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Article

Feasibility of Integrating Urinary Proteomics and Machine Learning for Diagnosing Diabetic Nephropathy

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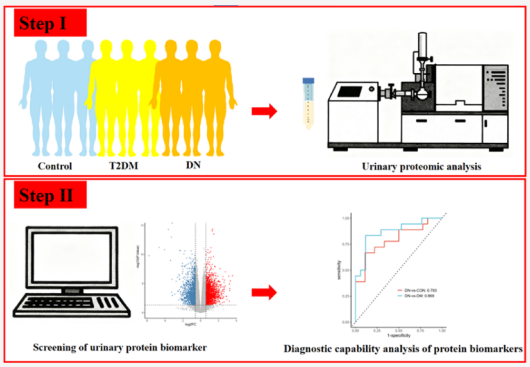
ABSTRACT: Diabetic nephropathy (DN) represents the predominant microvascular complication associated with diabetes mellitus; however, existing diagnostic techniques are inadequate. This study evaluated candidate urinary protein biomarkers for diagnosing DN. A cohort comprising 59 patients with type 2 diabetes, 60 patients with DN, and 60 healthy volunteers was recruited. Urine proteomics was utilized to investigate differential protein expression levels among various patient groups and to identify potential biomarkers in conjunction with data analysis from the gene expression omnibus database. Machine learning classification methods were utilized to construct differential diagnosis models for DN. The data set IPX0003092000 was used to validate these diagnostic models. Six potential biomarkers—SERPINF1, FABP4, CP, CFB, C4A, and A1BG—were identified. The diagnostic models for DN, constructed by using machine learning algorithms, demonstrated robust diagnostic performance. Notably, models employing the glmnet, plr, and ranger classification methods achieved AUC values exceeding 0.800 in both the training and test data sets. In the validation cohort, the AUC values for models constructed using the ranger, glmnet, and plr methods were 0.928, 0.942, and 0.850, respectively. We evaluated six candidate urinary biomarkers (SERPINF1, FABP4, CP, CFB, C4A, and A1BG) using urinary proteomics and developed a diagnostic model for DN using machine learning algorithms.



KEYWORDS: *diabetic nephropathy, urinary biomarkers, proteomic, machine learning algorithms, diagnostic model*

1. INTRODUCTION

Diabetic nephropathy (DN) represents a prevalent microvascular complication in individuals diagnosed with diabetes mellitus (DM).[1] The worldwide incidence of DN has experienced a significant increase over recent decades, primarily attributable to the heightened prevalence of type 2 diabetes mellitus (T2DM).[2] Recent data indicate that 463 million adults globally have been diagnosed with DM, with projections suggesting an increase to 700 million by the year 2045.[3] It is estimated that approximately 40% of individuals with DM will develop DN, which is the primary cause of diabetic end-stage renal disease (ESRD).[1,4] Consequently, early diagnosis and intervention are essential for decreasing the prevalence of DN and enhancing patient outcomes.

At present, the "gold standard" for diagnosing DN involves conducting a renal biopsy followed by histopathological examination. However, as an invasive procedure, kidney biopsy carries potential risks, including bleeding and infection.[5] Clinically, the urinary albumin-to-creatinine ratio (UACR) serves as a valuable diagnostic indicator for early DN by reflecting the dynamic changes in renal microvascular injury in patients.[6] In addition, the diagnosis of DN primarily relies on estimated glomerular filtration rate (eGFR) measurements and the patient's clinical features.[5,7] However, the evaluation of urinary albumin presents certain limitations, as albumin levels may remain within normal ranges during the early stages of DN.[8] Furthermore, elevated urinary albumin can result from other conditions, such as acute kidney injury, glomerulonephritis, and cardiovascular disease.[6] Notably, some patients with DM exhibit a marked reduction in eGFR and glomerular lesions despite maintaining a normal urinary albumin excretion rate.[9] Therefore, there is a pressing need to explore novel candidate biomarkers to enhance the diagnostic accuracy of DN.

A diverse array of biomarkers (including genes, proteins, and metabolites) is now considered beneficial for the early diagnosis of kidney injury in patients with DM, thereby offering novel insights into the clinical diagnosis of DN.[10] These urinary

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biomarkers not only indicate the presence of renal injury but also suggest a potential association with systemic inflammation, oxidative stress, and metabolic disturbances in affected patients.[11] For example, long noncoding RNAs, microRNAs, inflammatory factors, and proteins in urine are emerging as promising biomarkers for DN.[10,12] Investigating these novel biomarkers may enhance the accuracy of early stage DN diagnosis, thereby providing critical opportunities for timely clinical intervention.

In this study, we constructed a DN diagnostic model using machine learning algorithms. Machine learning, also known as artificial intelligence, aims to create computer systems that learn and adapt from experience.[13] Machine learning algorithms build predictive models by identifying clinical variables and learning decision rules from data.[13] Implementing machine learning algorithms helps identify appropriate candidates for evaluation and avoid routine clinical steps.[13] Xu et al.[14] conducted a systematic review and meta-analysis of risk prediction models for DN in Chinese patients with T2DM. They proposed that future efforts should focus on developing high-performance, user-friendly prediction models based on machine learning methods for clinical applications.[14]

This study sought to identify candidate urinary biomarkers associated with the diagnosis of DN in T2DM patients using urinary proteomics and to investigate their potential application in DN diagnosis. This study seeks to facilitate the diagnosis of DN, with a particular focus on identifying DN in patients with T2DM. The findings provide a reference basis for the complementary diagnosis of DN.

2. MATERIALS AND METHODS

2.1. Patient Recruitment and Sample Preparation

In this study, a cohort of 59 patients diagnosed with T2DM and 60 patients with DN were selected from those treated at the First People's Hospital of Xiaoshan District, Hangzhou, between May 2022 and May 2023. Additionally, a control (CON) group comprising 60 healthy individuals, with no history of diabetes, malignancies, or major diseases, was recruited from the hospital's checkup center. The inclusion criteria for the T2DM group were as follows: (i) fulfillment of the World Health Organization's diagnostic criteria for T2DM;[15] (ii) age between 18 and 80 years; and (iii) availability of comprehensive clinical information. The exclusion criteria included: (i) presence of other renal, cardiovascular, hypertension, cerebrovascular, endocrine, or immune system diseases; and (ii) status as pregnant or lactating women. The DN group (n = 60) required meeting T2DM and DN criteria, being 18−80 years old, and having full clinical information. The exclusion criteria for the DN group mirrored those applied to the T2DM group. Clinical data were collected from healthy individuals, DM patients, and DN patients. This study received approval from the Ethics Committee of the First People's Hospital of Xiaoshan District, Hangzhou (approval no. 2022-025), and informed consent was obtained from all participants.

2.2. Creatinine (SCr) Testing and eGFR Calculation

Serum samples were collected from all of the individuals. SCr levels were measured using a SCr test kit (creatine oxidase method) purchased from Medical system Biotechnology Co., Ltd. (Ningbo, China), following the kit's instructions on an AU5800 automatic biochemical analyzer (Beckman Coulter, Brea, CA, USA). Finally, eGFR values were calculated using the CKD-EPI formula, incorporating the patient age and gender.

eGFR Calculation Formula:

Female:

$$\text{SCr} \le 0.7\ \text{mg/dl: eGFR} = 144 \times (\text{Scr}/0.7)^{-0.329} \times (0.993)^{\text{age}}$$

$$\text{SCr} > 0.7\ \text{mg/dl: eGFR} = 144 \times (\text{Scr}/0.7)^{-1.209} \times (0.993)^{\text{age}}$$

Male:

$$\text{SCr} \le 0.9\ \text{mg/dl: eGFR} = 144 \times (\text{Scr}/0.9)^{-0.411} \times (0.993)^{\text{age}}$$

$$\text{SCr} > 0.9\ \text{mg/dl: eGFR} = 144 \times (\text{Scr}/0.9)^{-1.209} \times (0.993)^{\text{age}}$$

2.3. Urine Collection

A midstream clean urine sample was randomly obtained from participants in the morning and collected into a specimen cup. The sample underwent centrifugation at 3000g at 4 °C for 15 min (min). Subsequently, 8 mL of the supernatant was aspirated and transferred into numbered centrifuge tubes, rapidly frozen in liquid nitrogen for 15 min, and subsequently stored at −80 °C.

