0.01
	t4	N/A	3.37±1.76	3.92±1.77 [#]	15.501	< 0.01
Glu	Baseline	5.29±0.35	11.18±9.06*	13.44±13.92* [#]	28.884	< 0.01
	t1	N/A	10.23±8.23	14.77±14.01 [#]	38.084	< 0.01
	t2	N/A	10.06±9.16	11.27±12.09 [#]	19.364	< 0.01
	t3	N/A	10.02±7.20	11.88±12.05 [#]	26.536	< 0.01
	t4	N/A	10.14±7.75	10.76±11.26 [#]	21.618	< 0.01
HDL	Baseline	1.25±0.08	1.07±0.08*	1.14±0.11* [#]	153.852	< 0.01
	t1	N/A	1.08±0.06	1.15±0.10 [#]	170.774	< 0.01
	t2	N/A	1.11±0.06	1.21±0.10 [#]	130.853	< 0.01
	t3	N/A	1.12±0.06	1.22±0.08 [#]	154.846	< 0.01
	t4	N/A	1.13±0.06	1.23±0.08 [#]	116.317	< 0.01
HbA1c	Baseline	4.61±0.57	6.09±0.36*	9.22±2.73* [#]	314.269	< 0.01
	t1	N/A	5.91±0.30	8.86±2.55 [#]	313.239	< 0.01
	t2	N/A	5.64±0.34	7.91±2.53 [#]	190.171	< 0.01
	t3	N/A	5.52±0.29	7.25±2.48 [#]	126.294	< 0.01
	t4	N/A	5.43±0.31	7.13±2.26 [#]	143.911	< 0.01
LDL	Baseline	2.72±0.47	2.92±0.71	2.93±1.73	1.670	0.189
	t1	N/A	2.78±0.69	2.88±2.01	0.809	0.446
	t2	N/A	2.73±0.68	2.56±1.71	0.906	0.405
	t3	N/A	2.75±0.67	2.48±1.61 [#]	3.077	0.047
	t4	N/A	2.61±0.63	2.54±1.61	0.941	0.391
NAG	Baseline	2.10±0.97	2.53±0.63*	2.80±2.10* [#]	9.764	< 0.01
	t1	N/A	2.39±0.61	2.22±1.84	1.639	0.195
	t2	N/A	2.18±0.61	2.00±1.52	1.415	0.244
	t3	N/A	2.11±0.59	1.79±1.61 [#]	6.668	0.001
	t4	N/A	2.08±0.55	2.02±1.57	0.323	0.724
Scr	Baseline	59.99±16.37	60.18±16.41*	85.69±16.36* [#]	122.181	< 0.01
	t1	N/A	61.08±15.72	82.86±15.12 [#]	97.759	< 0.01
	t2	N/A	57.27±14.33	73.53±14.19 [#]	44.224	< 0.01
	t3	N/A	56.82±14.31	70.92±13.05 [#]	39.856	< 0.01
	t4	N/A	55.00±13.86	68.88±14.82 [#]	31.681	< 0.01

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TC	Baseline	4.62±1.17	4.95±1.17	4.79±1.16	3.025	0.051
	t1	N/A	4.90±1.06	4.91±1.09	2.646	0.072
	t2	N/A	4.62±1.01	4.59±1.03	0.116	0.891
	t3	N/A	4.68±0.90	4.52±0.90	1.391	0.252
	t4	N/A	4.53±1.00	4.48±1.01	0.127	0.881
TG	Baseline	1.68±0.25	2.02±1.62*	2.14±1.33* [#]	5.771	0.003
	t1	N/A	1.86±1.55	1.89±1.24	2.238	0.108
	t2	N/A	1.88±1.43	1.83±1.20	1.787	0.169
	t3	N/A	1.78±1.27	1.62±1.04	1.251	0.287
	t4	N/A	1.89±1.35	1.82±1.01	2.238	0.108
TNF-α	Baseline	7.62±0.42	7.56±0.41	7.66±0.36	2.416	0.091
	t1	N/A	7.39±0.44	7.27±0.39 [#]	38.864	< 0.01
	t2	N/A	6.99±0.40	6.51±0.34 [#]	321.469	< 0.01
	t3	N/A	6.82±0.35	6.10±0.31 [#]	731.139	< 0.01
	t4	N/A	6.77±0.39	6.01±0.28 [#]	747.914	< 0.01
β ₂ -MG	Baseline	0.22±0.23	1.67±0.52*	0.87±0.39 [#]	503.091	< 0.01
	t1	N/A	1.51±0.55	0.83±0.41 [#]	333.562	< 0.01
	t2	N/A	1.40±0.55	0.73±0.36 [#]	281.757	< 0.01
	t3	N/A	1.41±0.50	0.68±0.31 [#]	379.052	< 0.01
	t4	N/A	1.45±0.52	0.64±0.32 [#]	397.872	< 0.01

Note: Group comparisons were performed using one-way ANOVA followed by Tukey/Bonferroni post-hoc tests. t1: 6 month after enrollment (±3 days); t2: 12 month after enrollment (±7 days); t3: 18 months after enrollment (±7 days); t4: 24 months after enrollment (±7 days). Compared with the control group at baseline, *P < 0.017; compared with the Non-DKD group, [#]P < 0.017. BUN, Blood urea nitrogen; FDP, Fibrin Degradation Product; Fbg, Fibrinogen; Glu, Glucose; HDL, High-Density Lipoprotein; HbA1c, Glycated hemoglobin A1c; LDL, Low-Density Lipoprotein; NAG, N-Acetyl-β-D-Glucosaminidase; Scr, Serum creatinine; TC, Total Cholesterol; TG, Triglyceride; TNF-α, Tumor necrosis factor-α; β₂-MG, β₂-Microglobulin; DKD, Diabetic Kidney Disease.

