# Detection of N-glycoprotein associated with IgA nephropathy in urine as a potential diagnostic biomarker using glycosylated proteomic analysis

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**Abstract.** The aim of the present study was to elucidate the potential diagnostic value of urinary N-glycoprotein in patients with IgA nephropathy (IgAN) using mass spectrometry (MS). All procedures were performed between June 2021 and June 2023 at Guangan People's Hospital (Guangan, China). Fresh mid-morning fasting midstream urine samples were collected from a total of 30 patients with IgAN and 30 sex- and age-matched healthy volunteers. Data acquired from 6 participants are available through ProteomeXchange with the identifier PXD041151. By comparison between the IgAN group (n=3) and healthy controls (n=3) and selection criteria of $P<0.05$ and $|\log \text{fold-change}|>2$, a total of 11 upregulated and 22 downregulated glycoproteins in patients with IgAN were identified. The results of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses suggested that glycoproteins are involved in various functions, such as the regulation of cell growth, cell adhesion, cellular component organization and protein binding, as well as multiple pathways, including p53, Notch and mTOR signaling pathways. The urine levels of afamin were further measured by ELISA in a validation cohort to assess the diagnostic performance of the single indicator model. In conclusion, MS-based proteomics of urinary glycoproteins may be an alternative option for diagnosing patients with IgAN. Biomarkers of IgAN may include, but are not limited to, CCL25, PD-L1, HLA-DRB1, IL7RD and WDR82. In addition, the levels of urinary AFM indicators are of diagnostic value for IgAN.

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## Introduction

IgA nephropathy (IgAN) is the most common type of glomerulonephritis (GN) worldwide and is now one of the leading causes of end-stage renal disease (1,2). The prevalence of IgAN varies depending on the level of biopsy techniques, socioeconomic factors, geographic and environmental influences and ethnic differences (3). IgAN has become a serious threat to individuals and a huge burden to society, particularly in countries in East Asia and the Pacific region (4-6).

Invasive renal biopsy is indispensable for both diagnosis and prognosis prediction in patients with IgAN, mainly based on positive staining for IgA-dominant immune complex deposits in the interstitium by histopathology or immunofluorescence (7,8). According to the Oxford classification, endocapillary hyperplasia, interstitial cell hyperplasia, segmental glomerulosclerosis and tubular atrophy or interstitial fibrosis are considered independent predictors of prognosis for patients with IgAN (9-11). A major finding in patients with IgAN is the presence of circulating and glomerular immune complexes, including galactose-deficient IgA1, as IgG autoantibodies against hinge region O-glycans, and complement C3 (12). On the one hand, IgA-containing complexes cause renal damage mainly through glomerular inflammation and thylakoid hyperplasia. On the other hand, abnormal activation of the renin-angiotensin-aldosterone system and complement also leads to glomerulosclerosis, tubulointerstitial fibrosis and eventual loss of renal function (13,14). The clinicopathological manifestations of IgAN vary from asymptomatic urinary abnormalities, proteinuria, hypertension, reduced glomerular filtration rate (GFR) and nephrotic syndrome to progressive GN (14,15). Renal outcomes in patients with IgAN are related to the histological grade and findings at the time of diagnosis (10,16-18). Early detection, early diagnosis and early intervention are necessary and important measures in patients with IgAN. However, most patients with IgAN have a chronic onset and feature reduced GFR, mild to moderate proteinuria, persistent microhematuria and hypertension at the time of first diagnosis. Certain long-term follow-up studies have estimated

that 25% of patients with IgAN develop renal failure after 10 years and up to 50% after 30 years (19,20). Currently, there is still a lack of simple, feasible and non-invasive methods as alternative options to renal biopsy for the diagnosis, classification and prognosis prediction of IgAN.

Proteins and peptides have critical roles, with multifunctional and physical properties as hormones, enzymes, channels or transporters (21). During post-translational modifications, glycoproteins are modified by N- and O-linked glycosylation through the attachment of sugars to asparagine or serine (Ser)/threonine (Thr) residues (22,23). The two most common types of glycosylation are O-glycosylation and N-glycosylation. Eukaryotic O-glycosylation indicates the attachment of sugars to Ser or Thr residues by enzymes of the endoplasmic reticulum and Golgi apparatus. N-glycosylation in eukaryotes indicates the attachment of N-glycans to N-X-S/T/C sequences. Glycosylated proteins have a variety of integral roles in physiological regulation, such as DNA damage repair, cell growth, cell migration and immune responses, as well as pathophysiological conditions, including cancer, neurodegenerative diseases, cardiometabolic disorders and immune disorders (24-26). Previous studies have indicated that abnormal glycosylated glycoproteins are aberrantly expressed in serum and excreted into the urine with reduced renal selective permeability in patients with IgAN (27-30). Glycoproteins may have the potential to be used as diagnostic markers for IgA nephropathy.