2.4. Protein Extraction

The collected urine samples were removed from a −80 °C refrigerator and thawed on ice. Equal volumes of methanol and 1/4 volume of chloroform were added, mixed thoroughly, and placed at 4 °C for 5 min. After high-speed centrifugation, the supernatant was discarded, equal volumes of methanol were added again and mixed and centrifuged, and the supernatant was discarded. The extracted proteins in the centrifuge tubes were dried in the air, and premixed lysis solution (1% SDC, 1% protease inhibitor) was slowly added to the sample tubes and sonicated in an ultrasonic crusher. Then, centrifugation was performed at 4 °C for 10 min (15,000g) to remove impurities. Finally, the concentration of extracted proteins was measured by using the BCA method.

2.5. Protein Digestion

Depending on the concentration of extracted proteins, an equal amount of protein was taken from each sample, and the volume was made up to unity with the lysate. Then, dithiothreitol (DTT, 5 mM) was added and reduced at 56 °C for 30 min. Next, iodoacetamide (IAA, 15 mM) was added, and the reaction was carried out at room temperature for 15 min in the dark. Then, trypsin was added at a ratio of 1:50 (protease: protein, m/m) and digested overnight at 37 °C. Then, trypsin was added at a ratio of 1:100 (protease: protein, m/m), and the enzymatic digestion was continued at 37 °C for 4 h. At the end of the reaction, the samples were acidified by adding an appropriate amount of 10% trifluoroacetyl to adjust the pH to between 2 and 3. Subsequently, the samples were desalted using Strata X (Phenomenex, Torrance, CA, USA) and vacuum freeze-dried for use. Finally, the peptides were fully resolubilized and quantified using the Pierce Quantitative Peptide Assays & Standards kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC−MS/MS) Analysis

The analysis utilizes the nanoflow rate Vanquish Neo system (Thermo Fisher Scientific) for chromatographic separation, and samples post high-efficiency liquid chromatography separation are subjected to Data Independent Acquisition (DIA) mass spectrometry using the Astral high-resolution mass spectrometer (Thermo Fisher Scientific). The detection mode is positive ion, with a precursor ion scan range of 380−980 m/z and a primary mass spectrometry resolution of 240,000 at 200 m/z. Normalized AGC Target is set at 500%, with a Maximum IT of 5 ms. MS2 employs the DIA data acquisition mode, with 299 scan windows set, an Isolation Window of 2 m/z, HCD Collision Energy set at 25 ev, Normalized AGC Target at 500%, and a Maximum IT of 3 ms.

2.7. Data Analysis

The DIA data is processed using the DIA-NN software. The software parameters are set as follows: the enzyme used is trypsin, with a maximum missed cleavage site set to 2. Fixed modification is Carbamidomethyl (C), and dynamic modifications are set to Oxidation (M) and Acetyl (Protein N-term). Proteins identified through database retrieval must pass the set filtering parameter of FDR <1%.

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Table 1. Clinical Characteristics of Healthy Control, DM, and DN Patients

	CON	DM	DN	*P* DM vs DN
number	60	59	60	N/A
age (year)	46.17 ± 17.09	45.68 ± 14.09	56.05 ± 14.03	0.000
sex (male)	44 (73.33%)	42 (71.19%)	37 (61.67%)	0.270
(female)	16 (26.67%)	17 (28.81%)	23 (38.33%)	
BMI (kg/m2)	24.40 ± 4.46	25.91 ± 4.44	25.30 ± 5.00	0.500
blood glucose	4.72 ± 0.80	12.42 ± 6.06	10.26 ± 5.60	0.046
diabetes duration (years)	N/A	4.19 ± 6.04	11.83 ± 8.48	0.000
HbA1c (%)	N/A	10.51 ± 2.82	10.01 ± 2.53	0.307
eGFR (mL/min)	103.10 ± 15.87	115.40 ± 25.26	94.18 ± 41.72	0.000

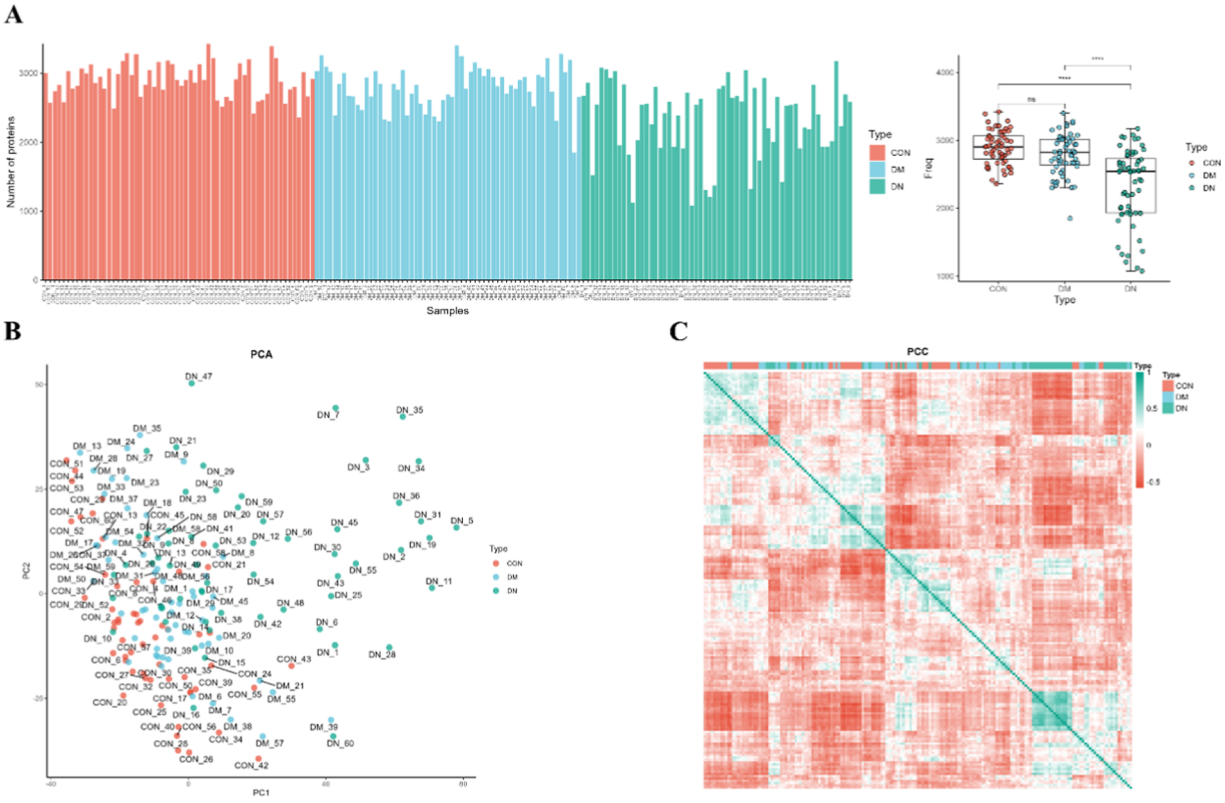

Figure 1. Proteomic features of urine from patients. (A) Frequency distribution analysis of urinary proteins in the CON, DM, and DN groups. (B) PCA analysis of total proteins in the CON, DM, and DN groups. (C) PCC analysis of total proteins in the CON, DM, and DN groups.

2.8. Proteomics Data Normalization and Treatment for Missing Values

Raw protein intensity values were processed before statistical analysis. Specifically, the intensity values were first subjected to a log 2 transformation to stabilize variance and approximate a normal distribution. Subsequently, centering was performed on a per-protein basis across all samples to minimize interprotein scale differences. To further reduce technical variability and correct for systematic biases introduced by differences in sample loading during mass spectrometry acquisition, median normalization was applied across samples.