Table 3. Kruskal-Wallis test results of Hcy, Cys-C, and mAlb of the three groups

Indicator	Time Point	Control group (n = 150)	Non-DKD group (n = 150)	DKD group (n = 150)	H	P
Hcy	Baseline	9.20±2.18	15.87±3.61*	22.43±4.85* [#]	477.563	< 0.01
	t1	N/A	15.26±3.02	21.63±4.43 [#]	551.58	< 0.01
	t2	N/A	14.65±3.51	18.79±4.19* [#]	304.298	< 0.01
	t3	N/A	13.99±2.86	17.37±4.06 [#]	271.212	< 0.01
	t4	N/A	14.27±3.19	16.80±4.32 [#]	209.059	< 0.01
Cys-C	Baseline	0.80±0.22	1.23±0.32*	1.85±0.40* [#]	412.294	< 0.01
	t1	N/A	1.23±0.30	1.72±0.38 [#]	343.032	< 0.01
	t2	N/A	1.18±0.31	1.54±0.36 [#]	225.961	< 0.01
	t3	N/A	1.11±0.32	1.46±0.33 [#]	185.857	< 0.01
	t4	N/A	1.11±0.32	1.41±0.33 [#]	158.631	< 0.01
mALB	Baseline	16.86±5.59	47.87±12.51*	182.63±47.36* [#]	1438.107	< 0.01
	t1	N/A	44.53±11.07	160.05±43.48 [#]	1293.268	< 0.01
	t2	N/A	40.12±10.97	130.13±35.07 [#]	1173.525	< 0.01
	t3	N/A	38.21±11.33	115.91±33.58 [#]	975.555	< 0.01
	t4	N/A	37.23±10.25	100.83±33.24 [#]	704.126	< 0.01

Note: t1: 6 months after enrollment (±3 days); t2: 12 months after enrollment (±7 days); t3: 18 months after enrollment (±7 days); T4: 24 months after enrollment (±7 days). Compared with the control group (at baseline), *P < 0.017; compared with the Non-DKD group, [#]P < 0.017. Hcy, Homocysteine; Cys-C, Cystatin C; mALB, microalbumin; DKD, Diabetic Kidney Disease.

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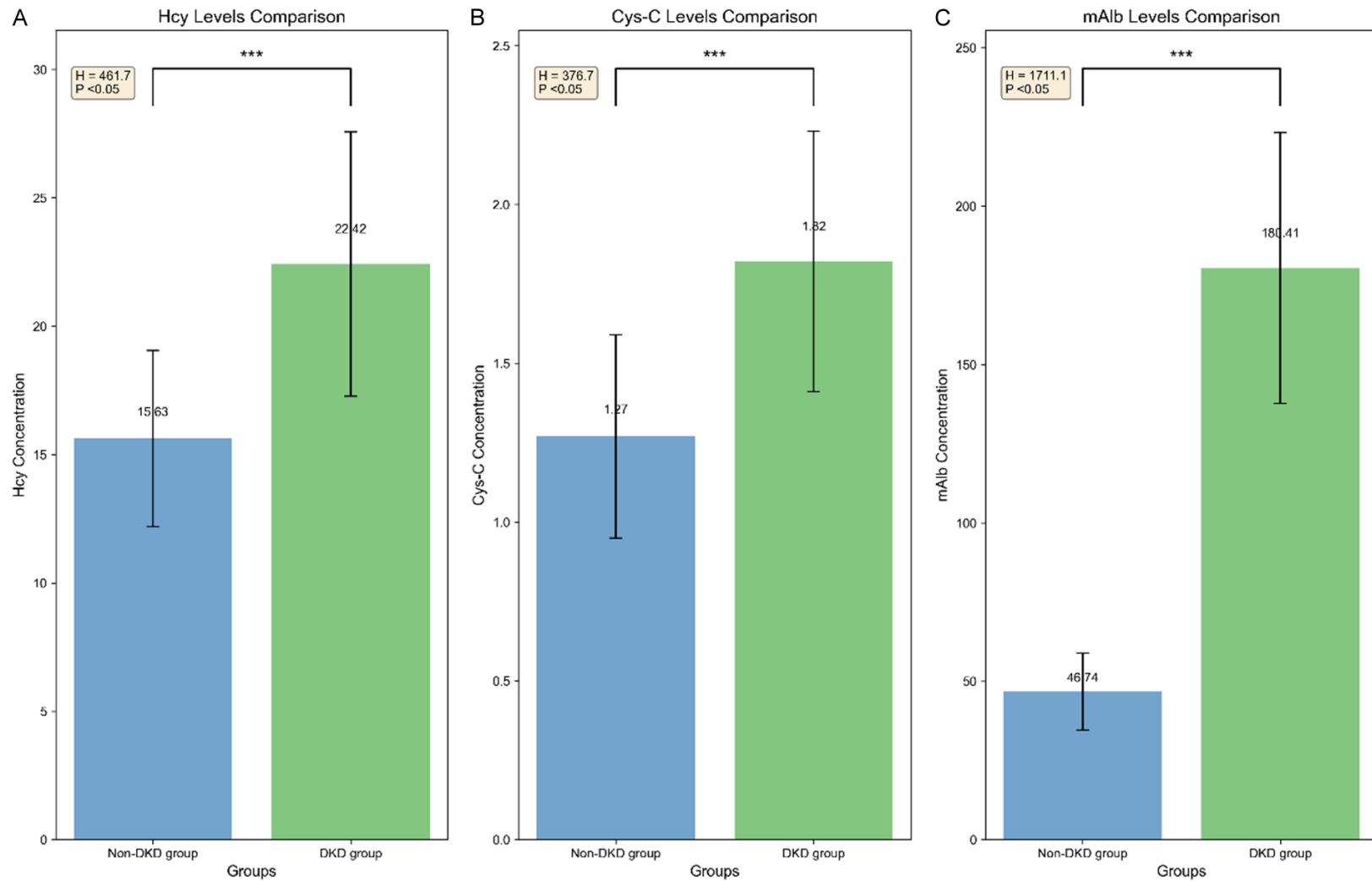


Figure 1. Grouped bar plots with pairwise comparisons of Hcy, Cys-C, and mAlb levels between Non-DKD group and DKD group. A. Comparison of Hcy levels. The Hcy concentration in the DKD group was significantly higher than that in the Non-DKD group. By Kruskal-Wallis test, ($H = 461.7$, $P < 0.05$). B. Comparison of Cys-C levels. The Cys-C concentration in the DKD group was significantly higher than that in the Non-DKD group. By Kruskal-Wallis test, ($H = 376.7$, $P < 0.05$). C. Comparison of mAlb levels. The mAlb concentration in the DKD group was significantly higher than that in the Non-DKD group. By Kruskal-Wallis test, ($H = 1711.1$, $P < 0.05$). Note: Group comparisons were performed using one-way ANOVA followed by Tukey/Bonferroni post-hoc tests, *** $P < 0.001$. Hcy, Homocysteine; Cys-C, Cystatin C; mAlb, microalbumin.