Mass spectrometry (MS) is an advanced experimental high-throughput technique that allows researchers to explore biomolecules (22). Glycoproteomics is an MS-based proteomics approach that has been used to identify glycoproteins involved in physiological processes, signaling pathways and pathological development (25). High-throughput site- and structure-specific characterization of different N-glycosylations under pathological conditions using MS-based N-glycoproteomics has become a common approach for the discovery of putative disease biomarkers. Recently, it has further been found to be involved in the regulation of various renal diseases, including diabetic kidney disease, IgAN, renal cell carcinoma, lupus nephritis and glomerular basement membrane resistant disease (31). Abnormal glycoproteins are excreted into the urine, which may be collected noninvasively and are a good source for patients with renal disease. Therefore, it is important to perform glycoproteomics analysis of the urine of patients with IgAN to find unique glycosylated proteins as specific diagnostic biomarkers for IgAN. In the present study, the urine of patients with IgAN was examined to identify potential urinary proteins as specific biomarkers of IgAN.

## Materials and methods

*Materials*. Reagents for glycoproteomics sample preparation were used as follows. The bicinchoninic acid assay kit was purchased from Real-Times Biotechnology Co. Coomassie Brilliant Blue staining solution was obtained from Wuhan Servicebio. N-glycosaminidase was obtained from New England Biolabs Co. Liquid chromatography tandem mass spectrometry (LC-MS/MS) grade ultrapure water, dithiothreitol (DTT), iodoacetamide, sequencing grade trypsin, LC-MS grade acetonitrile (ACN) and LC-MS grade formic acid were obtained from Thermo Fisher Scientific, Inc. Hydrop interaction liquid chromatography (HILIC) columns and Venusil® HILIC were purchased from Agela Technologies. $H_2{}^{18}O$ (also called water-$^{18}O$) reagents and other chemicals were purchased from Sigma-Aldrich (Merck KGaA). ProteoMiner™ enrichment kits for concentrating low-abundance proteins were purchased from Bio-Rad Laboratories, Inc. In addition, acetone was purchased from Xilong Scientific Chemical Co., Ltd. and water-saturated phenol was obtained from Shanghai Zeya Biological Co., Ltd.

*Study population and procedures*. This study was conducted at the Department of Nephrology, Guangan People's Hospital (Guangan, China). All participants were enrolled between June 2021 and June 2023.

The inclusion criteria were as follows: i) IgAN was defined as immunofluorescence and light microscopic IgA deposition in the thylakoid region with or without capillary rings, combined with or without IgG, IgM and C3 deposition according to the Kidney Diseases: Improving Global Outcomes guidelines (32). IgAN caused by other diseases was excluded by clinical manifestations and other laboratory findings. ii) Subjects were selected from patients with IgAN confirmed by first renal biopsy at our institution, without glucocorticoid and immunosuppressive therapy. iii) Patients agreed to participate in this study and signed an informed consent form.

The exclusion criteria were as follows. i) IgAN complicated by membranous nephropathy, light chain deposition, thin basement membrane nephropathy and other types of renal disease. ii) Pathological changes of IgAN caused by other diseases, such as systemic lupus erythematosus, Henoch-Schonlein purpura nephritis or hepatitis B virus-associated nephritis. iii) IgAN complicated by severe cardiopulmonary disease, or tumors (33).

Finally, 30 patients diagnosed with IgAN without other renal disease were recruited as the IgAN group. Another 30 sex- and age-matched healthy volunteers were also recruited as a healthy control group. These healthy volunteers were individuals who underwent physical examinations at our hospital with no history of renal disorder. They were further screened for kidney disease by detailed clinical and laboratory examinations, such as creatinine, urinary protein, blood pressure and urinalysis. No trace amounts of blood or protein were detected of these healthy volunteers at study entry.

The trial was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Guangan People's Hospital (Guangan, China; approval no. 2021009). This committee supervised the whole process of project implementation. All participants signed an informed consent form before enrollment. General data and medical information were collected from the participants.

*Collection and preservation of urine*. Approximately 20 ml of fresh mid-morning fasting urine samples were collected at the time of biopsy from patients with IgAN prior to receiving hormonal or immunosuppressive therapy during hospitalization. Similarly, ~20 ml of fresh mid-morning fasting urine samples were collected in healthy volunteers. All samples were collected in sterile containers, placed at room temperature,

centrifuged at 2,000 x g for 20 min over 1 h, and the supernatant was separated to remove debris and cells. The centrifuged urine samples were then stored at -80˚C for further analysis.

*Urine sample preparation and procedures*. Novogen was commissioned to perform all procedures, including total proteins extraction, protease digestion and HILIC enrichment of glycopeptides, as well as bioinformatics analysis.