Proteins exhibiting a high degree of missingness (>50%) and nonuniform distribution of missing data across groups were excluded to reduce the likelihood of false-positive results. All samples were processed within a single batch, and replicate analyses indicated no significant batch effects; consequently, no correction for batch effects was implemented.

2.9. Data Download

The data sets GSE142025 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142025), GSE217709 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE217709), GSE166239 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE166239), GSE185011 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE185011), and GSE30122 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30122) were procured from the Gene Expression Omnibus (GEO) database and employed to identify differentially expressed genes in patients with DM and DN. Additionally, the data set IPX0003092000 (https://www.iprox.cn/page/project.html?id=IPX0003092000) is accessible from the iProX database and serves as a resource for the validation of diagnostic models for DN.

2.10. Bioinformatics Analysis

In this study, the limma R package (version 3.58.1) was used to identify differentially expressed proteins in different groups. The R packages "ggplot2 (version 3.5.1)" and "pheatmap (version 1.0.12)" were used to establish the heat map and analyze the differentially expressed proteins. Fisher's exact test was used to analyze functional enrichments (KEGG, biological Process, cellular component, molecular function). Furthermore, we performed protein−protein interaction (PPI) network analysis on the differentially expressed proteins encoded using the

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J. Proteome Res. 2026, 25, 1292−1304

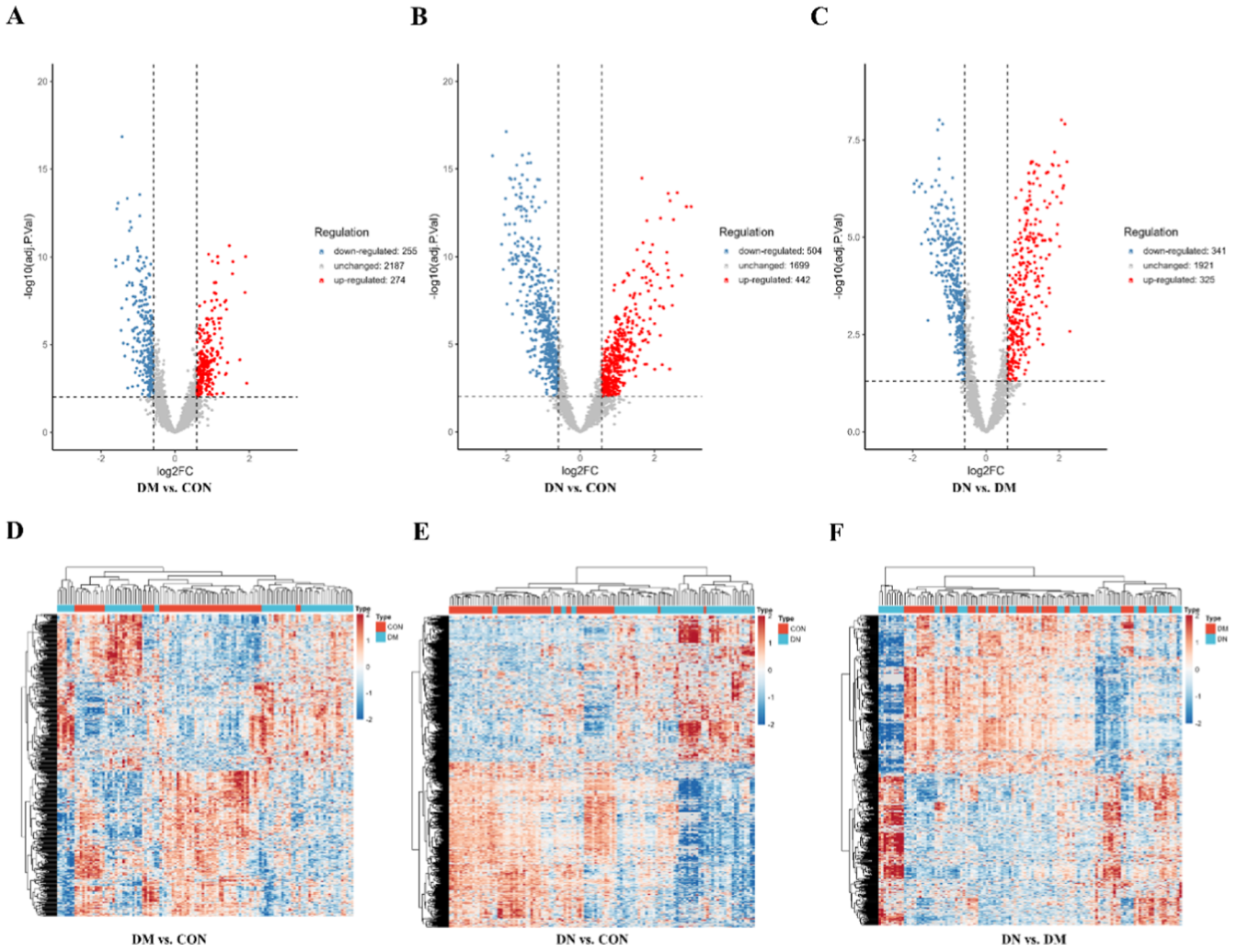

Figure 2. Analysis of differentially expressed proteins between different groups (DM vs CON, DN vs CON, and DN vs DM). Volcano plots (A−C) and heat maps (D−F) were used to analyze the changes in protein expression between different groups. (A,D) for DM vs CON; (B,E) for DN vs CON; (C,F) for DN vs DM.

STRING database. By applying the above bioinformatics methods, we conducted a systematic analysis of the urinary proteomics data from this study and publicly available data sets obtained from the GEO database to identify potential biomarkers for the diagnosis of DN.

2.11. Machine Learning Methods

In this study, to evaluate the performance of potential biomarkers in diagnosing DN, we constructed a DN diagnostic model using six different machine learning algorithms. Six different machine learning classification methods were implemented in the *R* package caret (7.0.1), including Stochastic Gradient Boosting (gbm), glmnet, Penalized Logistic Regression (plr), Support Vector Machines with Radial Basis Function Kernel (svmRadial), Naive Bayes (naive_bayes), and Random Forest (ranger). First, we included 179 samples: CON group ($n = 60$), the DM group ($n = 59$), and the DN group ($n = 60$). We constructed two diagnostic models: DN vs CON and DN vs DM. All samples were randomly divided into train and test sets at a 7:3 ratio. Six different machine learning classification methods (gbm, glmnet, plr, svmRadial, naive_bayes, and ranger) were employed for model construction. Model performance was evaluated using ROC curves via a 10-fold cross-validation strategy. Based on AUC values from the test set, models constructed using glmnet, plr, and ranger were selected for comparative performance analysis against clinical diagnostic indicators (eGFR). Finally, external validation of the DN vs CON diagnostic models built with glmnet, plr, and ranger was performed using the external data set IPX0003092000.

2.12. Statistical Analysis

The output data were analyzed by using *R* tools. Student's *t*-test was used to screen the differentially expressed proteins (fold change ≥1.5, $P < 0.05$). Receiver operating characteristic (ROC) curves and precision-recall curves were generated using the pROC (version 1.18.5) and ROCR packages, respectively.

3. RESULTS

3.1. Clinical Characteristics of the Patients Were Analyzed

Table 1 shows clinical data such as age, sex, body mass index (BMI), blood glucose, diabetes duration, and glycated hemoglobin (HbA1c). The CON group's age was similar to the DM group, but the DN group was older ($P = 0.001$). Blood glucose was higher in the DM and DN groups than in the CON group, with a significant difference between the DM and DN groups ($P < 0.05$). The DN group had a longer diabetes duration than the DM group ($P < 0.001$), linking longer diabetes to DN development. Moreover, eGFR was lower in DN compared to DM and CON groups, with a significant difference between DM and DN ($P < 0.001$). No significant differences were observed in BMI, gender, or HbA1c between the DM and DN groups. These findings indicate that patients with DN tend to be older and have a longer duration of diabetes.