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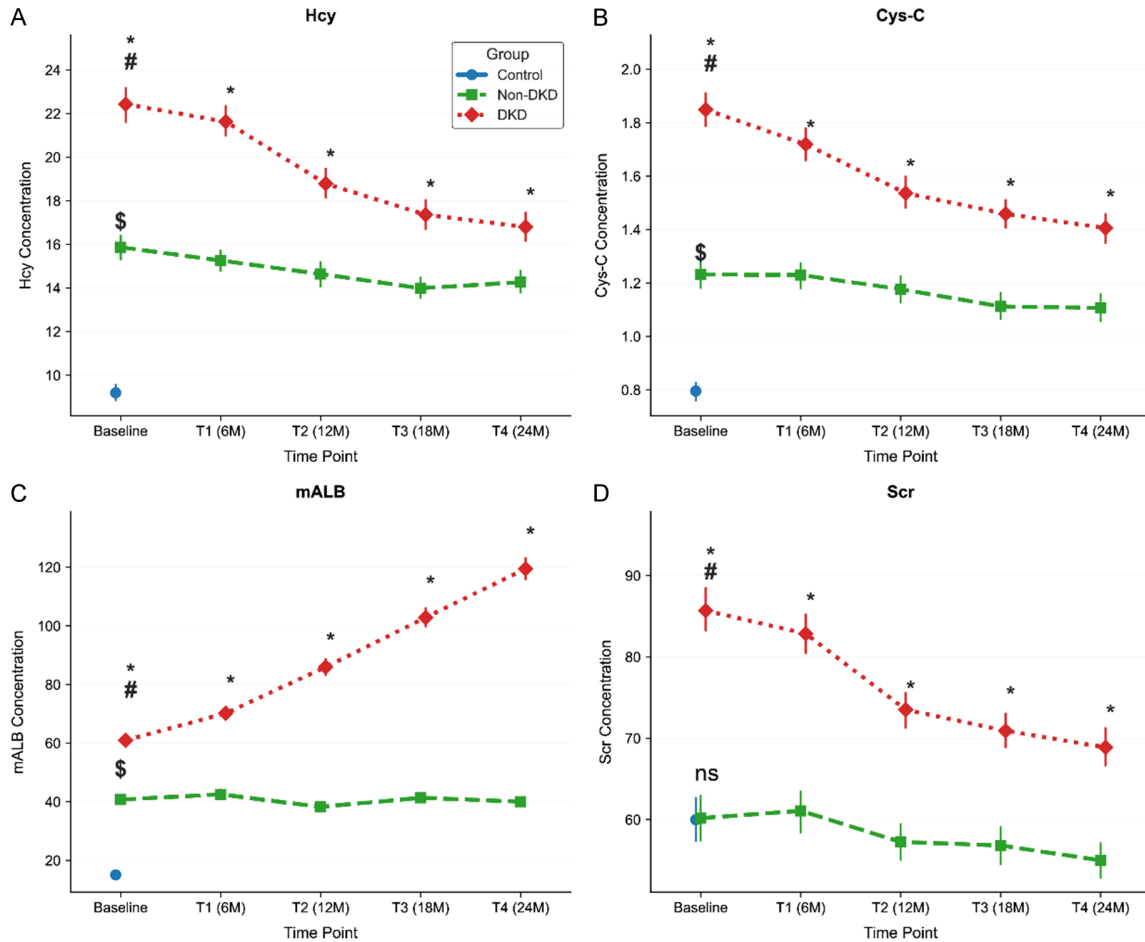


Figure 2. Longitudinal changes in biomarkers across different groups over 24 months. A. Time course of homocysteine (Hcy) concentration in controls (at baseline), Non-DKD, and DKD groups over 24 months. B. Time course of cystatin C (Cys-C) concentration in controls (at baseline), Non-DKD, and DKD groups over 24 months. C. Time course of microalbumin (mAlb) concentration in controls (at baseline), Non-DKD, and DKD groups over 24 months. D. Time course of serum creatinine (Scr) concentration in controls (at baseline), Non-DKD, and DKD groups over 24 months. Non-DKD group compared with the control group (at baseline), \$P < 0.017; DKD group compared with the control group (at baseline), #P < 0.017; DKD group compared with the Non-DKD group, *P < 0.017; Non-DKD group compared with the control group (at baseline), ns (P > 0.017). Hcy, Homocysteine; Cys-C, Cystatin C; mAlb, microalbumin; Scr, Serum creatinine.

patterns, with DKD patients exhibiting pronounced upward trends in mAlb, persistently elevated but declining Hcy and Cys-C, and dynamic changes in BUN, IL-6, and TNF- α , compared to the stable profiles in non-DKD (**Figure 3**).

Longitudinal trends in biomarker change rates across groups

Longitudinal changes in key biomarkers - Hcy, Cys-C, mAlb, Scr, and BUN - were compared between the Non-DKD and DKD groups over a 24-month period (**Figure 4**). The DKD group exhibited a significantly steeper decline in the

Hcy change rate (mean: -1.553) compared to the Non-DKD group (mean: -0.448), although absolute Hcy levels remained higher in the DKD group throughout the study period (**Figure 4A**). For Cys-C, the DKD group showed a more pronounced reduction in the rate of change (mean: -0.115) than the Non-DKD group (mean: -0.037), consistent with persistent glomerular filtration impairment (**Figure 4B**). A marked divergence was observed in mAlb trajectories: the DKD group demonstrated a sharp increase in mAlb change rate (mean: 14.967), whereas the Non-DKD group maintained stable and low levels (mean: -0.262), reflecting progressive damage to the glomeru-