*Extraction of total protein*. Urine samples from three patients with IgAN and three healthy controls were removed from the -80˚C refrigerator and thawed rapidly at room temperature. Each sample (15 ml) was ultrafiltered and concentrated to ~1 ml using a 10KD centrifugation device. The retentate of the concentrated samples was transferred to a 1.5-ml centrifuge tube. RIPA lysis buffer (100 mM ammonium bicarbonate/8 M urea, 0.2% SDS, pH=8) was added to this filtrate for protein lysis. After mixing the top and bottom phases in the centrifuge tube, the mixture was placed on ice and sonicated for 8 min. The solution was pre-cooled at 4˚C and centrifuged at 12,000 x g for 20 min. Subsequently, the supernatant was transferred into a new 1.5-ml centrifuge tube, DTTred at a concentration of 10 mM was added and the mixture placed at 56˚C for 1 h. A sufficient amount of iodoacetamide was added and the sample was incubated in the dark for 1 h. 4 times volume of precooled acetone at -20˚C was then added to the tubes. The samples were centrifuged at 12,000 x g for 15 min and the precipitate was collected. Then, 1 ml precooled acetone at -20˚C was further added to the tubes for resuspension. The samples were again centrifuged at 12,000 x g for 15 min and the precipitate was collected and air-dried at room temperature. Finally, a solution [6 M urea, 100 mM triethylammonium bicarbonate (TEAB), pH=8.5] was added to dissolve the precipitate for subsequent MS assay.

*Protease digestion and HILIC enrichment of glycopeptides*. An appropriate amount of protein lysate (8 M urea, 100 mM TEAB, pH=8.5) was added to the lysed protein samples, not exceeding 500 $\mu$l in total. After initial digestion with sequencing-grade trypsin for 4 h, a secondary digestion with a combination of sequencing-grade trypsin and $CaCl_2$ enzyme was subsequently performed overnight. The digested samples were acidified with formic acid and centrifuged at 12,000 x g for 20 min at 4˚C in an ultracentrifuge. The supernatant was washed by 0.1% formic acid with 3% acetonitrile three times with a C18 column (Peptide Desalting Spin Columns; cat. no. 89852; Pierce™; Thermo Fisher Scientific, Inc.). Next, the eluate (0.1% formic acid with 70% acetonitrile) was added to this C18 column for collection of the eluate, which was then lyophilized. The peptide-containing sample was then redissolved in 80% ACN and 0.1% trifluoroacetic acid (TFA), passed through the HILIC column, rinsed with 0.1% TFA and dried thermally at 45˚C with vacuum treatment. The precipitate was then suspended in 50 mM ammonium bicarbonate buffer dissolved in $H_2{}^{18}O$. 2 $\mu$l Peptide-N-glycosidase F (cat. no. 11365169001; Merck KGaA) dissolved in $H_2{}^{18}O$ was added. The mixture was shaken overnight at 37˚C and dried thermally at 45˚C with vacuum treatment.

*LC-MS*. Phase A (0.1% formic acid and 2% ACN aqueous solution) and phase B (0.1% formic acid and 95% ACN aqueous solution) were prepared for LC-MS. The thawed dry powder was phase-solubilized with the appropriate amount of solution A and centrifuged at 13,400 x g for 5 min at room temperature. From each sample, 1 $\mu$g of supernatant was used for quality assurance. An EASY-nLC™ upgraded ultra-performance LC system (Thermo Fisher Scientific, Inc.) with an in-house made autonomous pre-column (2 cm x75 $\mu$m x3 $\mu$m) and a home-made autonomous analytical column (15 cm x150 $\mu$m x1.9 $\mu$m) was used, using a linear gradient elution as listed in Table I. Q Exactive™ HF-X (Thermo Fisher Scientific, Inc.) and Nanospray Flex™ (Thermo Fisher Scientific, Inc.) positive and negative electron spray ionization ion sources were used. The ion spray voltage was set to 2.3 kV and the temperature of the ion transport tube was set to 320˚C. The mass spectra were acquired in data-dependent acquisition mode with a scan range of 350-1,500 m/z. The primary and secondary MS resolution, maximum volume and maximum injection time were set separately for the final generation of raw MS data.

Table I. Liquid chromatography elution gradient table.

| Time, min | Flow rate, nl/min | Mobile phase A, % | Mobile phase B, % |
|---|---|---|---|
| 0 | 600 | 95 | 5 |
| 2 | 600 | 90 | 10 |
| 107 | 600 | 60 | 40 |
| 112 | 600 | 50 | 50 |
| 115 | 600 | 10 | 90 |
| 120 | 600 | 0 | 100 |

Identification and MS quantification were performed using Mascot 2.5 software (Matrix Science Ltd.) and Proteome Discoverer 2.5 software (Thermo Fisher Scientific, Inc.). Independent samples t-tests were performed to investigate the differences in protein expression levels between the IgAN (n=3) group and the healthy control group (n=3). The fold change (FC) was calculated as the relative ratio of the mean protein expression levels in the urine samples of the two groups. Differentially expressed urinary glycoproteins were selected using |logFC|>2 as screening criteria. $P<0.05$ was considered to indicate a statistically significant difference, while $P<0.01$ was considered to indicate a highly statistically significant difference.