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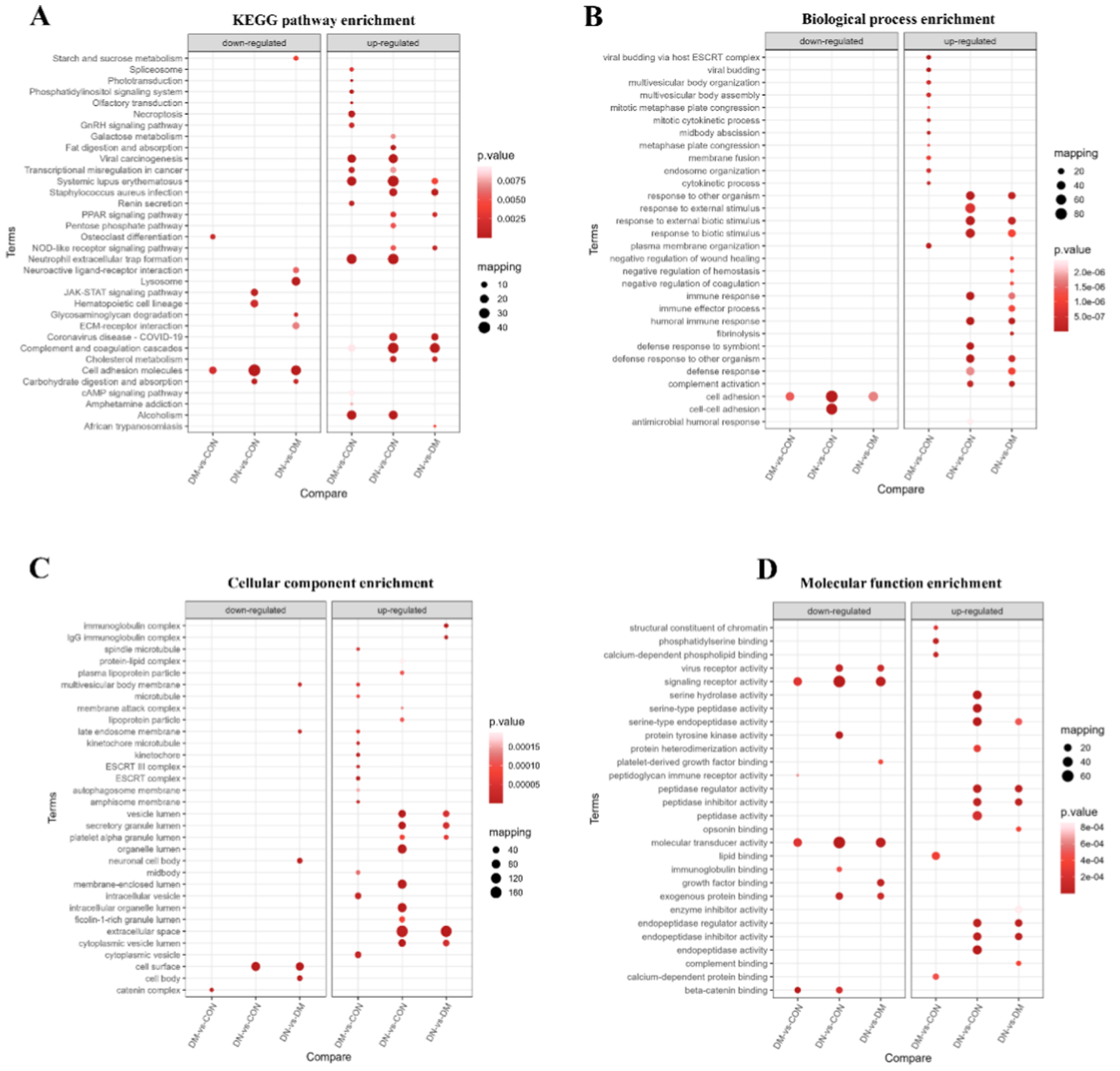

Figure 3. Functional analysis of differentially expressed proteins. (A) KEGG function enrichment analysis was performed for the differentially expressed proteins (DM vs CON, DN vs CON, and DN vs DM). (B–D) GO function enrichment analysis was performed for the differentially expressed proteins (DM vs CON, DN vs CON, and DN vs DM).

3.2. Proteomic Features of Urine from Patients

Figure 1 shows our initial analysis of urine protein counts across the groups. The frequency distribution shows no significant difference between the CON and DM groups, but the DN group has notably lower protein counts (Figure 1A, $P < 0.001$). The PCA scatter plot indicates a clear separation between the CON and DN groups, suggesting significant differences in protein expression, while the DM and DN groups are less distinct (Figure 1B). The Pearson correlation coefficient (PCC) results show group correlations (Figure 1C). The heat map revealed substantial heterogeneity in protein expression, particularly between the DN group and the others, suggesting potential biomarkers for clinical DN studies (Figure 1C).

3.3. Screening of Differentially Expressed Proteins

To identify statistically significant differentially expressed proteins, volcano plots were constructed (fold change = 1.5, $P < 0.05$) Figure 2A–C shows that, compared to the CON group, the DM group had 255 down-regulated and 274 up-regulated proteins, while the DN group had 504 down-regulated and 442 up-regulated proteins ($P < 0.01$). Compared to the DM group, the DN group had 341 down-regulated and 325 up-regulated proteins ($P < 0.05$). Unsupervised hierarchical clustering analyses highlighted significant protein expression between the CON, DM, and DN groups, indicating notable changes as DM progresses to DN (Figure 2D,E). These findings provide insights into urinary protein expression alterations, enhancing our understanding of the biological mechanisms distinguishing DM from DN.

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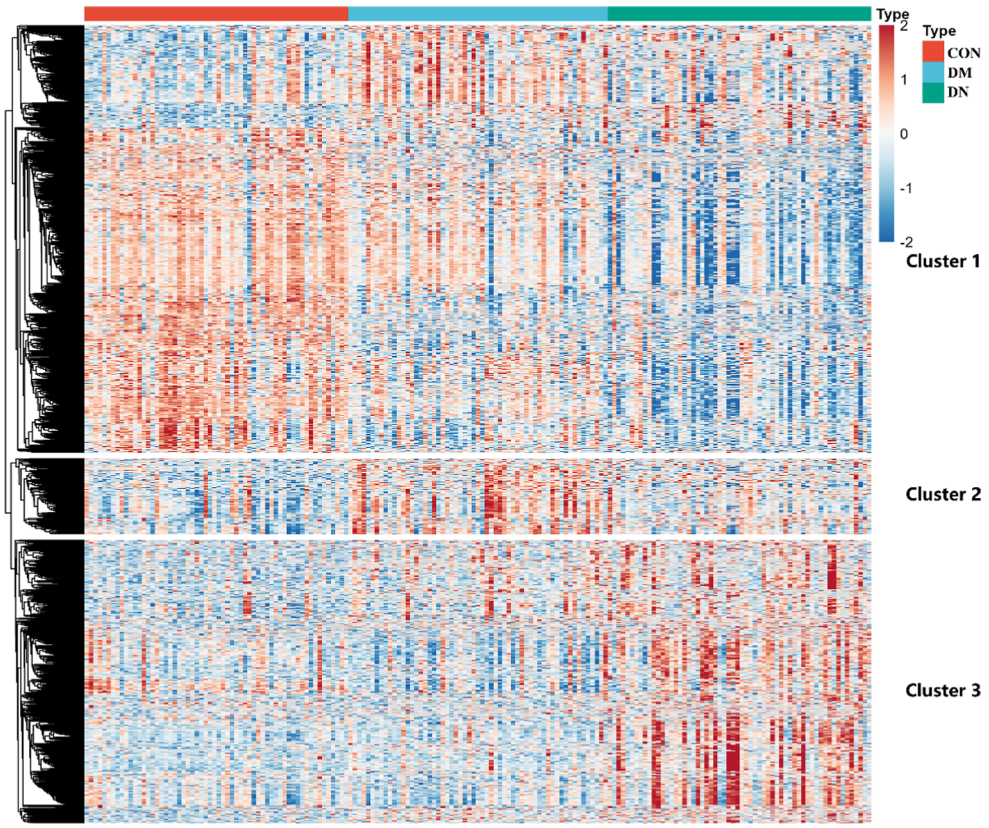

Figure 4. Hierarchical clustering and heatmap analyses indicated that protein expression in the Cluster 3 module increased with DM progression, peaking in DN patients.