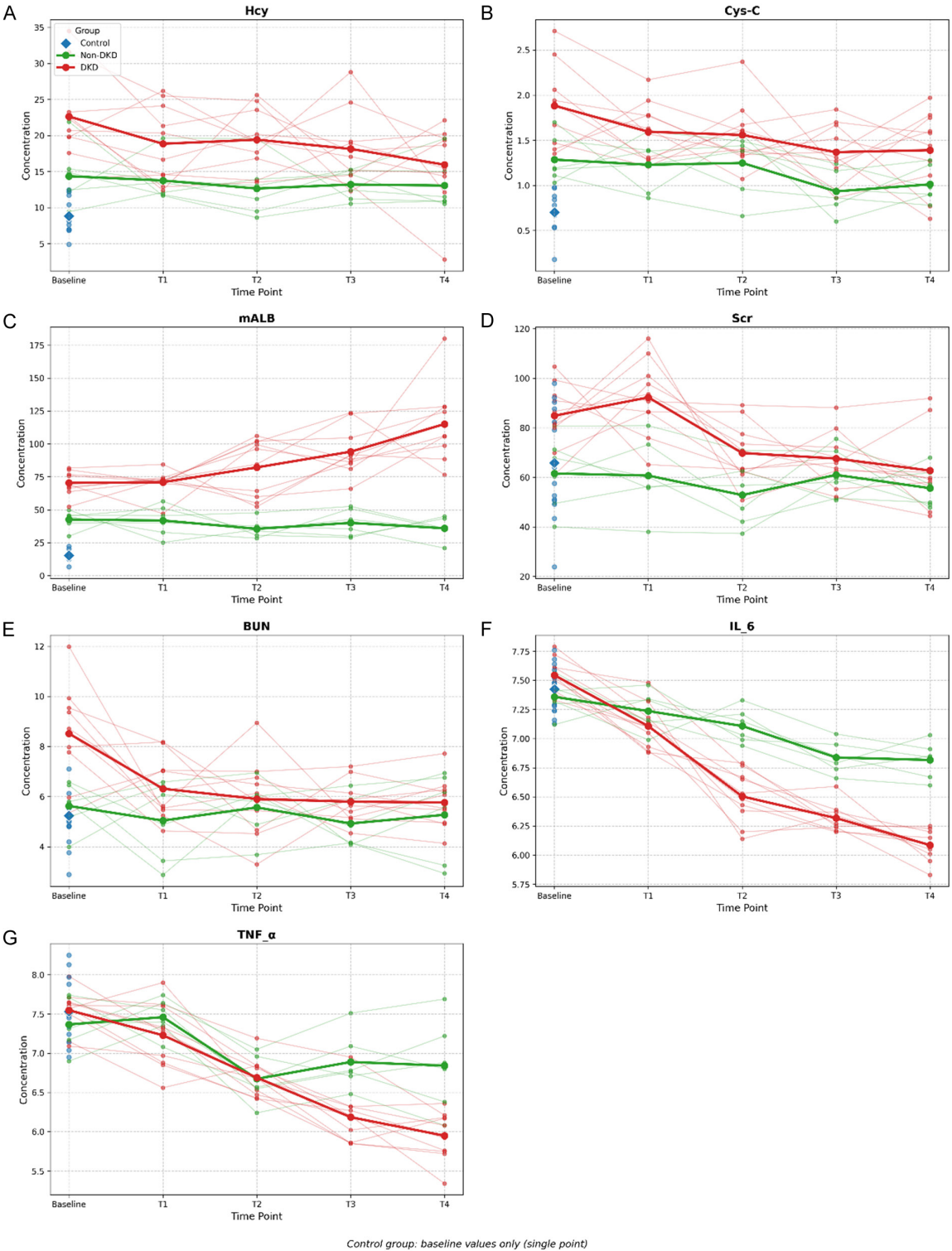


Figure 3. Individual biomarker trajectories in controls (at baseline), Non-DKD, and DKD groups over 24 months. A. Hcy Individual Trajectories: Tracks homocysteine (Hcy) concentration changes for Non-DKD (green), and DKD (red) groups across 24 months. B. Cys-C Individual Trajectories: Displays cystatin-C (Cys-C) dynamics, color-coded by group (Non-DKD: green; DKD: red) over time. C. mALB Individual Trajectories: Shows microalbumin (mALB) trends, with DKD (red) exhibiting a clear upward pattern vs. stable Non-DKD (green). D. Scr Individual Trajectories: Illustrates serum creatinine (Scr) concentration variability, color-coded by group (Non-DKD: green; DKD: red). E. BUN Individual Trajectories: Depicts blood urea nitrogen (BUN) fluctuations, with DKD (red) starting higher and showing

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distinct trends. F. IL-6 Individual Trajectories: Tracks interleukin-6 (IL-6) changes, highlighting DKD (red) downward trajectory vs. stabler Non-DKD (green). G. TNF- α Individual Trajectories: Displays tumor necrosis factor- α (TNF- α) dynamics, with DKD (red) showing pronounced decline over 24 months. Hcy, Homocysteine; Cys-C, Cystatin C; mALB, microalbumin; Scr, Serum creatinine; BUN, Blood urea nitrogen; IL_6, Interleukin-6; TNF- α , Tumor necrosis factor- α .