*Bioinformatics analysis*. Differential glycoproteins were analyzed through the DAVID database (http://david.abcc.ncifcrf.gov/). Gene Ontology (GO) functional prediction analysis was performed, including functional terms in the categories biological process (BP), cellular component (CC) and molecular function (MF). Furthermore, the DAVID database was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Protein-protein interaction network of differential glycoproteins was visualized in the String DB database (https://cn.string-db.org/).

*Determination of afamin (AFM) in urine*. The expression level of AFM in urine of both 30 healthy controls and 30 patients with IgAN were determined by ELISA. The Human Afamin DuoSet ELISA kit was acquired from Novus Biologicals

Table II. Characteristics of the enrolled participants for glycosylation proteome analysis.

| Characteristic | IgAN1 | IgAN2 | IgAN3 | HC1 | HC2 | HC3 |
|---|---|---|---|---|---|---|
| Age, years | 24 | 55 | 35 | 41 | 29 | 34 |
| Sex | F | M | F | F | F | F |
| Creatinine, $\mu$mol/l | 67 | 183 | 63 | 84 | 68 | 78 |
| Urea nitrogen, $\mu$mol/l | 5.46 | 11.5 | 5.37 | 5.6 | 5.4 | 6.0 |
| Hemoglobin, g/l | 139 | 142 | 137 | 125 | 127 | 130 |
| CRP, mg/l | 0.56 | 0.32 | 0.23 | 0 | 0 | 0 |
| Urine protein | +- | + | 3+ | - | - | - |
| 24-h urinary protein, g | 2.24 | 0.51 | 1.08 | - | - | - |
| Urine numbers of microscopic erythrocytes, /HP | 8-12 | 0 | 2-6 | - | - | - |
| Serum IgA, g/l | 3.06 | 3.58 | 2.32 | - | - | - |
| Serum IgE, IU/ml | 76.5 | 42.2 | 5 | - | - | - |
| Mesangial hyperplasia | Yes | Yes | Yes | - | - | - |
| IgA mesangial deposition | Yes | Yes | Yes | - | - | - |
| Degree of segmental hardening, % (n/total) | 21.4 (3/14) | 35.2 (6/17) | 44.80 (13/29) | - | - | - |

IgAN, IgA nephropathy; IgAN1/2/3, cases 1/2/3 from IgAN group; M, male; F, female; HC, healthy control; CRP, C-reactive protein; HP, per high-power field of view.

Corp. (cat. no. DY8065-05). All procedures were performed according to the manufacturer's protocol.

*Statistical analysis*. For sample estimation, since similar data could not be found in published articles, a power analysis was performed with PASS 15 Power Analysis and Sample Size Software (2017) (NCSS, LLC) based on our results. A 1-β value of >0.8 was considered a criterion for the target power.

Values are expressed as mean ± SEM. Comparisons between two groups were assessed by unpaired Student's t-test (if continuous variables) and chi-square ($\chi^2$) test (if categorical variables). Statistical analysis was performed by SPSS version 19.0 (IBM Corp.). The receiver operating characteristic (ROC) curve was generated and the area under the ROC curve (AUC) was determined by SPSS software version 19.0 (IBM Corp.) based on the results of AFM expression levels in urine. P<0.05 was considered to indicate statistical significance.

## Results

*Data quality of urinary glycoprotein from IgAN and healthy controls*. Proteins and peptides were analyzed from the urine of patients with IgAN (n=3) and healthy volunteers (n=3) and used for subsequent MS analysis. The clinical characteristics of the above participants are listed in Table II. The age of enrolled patients with IgAN and healthy controls was 24, 55 and 35 years, as well as 41, 29, 34 years, respectively. Their corresponding sex was female, male, female, and female, female, female. Sex and age of these six enrolled participants in the IgAN group and the healthy control group was compared with respectively the $\chi^2$ test and t-test. No significant differences were found in both sex and age in 2 groups (P>0.05). A flowchart of the MS data quality analysis is provided in Fig. 1A. The results of the peptide information recombination indicated that the protein mass of the peptides detected in the samples ranged from 8-23 amino acids, with the peak of the mass precursor ion tolerance close to 0 (Fig. 1B and C). The results of the protein information recombination further suggested that the number of unique peptide sequences contained in all identified proteins as plausible proteins ranged from ~1 to 10 in their relative proportions shown (Fig. 1D). A total of 851 proteins and peptides were obtained, while the protein coverage was >1% (Fig. 1E). The molecular weight of the proteins was mainly between 10 and 20 kDa (Fig. 1F). Therefore, the data from the protein analysis in this experiment were indicated to be accurate and reliable.