3.4. Functional Analysis of Differentially Expressed Proteins

The KEGG pathway analysis revealed that the proteins exhibiting differential expression among the DM, DN, and CON groups are involved in regulating various biological pathways (Figure 3A). Specifically, the down-regulated proteins are implicated in pathways such as "starch and sucrose metabolism", "lysosome", "cell adhesion molecules", and "carbohydrate digestion and absorption". In contrast, the up-regulated proteins are associated with pathways including "systemic lupus erythematosus", "PPAR signaling pathway", and "NOD-like receptor signaling pathway", indicating a potential link between DN and stress response, metabolic disorders, and infection risk (Figure 3A). Overall, these results show that up-regulated pathways predominantly involve immune inflammation and metabolic reprogramming, exhibiting stronger signals; down-regulated pathways primarily focus on cellular processes such as basal metabolism and RNA processing/splicing, presenting a more dispersed or weaker trend (Figure 3A). GO Biological Process enrichment analyses further identified that the down-regulated proteins are involved in the regulation of "cell adhesion" and "cell−cell adhesion" processes, while the up-regulated proteins participate in the regulation of processes related to cell division and response to biological stimuli (Figure 3B). Overall, up-regulated pathways are significantly concentrated in processes related to immune/defense responses and reactions to external stimuli; down-regulated pathways are less numerous, primarily appearing in cell adhesion and intercellular/membrane-related processes (Figure 3B). These findings suggest that the progression of DN may involve immune system activation, disrupted cellular interactions, and altered cellular responsiveness to external stimuli. In addition, GO Cellular Component enrichment analyses revealed that the down-regulated proteins were predominantly associated with cellular components related to the cell surface and neuronal cell body. In contrast, the up-regulated proteins were primarily linked to the extracellular space and secretion-related cellular structures (Figure 3C). Moreover, the GO Molecular Function enrichment analysis indicated that the up-regulated molecular function pathways primarily cluster around protease activity/regulation, binding functions (including ligand/immune-related), and receptor/enzyme activity pathways, which may imply an enhanced regulation of enzyme activity by cells in response to environmental changes (Figure 3D). Down-regulated pathways, conversely, tend to lean more toward molecular recognition and binding/binding and signaling components (such as molecular sensor/receptor activity, lipid binding, and β-catenin binding), suggesting potential impairments in these cellular functions (Figure 3D).

3.5. Screening of Up-Regulated Proteins in DN

Hierarchical clustering and heatmap analyses indicated that protein expression in the Cluster 3 module increased with DM progression, peaking in DN patients (Figure 4). KEGG enrichment analysis of Cluster 3 proteins indicated their involvement in pathways related to systemic lupus erythematosus, neutrophil extracellular trap formation, the IL-17 signaling pathway, complement and coagulation cascades, and other inflammation and immunomodulation pathways (Figure 5A). A PPI network was created for significantly up-regulated proteins ($P < 0.05$) in the top 15 KEGG-enriched pathways (Figure 5B). Ultimately, 28 significantly up-regulated proteins in DN patients

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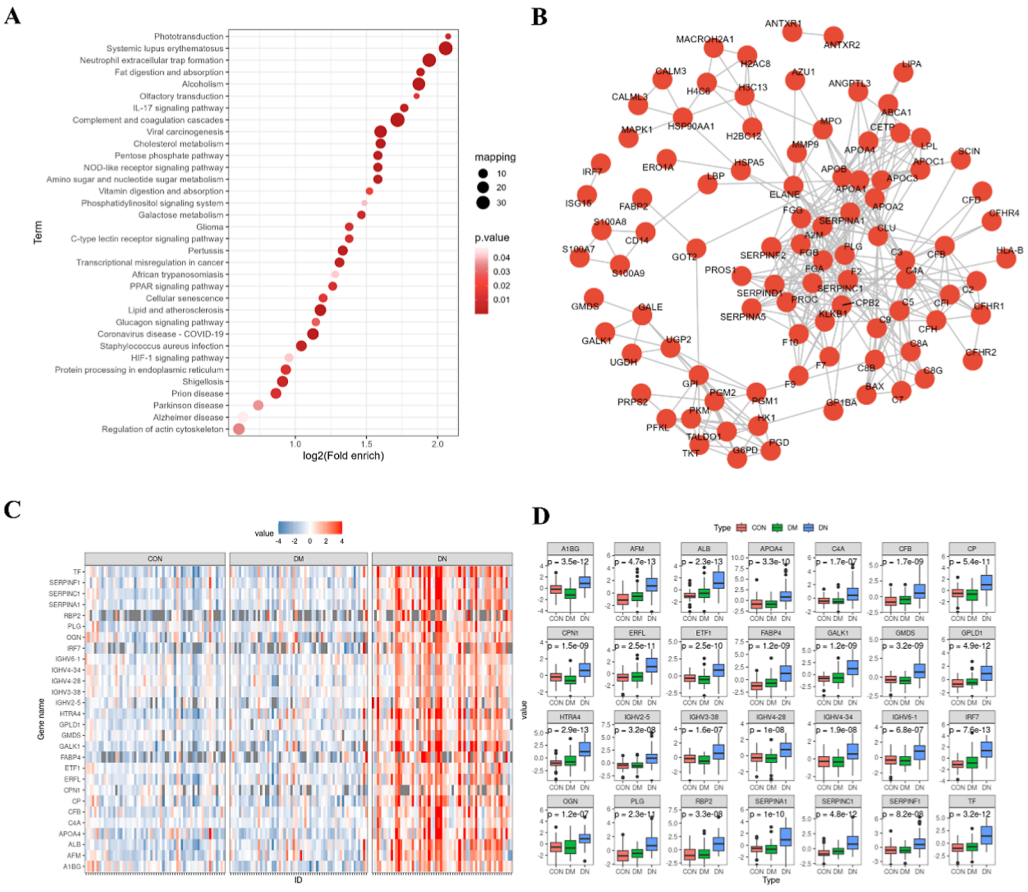

Figure 5. Screening of potential urinary biomarkers for DN. (A) KEGG enrichment analysis results for proteins in Cluster 3 modules of Figure 4. (B) PPI network of proteins that were significantly up-regulated in the top 15 pathways enriched by KEGG. (C,D) Twenty-eight proteins that were significantly up-regulated in the urine of DN patients were selected for the study of potential urinary biomarkers of DN.

were identified as potential urinary biomarkers for DN (Figure 5C,D).

3.6. Screening of Potential Urinary Protein Biomarkers for DN

To identify potential urinary protein markers for DN, we analyzed five GEO databases. A PCA scatter plot (Figure 6A−E) illustrates sample distribution, while Volcano plots (Figure 6F−J) highlight differentially expressed genes (FC ≥ 1.5, $P < 0.05$). In GSE142025, 1621 genes were up-regulated and 1511 down-regulated; GSE166239 had 1622 up-regulated and 1433 down-regulated genes; GSE185011 revealed 132 up-regulated and 41 down-regulated genes; GSE217709 had 238 up-regulated and 130 down-regulated genes; GSE30122 showed 427 up-regulated and 189 down-regulated genes (Figure 6F−J). Ultimately, six potential biomarkers—pigment epithelium-derived factor member 1 (SERPINF1), fatty acid-binding protein 4 (FABP4), ceruloplasmin (CP), complement factor B (CFB), complement C4-A (C4A), and α-1B-glycoprotein (A1BG)—were identified through cross-validation of our urine proteomic analysis with the results from the GEO database analysis (Figure 6K,L).