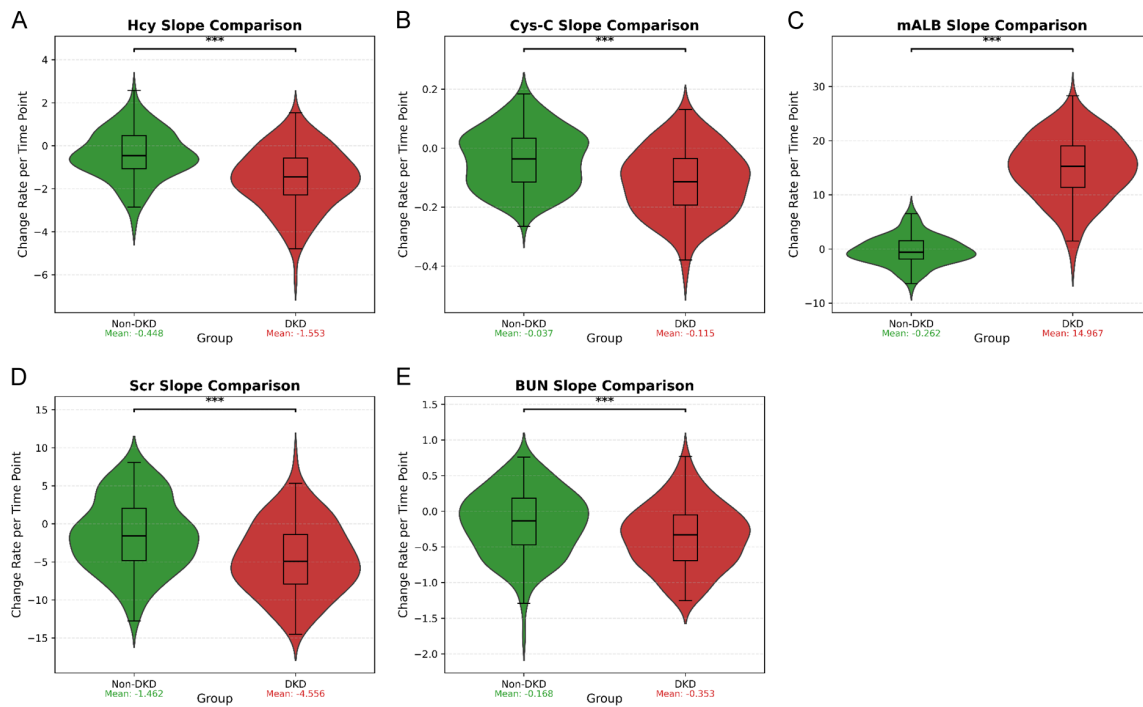


Figure 4. Comparison of biomarker change rates between groups. A. Hcy Slope Comparison: Displays the change rate distribution of homocysteine (Hcy). B. Cys-C Slope Comparison: Shows the change rate of cystatin C (Cys-C). C. mALB Slope Comparison: Depicts the change rate of microalbumin (mALB). D. Scr Slope Comparison: Presents the change rate of serum creatinine (Scr). E. BUN Slope Comparison: Illustrates the change rate of blood urea nitrogen (BUN). *** $P < 0.001$. Hcy, Homocysteine; Cys-C, Cystatin C; mALB, microalbumin; Scr, Serum creatinine; BUN, Blood urea nitrogen; DKD, Diabetic Kidney Disease; Non-DKD, Non-Diabetic Kidney Disease.

lar filtration barrier (**Figure 4C**). Similarly, the DKD group experienced a greater decline in Scr change rate (mean: -4.556 vs. -1.462 in Non-DKD; **Figure 4D**), indicating a more rapid trajectory of renal function deterioration. A more substantial reduction in BUN change rate was also observed in the DKD group (mean: -0.353 vs. -0.168; **Figure 4E**), further supporting impaired renal urea excretion capacity. Collectively, these longitudinal biomarker dynamics highlight distinct metabolic and renal functional trajectories in DKD, capturing progressive renal injury and differentiating DKD from Non-DKD over time.

LASSO regression for feature selection

LASSO regression was used to identify key predictive features, with coefficient trajectories

showing that stronger regularization shrank less relevant features (e.g., cytokine-related variables) to zero, while slope/ratio features exhibited greater sensitivity to regularization (**Figure 5A**). Cross-validation identified an optimal alpha value of 0.0126, balancing model complexity and fit (**Figure 5B**), which retained a subset of stable predictors with consistent contributions to the model.

Predictive performance of diagnostic models

Receiver operating characteristic (ROC) curves demonstrated that both logistic regression and XGBoost models achieved perfect discrimination for early DKD, with an area under the curve (AUC) of 0.930 (**Figure 6A**). Calibration curves confirmed strong agreement between predicted probabilities and observed out-

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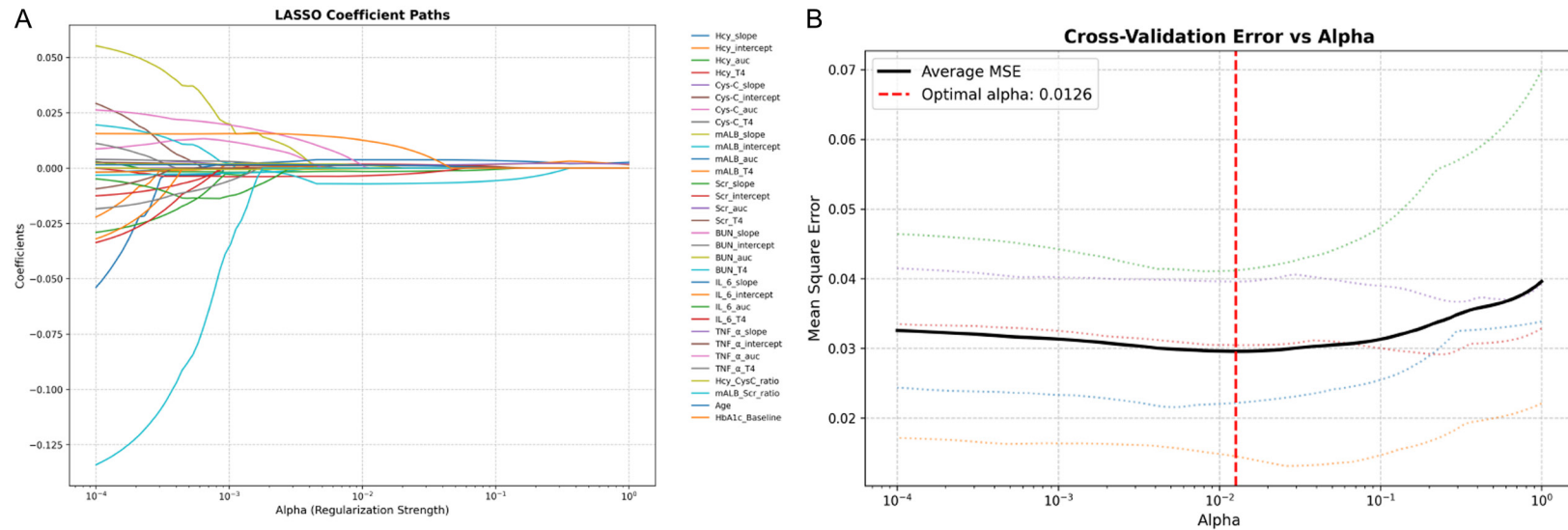


Figure 5. LASSO Coefficient Paths and LASSO Cross - Validation for Optimal Alpha Selection. A. Coefficient trajectories across alpha values illustrate feature selection. B. Plots mean squared error (MSE) vs. alpha, identifying optimal alpha = 0.0126 to balance regularization and model fit, guiding feature refinement for the analysis. Hcy, Homocysteine; Cys-C, Cystatin C; mALB, microalbumin; Scr, Serum creatinine; BUN, Blood urea nitrogen; IL_6, Interleukin-6; TNF_a, Tumor necrosis factor- α ; HbA1c, Hemoglobin A1c.