*Identification of differentially expressed urinary glycoproteins*. Urine samples from patients with IgAN (n=3) and healthy controls (n=3) were examined by the data-dependent acquisition LC-tandem MS label-free quantitative technique. A total of 851 N-glycoproteins were detected in the six urine samples mentioned above, of which 169 differential N-glycoproteins were found (Fig. 2A). Cluster analysis of the IgAN and healthy groups revealed high similarity of data patterns within the groups and low similarity of data patterns between the groups (Fig. 2B), both of which further justified the screening of differential proteins.

Compared to healthy controls, the IgAN group had 37 glycoproteins, of which 11 glycoproteins were specifically expressed in IgAN, including C4BPA, CABLES2, DSC2, FAM171A1, FAP, FOLR2, NFIB, SLC2A13, SLCO4C1, UNQ172 and WDR82 (Table III). Meanwhile, a total of 132 glycoproteins were downregulated in the IgAN group

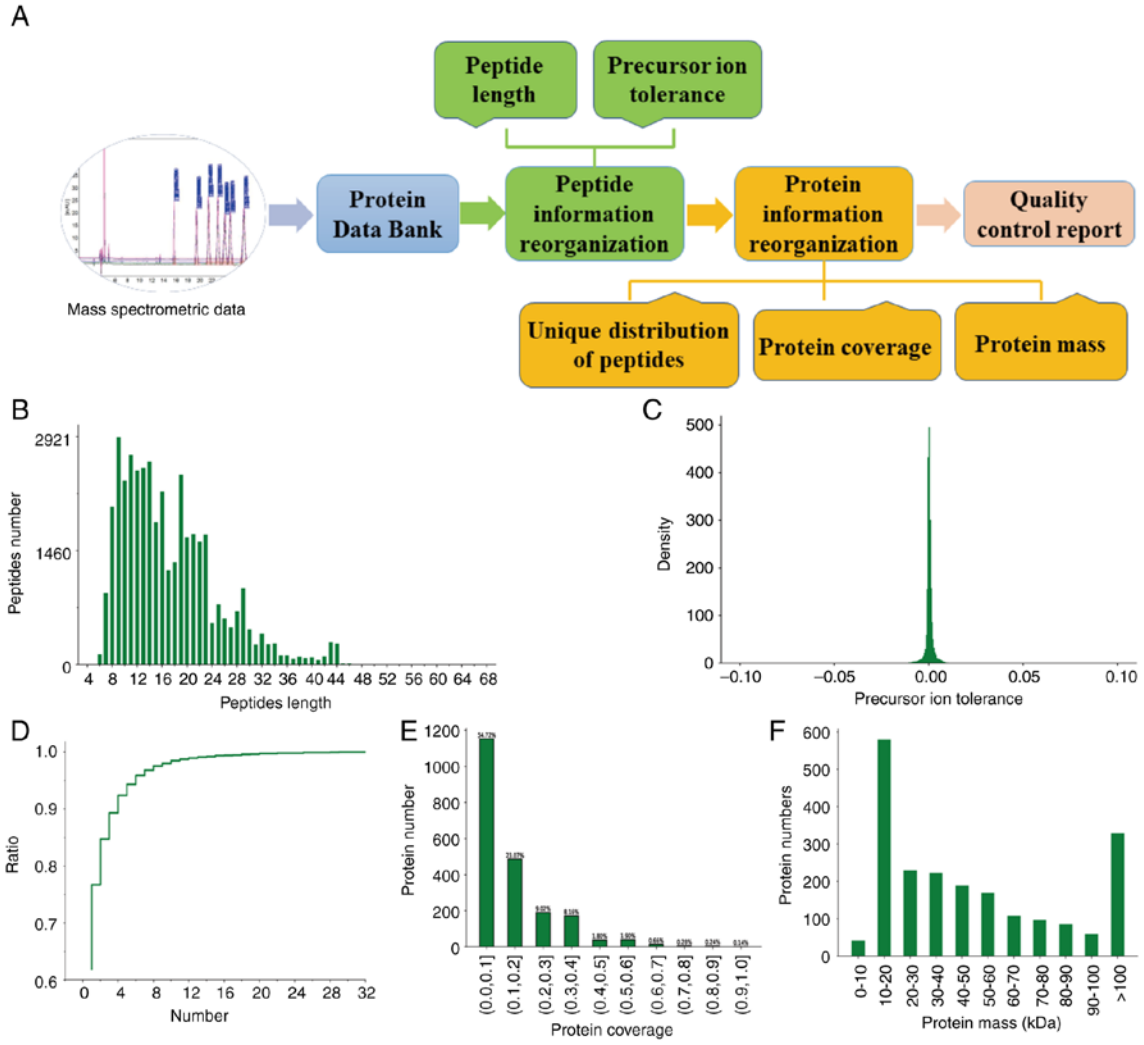


Figure 1. Data quality of urine glycoproteins from the IgAN group and healthy controls. (A) Flow chart of data quality from mass spectrometry. (B) Peptide length and relative peptide number in samples. (C) Density and precursor ion tolerance. (D) Number and relative proportion of unique peptides. (E) Protein coverage and protein number in samples. (F) Protein mass and protein number (n=3 per group). IgAN, IgA nephropathy.