3.7. Diagnostic Capability Analysis of Potential Urinary Protein Biomarkers

To evaluate the diagnostic effectiveness of the combined detection of six urinary protein biomarkers (SERPINF1, FABP4, CP, CFB, C4A, and A1BG) for DN, we developed a diagnostic model employing multiple machine learning methods. Results showed that these biomarkers performed exceptionally well in diagnosing DN (DN vs CON, and DN vs DM), with AUC values over 0.800 using glmnet, plr, and ranger methods (Table 2). Based on AUC values from the test set, models constructed using glmnet, plr, and ranger were selected for comparative performance analysis against clinical diagnostic indicators (eGFR). These models surpassed the eGFR clinical indicator in accuracy (Figure 7), highlighting the biomarkers' potential in DN diagnosis.

3.8. Diagnostic Capability Validation of Potential Urinary Protein Biomarkers

In the validation set (CON = 26, DN = 52), the AUC values for the three machine learning methods (ranger, glmnet, and plr) were 0.928, 0.942, and 0.850, respectively, while the precision-recall values were 0.968, 0.975, and 0.930 (Figure 8A−F, Table 3). These results suggest that detecting six potential urinary protein biomarkers is effective for diagnosing DN.

4. DISCUSSION

DN is now the leading cause of ESRD globally.[16] Serum creatinine and urinary albumin have limitations in accurately diagnosing DN, highlighting the need for more sensitive early biomarkers.[17,18] Liquid biopsy holds significant potential for clinical use in DN by identifying biomarkers in blood or urine to aid in disease staging, risk assessment, and prognosis prediction.[19,20]

Urine, a readily accessible biological fluid, is used to develop novel biomarkers for DN.[10] Wani et al.[21] conducted a comprehensive review evaluating established and novel urinary

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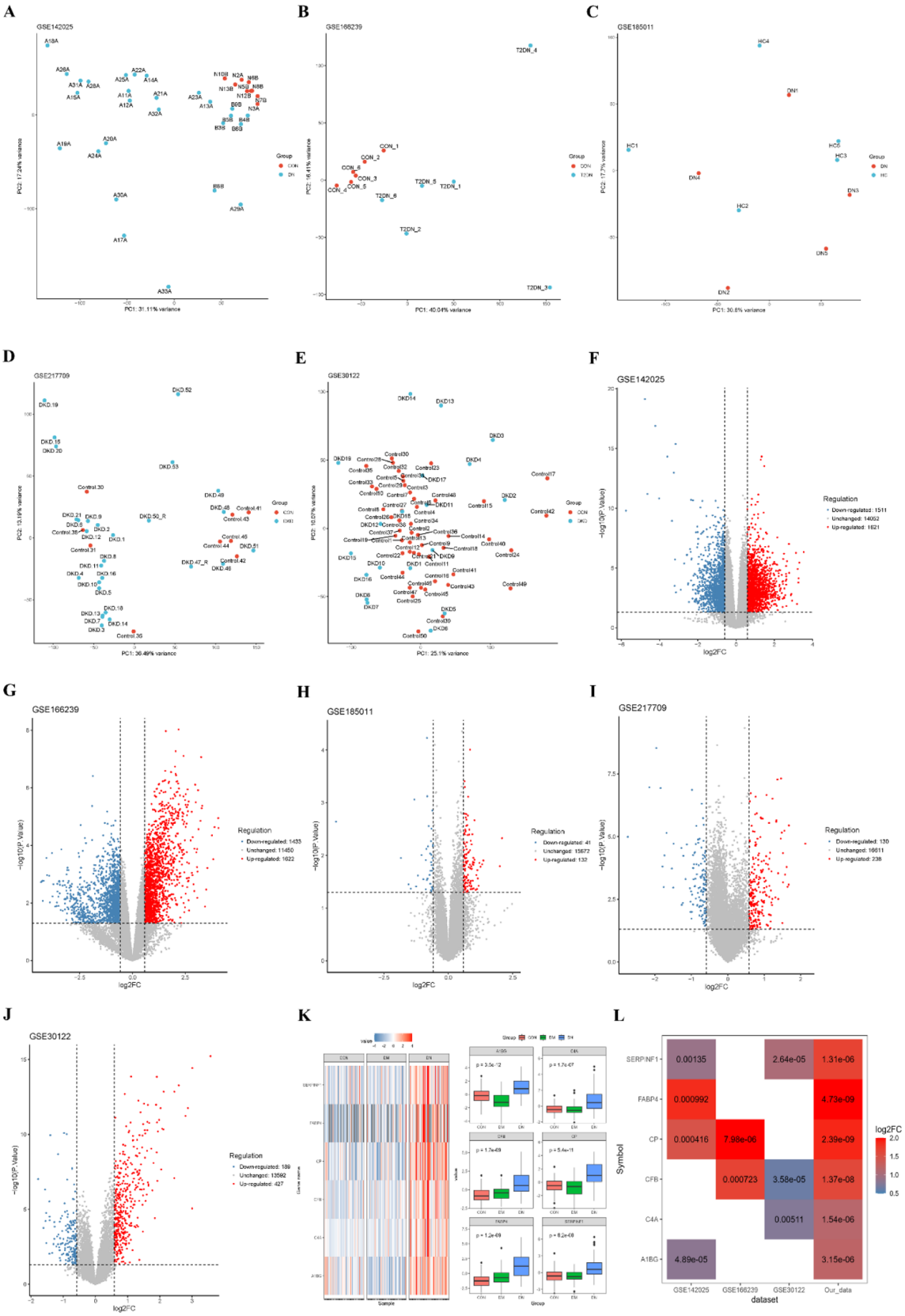

Figure 6. Screening of potential urinary protein biomarkers for DN. (A−E) The PCA scatter plot shows the distribution of each set of samples in the GEO database (GSE142025, GSE217709, GSE166239, GSE185011, and GSE30122) in the principal component space. (F−J) The volcano plot of differentially expressed genes between the control group and DN group in the GEO database (GSE142025, GSE217709, GSE166239, GSE185011, and GSE30122). (K−L) SERPINF1, FABP4, CP, CFB, C4A, and A1BG expressions in the results of our study (K) and in the GEO database.

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J. Proteome Res. 2026, 25, 1292−1304

Table 2. Performance of Six Urinary Protein Biomarkers in DN Diagnostic Models Constructed by Machine Learning Methods

group	machine learning method	AUC. test	AUC. train	specificity	sensitivity	accuracy	kappa	F1
DN vs CON	gbm	0.793	0.844	0.889	0.667	0.778	0.556	0.750
DN vs CON	glmnet	0.827	0.881	0.833	0.778	0.806	0.611	0.800
DN vs CON	plr	0.830	0.868	0.833	0.778	0.806	0.611	0.800
DN vs CON	svmRadial	0.772	0.817	0.833	0.722	0.778	0.556	0.765
DN vs CON	naive_bayes	0.796	0.841	0.778	0.778	0.778	0.556	0.778
DN vs CON	ranger	0.818	0.849	0.722	0.889	0.806	0.611	0.821
DN vs DM	gbm	0.869	0.753	0.882	0.833	0.857	0.715	0.857
DN vs DM	glmnet	0.879	0.846	1.00	0.667	0.829	0.660	0.800
DN vs DM	plr	0.889	0.813	0.941	0.778	0.857	0.715	0.848
DN vs DM	svmRadial	0.856	0.791	0.941	0.722	0.829	0.659	0.813
DN vs DM	naive_bayes	0.863	0.845	0.941	0.667	0.800	0.603	0.774
DN vs DM	ranger	0.886	0.826	0.941	0.722	0.829	0.659	0.813