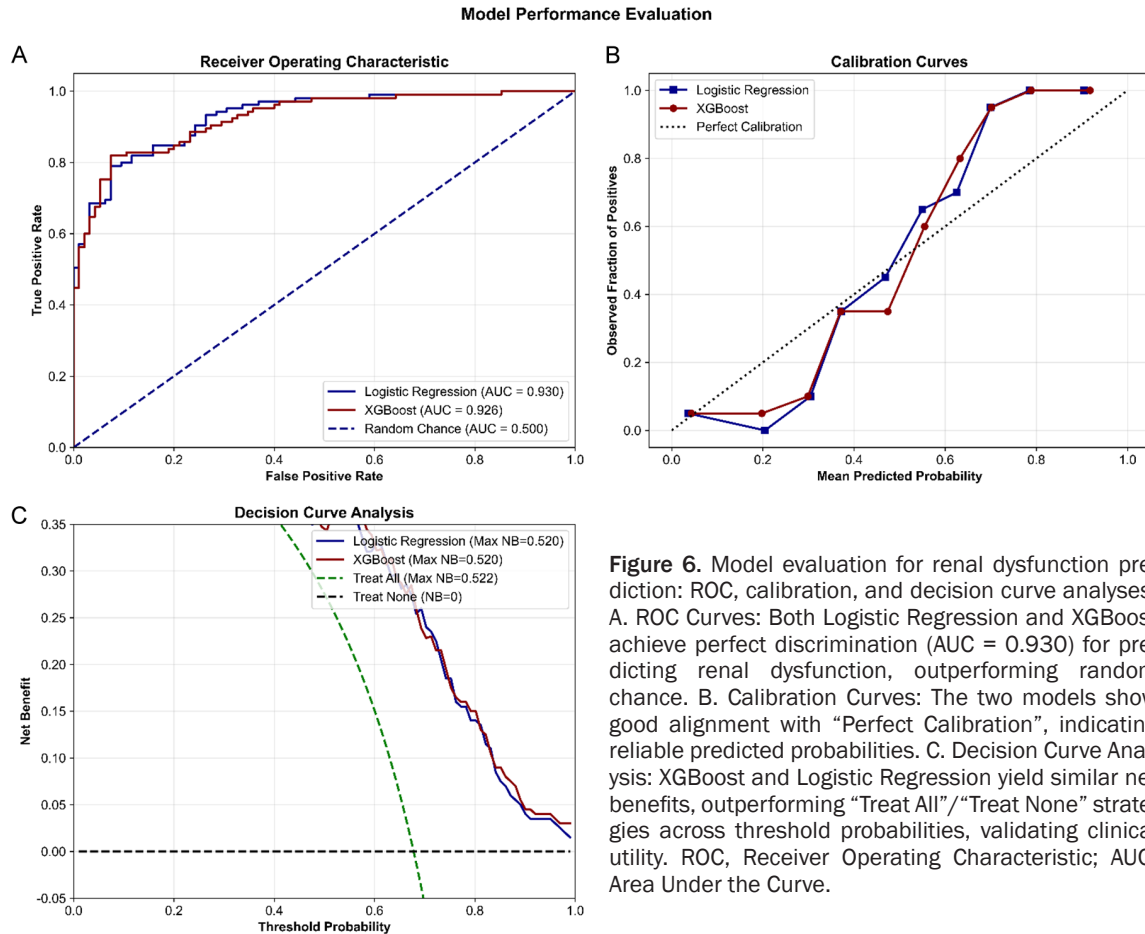


Figure 6. Model evaluation for renal dysfunction prediction: ROC, calibration, and decision curve analyses. A. ROC Curves: Both Logistic Regression and XGBoost achieve perfect discrimination (AUC = 0.930) for predicting renal dysfunction, outperforming random chance. B. Calibration Curves: The two models show good alignment with “Perfect Calibration”, indicating reliable predicted probabilities. C. Decision Curve Analysis: XGBoost and Logistic Regression yield similar net benefits, outperforming “Treat All”/“Treat None” strategies across threshold probabilities, validating clinical utility. ROC, Receiver Operating Characteristic; AUC, Area Under the Curve.

comes, indicating high calibration accuracy (Figure 6B). Decision curve analysis further showed that both models achieved a maximum net benefit of 0.520, outperforming “Treat All” or “Treat None” strategies across threshold probabilities (Figure 6C).

Key predictive features

Feature importance analysis in logistic regression identified mALB_T4 (24-month mAlb) and mALB_auc (area under the mAlb curve over 24 months) as the most influential predictors, with odds ratios significantly exceeding the reference level (Figure 7A). SHAP analysis further confirmed the predominant role of mAlb-related dynamic features in model predictions (Figure 7B). While Hcy and Cys-C showed relatively smaller individual contributions in the multivariate model, their combination with mAlb provided complementary value for early detection, as evidenced by the improved diagnostic performance of the combined model

compared to mAlb alone. These results highlight the central role of mAlb dynamics while acknowledging the additive value of incorporating multiple biomarkers for comprehensive DKD risk assessment.

Diagnostic efficacy of combined Hcy, Cys-C, and mAlb testing

To evaluate the diagnostic performance of combined Hcy, Cys-C, and mAlb in early DKD, ROC curves were constructed for individual markers and their combination. The combined detection of Hcy, Cys-C, and mAlb exhibited superior diagnostic efficacy compared to single markers: it achieved a sensitivity of 82.0%, specificity of 86.7%, and a AUC of 0.928. In contrast, individual markers showed lower performance: mAlb alone had an AUC of 0.868, Hcy alone had an AUC of 0.651, and Cys-C alone had an AUC of 0.842 (all $P < 0.001$ vs. combined). Pairwise comparisons of ROC curves confirmed that the combined

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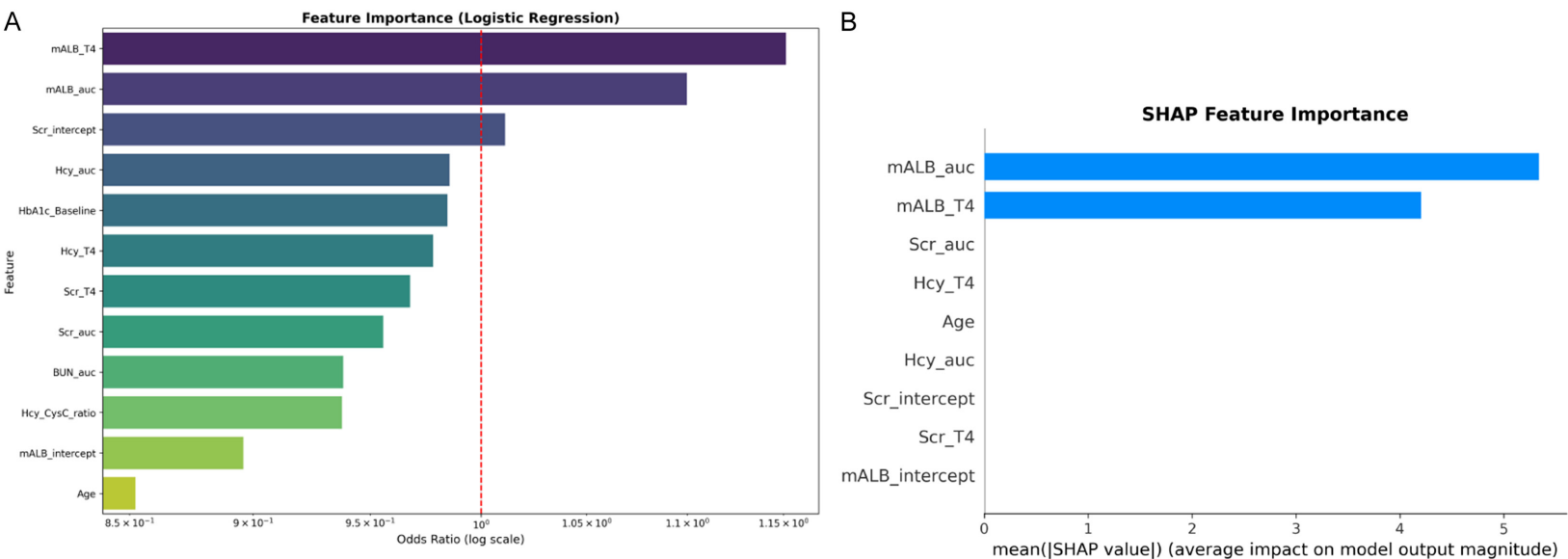