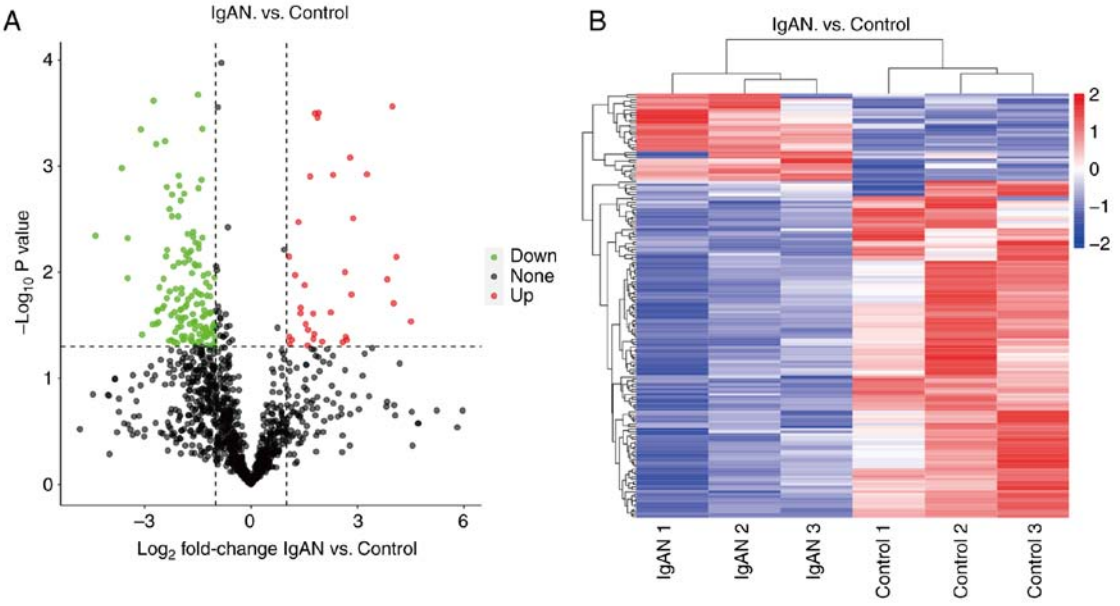


Figure 2. Identification of differentially expressed urinary glycoproteins in IgAN and healthy control groups using |log2 fold change|>2 and P<0.05 as filter criteria. (A) Visualization of the volcano plot in two groups. (B) Heat map with clustering of differential gene expression in the two groups (n=3 per group). IgAN, IgA nephropathy.

Table III. Upregulated N-glycoproteins (n=11) in IgAN vs. healthy controls.

| Protein | Gene | IgAN1 | IgAN2 | IgAN3 | HC1 | HC2 | HC3 |
|---|---|---|---|---|---|---|---|
| P04003 | C4BPA | $4.597x10^6$ | $1.184x10^6$ | $9.895x10^6$ | 0 | 0 | 0 |
| H0Y5H2 | CABLES2 | $2.116x10^5$ | $2.917x10^5$ | $2.589x10^5$ | 0 | 0 | 0 |
| A9X9L1 | DSC2 | $1.106x10^6$ | $8.126x10^5$ | $1.304x10^6$ | 0 | 0 | 0 |
| Q5VUB5 | FAM171A1 | $1.556x10^5$ | $4.516x10^4$ | $2.113x10^4$ | 0 | 0 | 0 |
| C9J131 | FAP | $3.034x10^5$ | $1.652x10^5$ | $2.427x10^5$ | 0 | 0 | 0 |
| F5H3Z4 | FOLR2 | $5.122x10^5$ | $2.554x10^5$ | $1.587x10^5$ | 0 | 0 | 0 |
| O00712 | NFIB | $2.869x10^5$ | $7.288x10^5$ | $6.267x10^5$ | 0 | 0 | 0 |
| Q96QE2 | SLC2A13 | $2.868x10^5$ | $1.313x10^5$ | $2.685x10^5$ | 0 | 0 | 0 |
| Q6ZQN7 | SLCO4C1 | $4.135x10^5$ | $1.348x10^5$ | $2.590x10^5$ | 0 | 0 | 0 |
| Q6UY50 | UNQ172 | $6.541x10^5$ | $2.338x10^5$ | $6.071x10^5$ | 0 | 0 | 0 |
| Q6UXN9 | WDR82 | $3.162x10^5$ | $6.517x10^5$ | $4.647x10^5$ | 0 | 0 | 0 |

All values are relative quantitative values IgAN, IgA nephropathy; IgAN1/2/3, cases 1/2/3 from IgAN group; HC, healthy control.