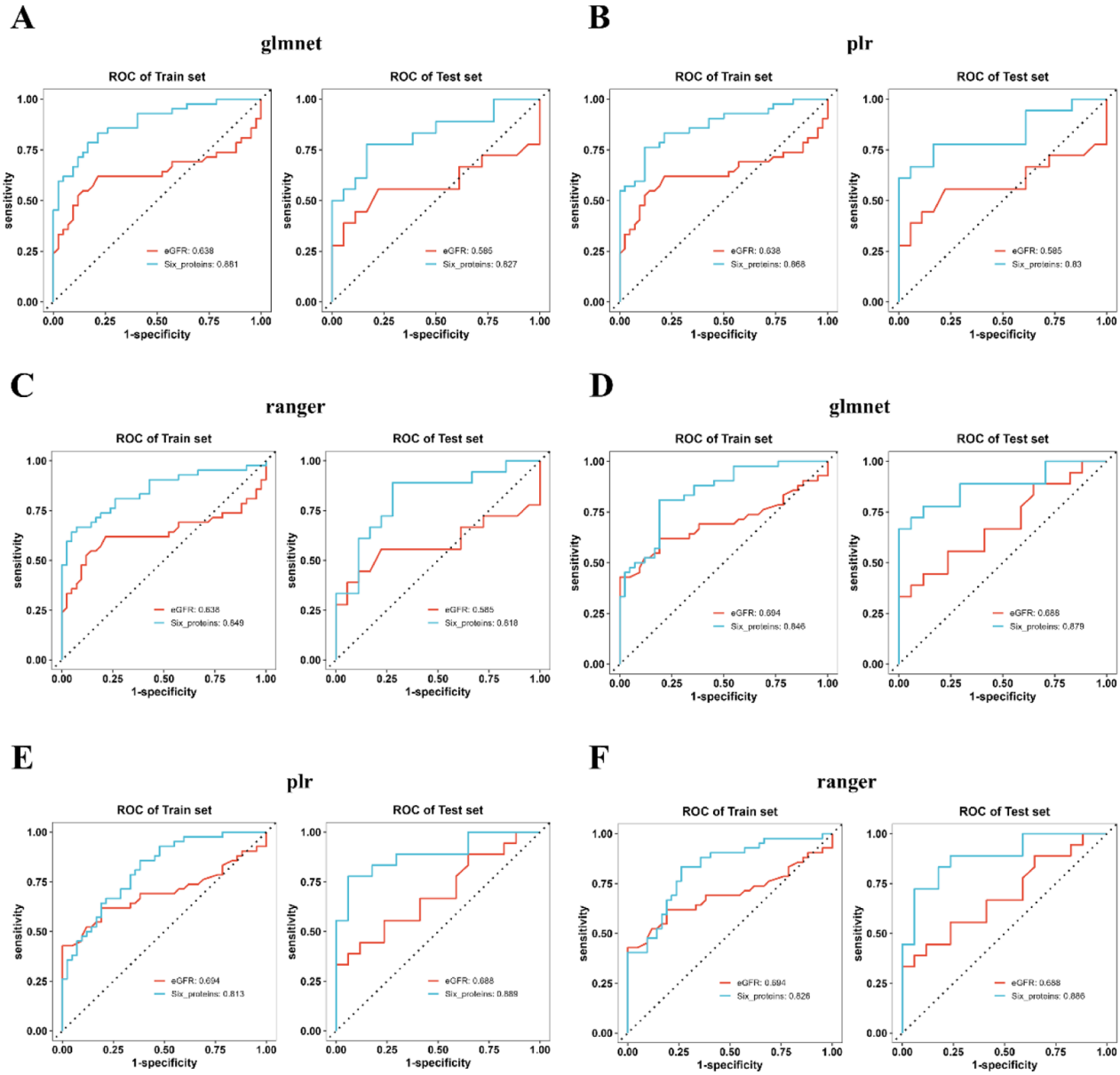

Figure 7. Comparative diagnostic performance of six urinary protein biomarkers and eGFR in diagnosing DN. (A−C) Comparative performance of six urinary protein biomarkers and eGFR in diagnosing DN vs CON (glmnet, plr, and ranger methods). (D,E) Comparative performance of six urinary protein biomarkers and eGFR in diagnosing DN vs DM (glmnet, plr, and ranger methods).

protein biomarkers, including Albumin, Transferrin, Immunoglobulins, CP, Type IV Collagen, Laminin, Glycosaminoglycan, Fibronectin, and Lipocalin-type Prostaglandin D2 Synthase. Huang et al.[22] discovered 417 peptides differing between DN

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J. Proteome Res. 2026, 25, 1292−1304

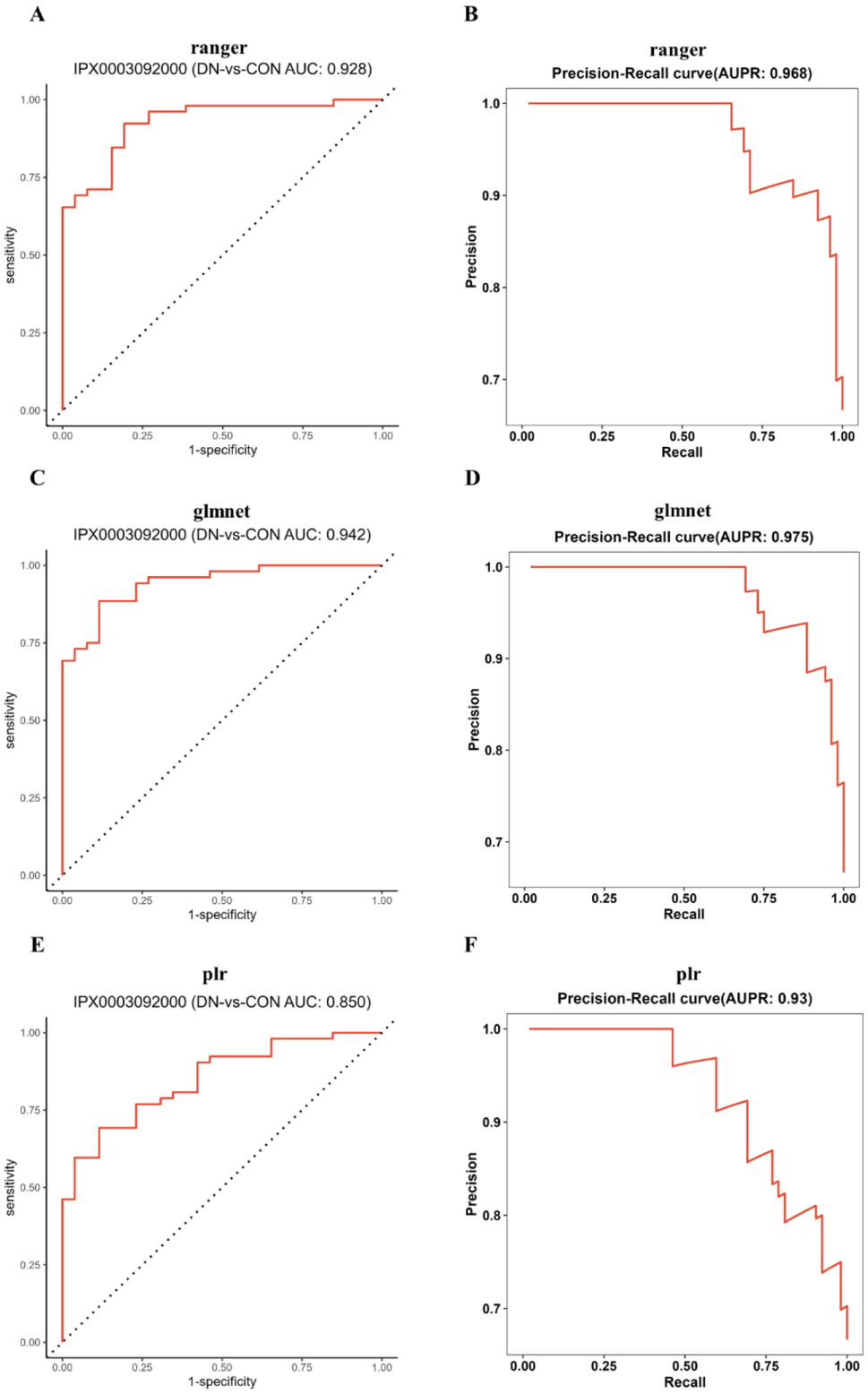

Figure 8. IPX0003092000 data set served as the validation cohort, where the performance of the DN diagnostic model was validated using three distinct machine learning classification methods (glmnet, plr, and ranger).

patients and controls. Yan et al.[16] found five potential biomarkers (KLK1, CSPG4, PLAU, SERPINA3, and ALB) in a study with T2DM patients and healthy controls.[16] Another study identified 73 low-molecular-weight proteins, with significant changes in 32, noting a decrease in V-ATPase subunit C1 and an increase in IKBIP, both linked to DN progression.[23] However, new biomarkers often lack specificity, consistent methods, or extensive validation, limiting their clinical use. This study evaluates candidate urinary protein biomarkers for diagnosing DN. Our study found 341 down-regulated and 325 up-regulated proteins in DN versus DM, suggesting significant changes as DM advances to DN.