Figure 7. Feature importance analysis for renal dysfunction prediction models. A. Logistic Regression Feature Importance: Bar plot shows odds ratios (log scale) of key features. mALB_T4 and mALB_auc rank highest, indicating strong influence on model predictions. B. SHAP Feature Importance: Plot quantifies average impact of features on model output (mean |SHAP value|). mALB_auc and mALB_T4 again top the list, confirming their critical role in predicting renal dysfunction. These results highlight mALB-related metrics as dominant predictors, aligning with DKD progression dynamics. Hcy, Homocysteine; Cys-C, Cystatin C; mALB, microalbumin; Scr, Serum creatinine; BUN, Blood urea nitrogen; TNF_α, Tumor necrosis factor-α; HbA1c, Hemoglobin A1c.

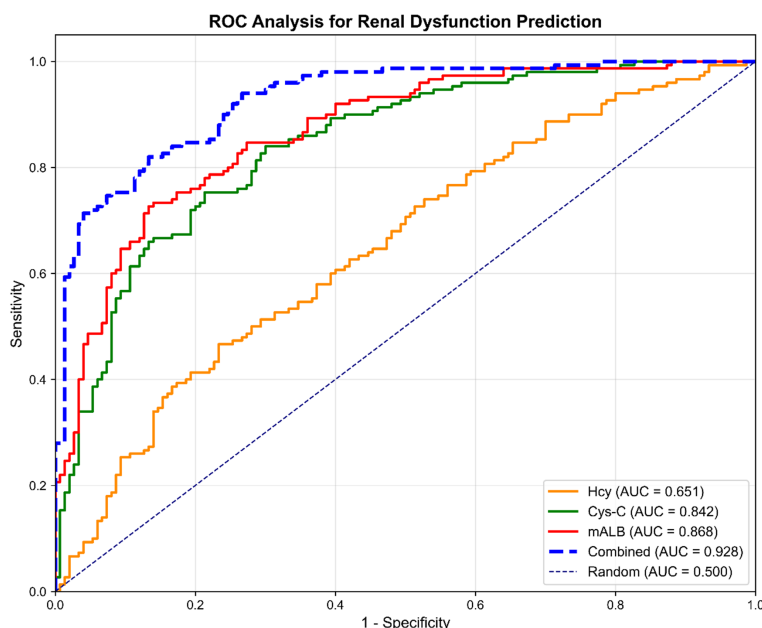


Figure 8. ROC analysis of key biomarkers (Hcy, Cys-C, mAlb) for predicting renal dysfunction in diabetic kidney disease. ROC, Receiver Operating Characteristic; Hcy, Homocysteine; Cys-C, Cystatin C; mAlb, microalbumin; AUC, Area Under the Curve.

model significantly outperformed each single marker, with the largest improvement in sensitivity and specificity. This indicates that integrating Hcy (reflecting metabolic disorder), Cys-C (indicating filtration dysfunction), and mAlb (representing filtration barrier damage) captures multidimensional pathological changes in early DKD, thereby minimizing false negatives and false positives associated with single markers (Figure 8).

Discussion

For the prevention and control of diabetic nephropathy, serum Hcy, Cys-C, and urine microalbumin testing have demonstrated unique advantages. On the one hand, these assays are relatively simple [7, 8]. Most primary medical institutions are equipped with conventional biochemical analyzers capable of detecting Cys-C and urinary microalbumin [7]. After systematic training, primary medical staff can readily perform the tests and interpret the results. This facilitates large-scale early screening of diabetic nephropathy at the grassroots level, which may substantially advance prevention and control efforts [9]. On the other hand, limited resources in primary healthcare, including insufficient professional

staff and suboptimal quality control systems, may compromise testing accuracy and reliability. For example, non-standardized procedures in sample collection, storage, and testing can easily introduce bias, thus affecting the accuracy of diagnosis [10].

Diabetic nephropathy management involves multiple medical disciplines, and these three biochemical indicators constitute a critical bridge between endocrinology, nephrology, and laboratory medicine. In endocrinology clinics, physicians can promptly identify early renal risk in patients with diabetes and make timely referrals to the nephrology department based on the changes in these indicators during routine glucose management [11]. Upon referral,

nephrologists integrate accurate laboratory data into a comprehensive renal function assessment and develop individualized, multi-dimensional treatment plans addressing blood pressure control, lipid regulation, optimization of glomerular filtration, and reduction of proteinuria. This multidisciplinary collaboration model not only enhances diagnostic and therapeutic efficiency but also ensures that patients receive continuous and high-quality medical services, effectively improving the overall patient experience [12].

In contemporary medical practice, diabetic nephropathy has become a major contributor to disability and mortality among diabetic patients, making early and accurate diagnosis the cornerstone for improving prognosis. Recent studies have highlighted serum Hcy, Cys-C, and urine mAlb as key biochemical indicators for early diagnosis of diabetic nephropathy. These findings not only offer new hope for clinical management but also present challenges in translating research into practice [13].