Table IV. Downregulated glycoproteins (n=22) in IgAN vs. healthy controls.

| Protein | Gene | IgAN1 | IgAN2 | IgAN3 | HC1 | HC2 | HC3 |
|---|---|---|---|---|---|---|---|
| A0A087X0I3 | ADA2 | 0 | 0 | 0 | $7.607x10^5$ | $1.089x10^6$ | $3.845x10^5$ |
| Q9UKQ2 | ADAM28 | 0 | 0 | 0 | $6.702x10^5$ | $2.751x10^6$ | $7.011x10^5$ |
| A0A1B0GTY3 | ADAMTS2 | 0 | 0 | 0 | $6.190x10^6$ | $9.887x10^6$ | $7.362x10^6$ |
| Q5VTW1 | ADAMTS4 | 0 | 0 | 0 | $7.387x10^5$ | $8.900x10^5$ | $7.367x10^5$ |
| Q08AK2 | CCL25 | 0 | 0 | 0 | $2.924x10^6$ | $4.164x10^6$ | $2.077x10^6$ |
| Q9NZQ7 | CD274 | 0 | 0 | 0 | $4.101x10^5$ | $2.571x10^5$ | $3.432x10^5$ |
| P39060 | COL18A1 | 0 | 0 | 0 | $1.259x10^5$ | $3.497x10^5$ | $9.608x10^5$ |
| P07585 | DCN | 0 | 0 | 0 | $1.899x10^6$ | $1.144x10^6$ | $8.998x10^5$ |
| A0A141AXF5 | FGFR2 | 0 | 0 | 0 | $2.356x10^5$ | $3.132x10^5$ | $3.644x10^5$ |
| P56159 | GFRA1 | 0 | 0 | 0 | $3.010x10^5$ | $5.502x10^5$ | $7.028x10^5$ |
| Q3LA85 | HLA-DRB1 | 0 | 0 | 0 | $4.852x10^5$ | $1.294x10^6$ | $1.461x10^5$ |
| P16871 | IL7R | 0 | 0 | 0 | $7.330x10^5$ | $3.957x10^5$ | $1.978x10^5$ |
| Q6UXK2 | ISLR2 | 0 | 0 | 0 | $2.039x10^5$ | $1.591x10^5$ | $2.138x10^5$ |
| O75197 | LRP5 | 0 | 0 | 0 | $6.139x10^4$ | $5.514x10^5$ | $2.122x10^5$ |
| O75086 | MMP23B | 0 | 0 | 0 | $5.638x10^5$ | $7.407x10^5$ | $6.469x10^5$ |
| Q6UXH9 | PAMR1 | 0 | 0 | 0 | $4.697x10^5$ | $3.056x10^5$ | $2.900x10^5$ |
| Q5FWE3 | PRRT3 | 0 | 0 | 0 | $3.049x10^5$ | $3.480x10^5$ | $4.729x10^5$ |
| M0QZH0 | RCN3 | 0 | 0 | 0 | $6.473x10^5$ | $1.363x10^5$ | $4.489x10^5$ |
| Q969Z4 | RELT | 0 | 0 | 0 | $2.317x10^5$ | $7.763x10^5$ | $5.149x10^5$ |
| C9J1V2 | SEMA3F | 0 | 0 | 0 | $3.541x10^5$ | $7.040x10^5$ | $5.402x10^5$ |
| Q9H173 | SIL1 | 0 | 0 | 0 | $1.917x10^5$ | $9.445x10^5$ | $2.743x10^5$ |
| Q8TER0 | SNED1 | 0 | 0 | 0 | $2.438x10^5$ | $1.948x10^5$ | $2.602x10^5$ |

IgAN, IgA nephropathy; IgAN1/2/3, cases 1/2/3 from IgAN group; HC, healthy control.

compared with the healthy controls, of which 22 glycoproteins were specifically expressed in IgAN, including ADA2, ADAM 28, ADAMTS2, ADAMTS4, CCL25, CD274 molecule [CD274, also known as programmed death-ligand 1 (PD-L1)], COL18A1, DCN, FGFR2, GFRA1, HLA-DRB1, IL7R, ISLR2, LRP5, MMP23B, PAMR1, PRRT3, RCN3, RELT, SEMA3F, SIL1, SNED1 (Table IV).