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J. Proteome Res. 2026, 25, 1292−1304

Table 3. Performance of Six Urinary Protein Biomarkers in DN Diagnostic Models Constructed by Machine Learning Methods in the Validation Set

group	machine learning method	AUC value	specificity	sensitivity	accuracy	kappa
DN vs CON	ranger	0.928	0.712	0.846	0.756	0.504
DN vs CON	glmnet	0.942	0.712	0.962	0.795	0.593
DN vs CON	plr	0.850	0.750	0.770	0.756	0.504

Based on differentially expressed proteins identified through proteomics screening, we further analyzed the regulatory roles of these molecules in relevant signaling pathways during the progression of DN. Recent studies have identified several pathways contributing to DN development, including PPAR, MAPK, and IL-17 signaling pathways.[24] Modulating the PPAR pathway, particularly with PPAR agonists like fenofibrate, can prevent DN by affecting macrophage polarization and endothelial function.[25,26] PPARγ agonists also help reduce hyperglycemia and insulin resistance.[27] Zhang's[28] study indicates that Yishen capsule may improve DN via the NOD-like receptor pathway. Our KEGG functional enrichment analysis also confirmed the involvement of PPAR and the NOD-like receptor pathway in DN progression. Research indicates that immune and inflammatory factors are pivotal in DN's development, with innate immune responses in the renal system and immune cells driving inflammation.[29] Single-cell sequencing studies also show that DN progression involves altered cell−cell interactions.[30] GO analysis in this study suggests that DN progression may involve immune activation, disrupted cellular interactions, impaired cellular function, and altered regulation of enzyme activity.

Based on the results of urine proteomics analysis, we further screened and identified potential candidate biomarkers. To better identify potential urinary protein markers for DN, we analyzed five expression profiling data sets from the GEO database alongside this study's findings. We identified six potential biomarkers: SERPINF1, FABP4, CP, CFB, C4A, and A1BG. SERPINF1, known for its antiangiogenic properties, is linked to insulin resistance and obesity, making it a potential biomarker for T2DM.[31,32] Recent studies show that in autosomal dominant polycystic kidney disease patients, serum SERPINF1 levels negatively correlated with eGFR and are linked to renal function decline.[31] FABP4, an adipokine from macrophages and adipocytes, is also expressed in injured glomerular endothelial cells.[33] Elevated FABP4 in renal tubules correlates with poor outcomes in DN patients, suggesting urinary FABP4 as a potential biomarker for glomerular injury in diabetics.[34] CP is the main copper-carrying and binding protein in blood, challenging to filter by the glomerulus.[35] Wang et al.[35] proposed urinary CP as a potential DKD marker, needing further study for clinical use. DN pathogenesis involves excessive alternative pathway activation, with CFB being crucial, and urinary CFB levels are associated with DN severity.[36,37] A1BG, a plasma glycoprotein, may serve as a potential diagnostic biomarker for immune-modulatory and inflammatory diseases.[38] Altman et al.[39] identified eight urinary proteins, including A1BG, for early DN diagnosis and risk assessment. C4A is vital in forming immune complexes and regulating inflammation.[40] Zhang et al.[41] conducted an analysis of glycated plasma proteins in patients with type 1 diabetes exhibiting both good and poor glycemic control and reported no significant differences in C4A levels. In this study, we observed significantly elevated concentrations of SERPINF1, FABP4, CP, CFB, C4A, and A1BG in the urine of DN patients.

Next, we constructed a multivariate predictive model for the clinical diagnosis of DN based on selected candidate urinary protein biomarkers. Recently, machine learning algorithms have been increasingly used in clinical diagnostics, outperforming traditional methods by effectively handling complex data and improving predictive accuracy through large data set processing and automatic feature selection.[42,43] Yan et al.[16] identified five potential biomarkers for DN by a machine learning method and found that the AUC for KLK1 in DN diagnosis was 0.541, CSPG4 was 0.525, PLAU was 0.554, SERPINA3 was 0.531, and ALB was 0.521. However, the patient numbers included in each group of the screening cohort in Yan et al.'s[16] study were small (n = 12), and the diagnostic performance of each biomarker requires further validation. Zhang et al.[44] developed a non-invasive model using machine learning techniques like Random Forest (RF) and Support Vector Machine (SVM) to differentiate DN from non-DN in Chinese patients. The model achieved AUCROC scores of 0.953 and 0.947 for RF and SVM, respectively, with external validation scores of 0.920 and 0.911.[44] Despite its effectiveness, the model's clinical application may be limited due to the large number of variables involved, such as diabetic retinopathy, DM duration, hemoglobin, and others.[44] Our study evaluated six candidate urinary protein biomarkers that distinguish DN, with machine learning models glmnet, plr, and ranger achieving AUC values above 0.800 in both training and test sets. In the validation cohort, AUC values were 0.928 for ranger, 0.942 for glmnet, and 0.850 for plr, with improved performance likely due to the use of tissue samples instead of urine.

This study is subject to several limitations. First, the research was constrained by a small sample size and the absence of a clinical validation cohort, preventing the validation of potential biomarkers through ELISA or western blot assays in an independent cohort to confirm their clinical utility. Second, this study developed a discovery and diagnostic model using urine proteomics, validated with external data sets. While biomarkers were identified from clinical sample analyses, their mechanisms in DN progression were not explored through in vitro or in vivo experiments. Third, this retrospective single-center study from China excluded participants with comorbidities, but unmeasured confounding factors may still be present, and the study population is demographically homogeneous. Finally, although the combined detection of six potential urinary biomarkers shows better discriminatory performance than eGFR, the difference is not statistically significant. Future work will include in vitro cell experiments and animal models to explore protein biomarkers' molecular mechanisms in DN, along with multicenter clinical trials to confirm the stability and efficacy of six potential urinary biomarkers.

In conclusion, this study employed urine proteomics to evaluate six candidate urinary biomarkers (SERPINF1, FABP4, CP, CFB, C4A, and A1BG) and developed a diagnostic model for the identification of DN using a machine learning algorithm. This model is designed to assist in the diagnosis of DN, particularly for identifying DN in patients with T2DM. The findings of this study provide new insights for the clinical supplementary diagnosis of DN.

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J. Proteome Res. 2026, 25, 1292−1304

■ ASSOCIATED CONTENT

Data Availability Statement

The data reported in this paper have been deposited in the OMIX, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix: accession no. OMIX012111).

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Author Contributions

Jun Chen and Junli Gao conceived the study. Jiangen Yu, Di Zhou, and Da Li designed and conducted the experiments. Jiangen Yu, Yubo Chen, Dezhen Wang, and Xiuhong Li analyzed the data. Jiangen Yu, Fangfang Chen, and Dan Zhao wrote the manuscript. All authors contributed to manuscript revisions and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

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All participants agreed to participate in this study and signed the written informed consent to participate in this study.

All participants agreed to participate in this study and agreed to have their data published in a journal article.

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