In recent years, increasing attention has been directed toward the combined diagnostic potential of these three markers [14, 15]. A large-scale prospective study involving over

1,000 patients with diabetes and up to 5 years of follow-up demonstrated that serum Hcy alone achieved a sensitivity of approximately 83% and a specificity of 89% for early diagnosis of diabetic nephropathy, while Cys-C alone achieved a sensitivity of 83% and a specificity of 87% [16]. The sensitivity and specificity of urine microalbumin alone for early DKD were 93% [17, 18]. However, when these three biomarkers were integrated into a combined diagnostic model, the sensitivity reached 82.0% and the specificity 86.7%. This demonstrates their strong synergistic value and enhances the ability to accurately identify patients in the early (or initial) stages of diabetic nephropathy.

Another scholar, Dr. McEwan, investigated the predictive value of dynamic monitoring of these three biochemical indicators for DKD progression [19, 20]. In a 3-year follow-up study involving 500 patients with newly diagnosed diabetes, it was found that patients with baseline serum Hcy levels in the high-normal range had a 3- to 4- fold higher risk of developing clinically diagnosed DKD within 1-2 years, compared with patients with stable indicators, if accompanied by a sustained upward trend in Cys-C and a gradual increase in urine microalbumin excretion, even before reaching the diagnostic threshold for DKD [21]. These findings suggest that regular dynamic monitoring of Hcy, Cys-C, and mAlb enables clinicians to predict disease development in advance, adjust treatment strategies in a timely manner, and secure valuable opportunities for intervention. In a related commentary, Dr. Johnson noted that “ambulatory monitoring is not merely a simple data record but a key to precision medicine, enabling timely intervention in the early stage of DKD” [22].

At the level of basic medical research, breakthroughs have also been made in elucidating the connection between these three biochemical indicators and the pathophysiology of DKD. Dr. Perkovic's research team reported that, in addition to its established role in vascular endothelial injury, elevated serum Hcy can activate specific intracellular signaling pathways, thereby upregulating fibrosis-related genes and accelerating renal fibrogenesis [23]. Cys-C has been shown to interact with receptors on glomerular podocytes, with the dynam-

ics of this binding reflecting subtle changes in glomerular filtration function in real time, thus providing mechanistic evidence for its role as an early warning biomarker of kidney injury. For urinary microalbumin, Dr. Kowalski's team further revealed that its increased excretion is regulated by a complex cytokine network involving multiple growth factors and inflammatory mediators, which collectively disrupt glomerular basement membrane barrier integrity. These findings have strengthened the scientific validity of Hcy, Cys-C, and mAlb as key indicators for the early diagnosis of DKD at the molecular level.

Cystatin C, a low-molecular-weight protein produced at a constant rate by nucleated cells, serves as a superior marker of GFR compared to creatinine, as it is independent of muscle mass and inflammatory status. In our cohort, Cys-C levels in DKD patients were markedly higher than those in patients with diabetes without nephropathy and healthy controls. This finding is consistent with prior evidence showing that Cys-C is highly sensitive to subtle declines in GFR, even during preclinical stages of renal dysfunction. The early rise in Cys-C may reflect impaired proximal tubular reabsorption capacity, a hallmark of diabetic nephropathy. Notably, the combination of Hcy and Cys-C provides complementary insights: while Hcy reflects metabolic and vascular injury, Cys-C captures early glomerular filtration dysfunction, thereby offering a more holistic view of renal pathology.

mAlb, the traditional gold standard for DKD screening, was also found to be significantly elevated in DKD patients compared to diabetic and healthy controls. However, mAlb's utility is limited by its susceptibility to confounding factors such as hypertension, infections, and transient hyperglycemia, which reduce its specificity to approximately 75% [20]. Our study addressed this limitation by combining mAlb with Hcy and Cys-C, thereby mitigating false positives and improving diagnostic accuracy. The Kruskal-Wallis test confirmed highly significant intergroup differences across all three markers. ROC analysis revealed an AUC of 0.868 for mAlb, 0.651 for Hcy, and 0.842 for Cys-C. Notably, the combined model achieved sensitivity and specificity exceeding those of individual tests, supporting the hypothesis that

multi-dimensional biomarker panels outperform single indicators in complex diseases like DKD.

The major limitation of this study is the relatively limited sample size, which significantly hampers the generalizability of the findings. A limited cohort can hardly capture the high heterogeneity among patients with diabetic nephropathy, and the diagnostic performance of these three biochemical markers may differ across regions, ethnicities, lifestyles, and genetic backgrounds. For example, in regions with high seafood consumption, baseline serum Hcy level may be lower due to dietary influences, potentially affecting diagnostic accuracy when applying population-wide reference values. Considering racial factors, the unique genetic profiles of certain ethnic minority populations may affect the metabolism and excretion of Cys-C, thereby reducing the applicability of diagnostic models derived from single-ethnic cohorts to multi-ethnic populations.

Given the complex etiology and pathophysiology of diabetic nephropathy, reliance on only three biochemical indicators is insufficient to achieve accurate diagnosis. Advances in genetic testing have shown that single nucleotide polymorphisms (SNPs) at specific gene loci are closely associated with DKD susceptibility. For example, variants of the SLC2A9 gene significantly alter renal glucose transport efficiency, thereby influencing individual risk of developing DKD. In addition, proteomic analyses of renal biopsy tissue provide insights into protein expression changes during renal injury at the molecular level, offering opportunities to identify novel diagnostic markers and therapeutic targets. In addition, hemodynamic monitoring, such as renal artery blood flow velocity and intraglomerular pressure, can further reflect the renal perfusion status in real time. When integrated with biochemical markers such as Hcy, Cys-C, and mAlb, these emerging approaches could construct a multidimensional framework for early and accurate DKD diagnosis, paving the way for personalized treatment strategies.

This study has several limitations. Its single-center, retrospective design and homogeneous cohort restrict generalizability. In addition, dietary and genetic factors were not assessed, and longitudinal biomarker interactions could

not be comprehensively evaluated. Future research should include prospective, multi-center studies with larger and more diverse populations, integrate biochemical, genetic, proteomic, and hemodynamic data, and combine novel technologies to further refine early diagnostic strategies for DKD.

Conclusion

This study demonstrates that the combined assessment of Hcy, Cys-C, and mAlb offers a reliable and accurate tool for the early diagnosis of DKD. By addressing the limitations of single-marker testing and offering a cost-effective, reproducible protocol, these findings carry significant implications for clinical practice and public health. Future multicenter studies with larger and more diverse populations are needed to validate these results, but the integration of multi-dimensional biomarkers has the potential to transform DKD diagnosis and management, ultimately improving patient outcomes worldwide.

Disclosure of conflict of interest

None.

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