Based on the cutoff criteria of $P<0.05$ and $|logFC|>2$ by comparison of the IgAN group and healthy controls, the top 5 significantly differentially expressed proteins between the two groups were listed. The top 5 upregulated differential glycoproteins are CP, CNDP1, BCHE, GPLD1 and AFM. Meanwhile, the top 5 downregulated differential glycoproteins were PTPRK, NRXN1, LAMA2, EFNA4 and PCDH19 (Table V).

Table V. Top 5 differentially expressed glycoproteins between IgAN compared with healthy controls.

| Protein | Gene | IgAN1 | IgAN2 | IgAN3 | HC1 | HC2 | HC3 | IgAN vs. HC P-value | IgAN vs. HC $log_2FC$ | Direction of differential expression of IgAN vs. HC |
|---|---|---|---|---|---|---|---|---|---|---|
| A5PL27 | CP | $7.326x10^8$ | $4.178x10^8$ | $7.332x10^8$ | $2.404x10^7$ | $2.459x10^7$ | $3.420x10^7$ | 0.029 | 4.507 | Up |
| Q96KN2 | CNDP1 | $1.770x10^7$ | $1.760x10^7$ | $2.258x10^7$ | $8.670x10^5$ | $1.593x10^6$ | $9.144x10^5$ | 0.007 | 4.100 | Up |
| F8WF14 | BCHE | $2.221x10^7$ | $2.632x10^8$ | $1.598x10^7$ | $5.090x10^5$ | $1.418x10^6$ | $2.036x10^6$ | 0.020 | 4.025 | Up |
| P80108 | GPLD1 | $3.145x10^7$ | $3.018x10^7$ | $3.830x10^7$ | $1.786x10^6$ | $3.259x10^6$ | $1.259x10^6$ | <0.001 | 3.986 | Up |
| P43652 | AFM | $5.535x10^8$ | $4.248x10^8$ | $6.108x10^8$ | $3.505x10^7$ | $4.663x10^7$ | $2.931x10^7$ | 0.012 | 3.840 | Up |
| Q15262 | PTPRK | $1.761x10^5$ | $1.237x10^5$ | $1.293x10^5$ | $2.608x10^6$ | $3.105x10^6$ | $3.264x10^6$ | 0.004 | -4.387 | Down |
| A0A1B0GVF4 | NRXN1 | $1.009x10^6$ | $7.591x10^5$ | $6.057x10^5$ | $1.057x10^7$ | $7.539x10^6$ | $8.374x10^6$ | 0.011 | -3.480 | Down |
| A0A087WX80 | LAMA2 | $6.116x10^5$ | $2.755x10^5$ | $1.531x10^5$ | $2.678x10^6$ | $4.665x10^6$ | $4.246x10^6$ | 0.005 | -3.478 | Down |
| P52798 | EFNA4 | $1.010x10^6$ | $2.692x10^5$ | $1.514x10^5$ | $3.683x10^6$ | $4.228x10^6$ | $4.377x10^6$ | <0.001 | -3.102 | Down |
| Q8TAB3 | PCDH19 | $7.577x10^5$ | $1.001x10^6$ | $4.406x10^5$ | $4.400x10^6$ | $8.282x10^6$ | $5.793x10^6$ | 0.039 | -3.070 | Down |

IgAN, IgA nephropathy; IgAN1/2/3, cases 1/2/3 from IgAN group; HC, healthy control; FC, fold change.

*Differential GO analysis of glycoproteins in urine*. GO annotations of all identified N-glycoproteins in the IgAN group and control group, in the categories CC, BP and MF, are provided in Fig. 3A. In addition, 169 differentially expressed proteins were classified as CC, BP and MF by GO functional enrichment analysis using the DAVID bioinformatics database (Fig. 3B-D). The results indicated that the differential N-glycoproteins of the IgAN group were mainly enriched in the membrane, as well as extracellular, intracellular and nuclear domains (Fig. 3A). The main function of downregulated N-glycoproteins in the category BP was regulating cell growth and cell adhesion, while that of the upregulated N-glycoprotein was prenylcysteine catabolic process (Fig. 3B and C). The main functional term in the category MF of downregulated N-glycoproteins was binding, including proteins and insulin-like growth factors, while that of the upregulated N-glycoprotein was transporter activity (Fig. 3B and C). According to the overall analysis in the category BP, differentially expressed glycoproteins had the main functions of regulating cell growth, cell adhesion and cellular component organization. In addition, according to the analysis in the category MF, differentially expressed N-glycoproteins had predicted functions of protein binding, insulin-like growth factor binding and other types of binding (Fig. 3D).

*KEGG analysis of differential urinary glycoproteins*. Further KEGG enrichment analysis was performed to examine the enrichment of proteomic pathways (Fig. 4). Of the 169 differentially expressed N-glycoproteins, the majority were significantly associated with metabolic pathways, indicating the metabolism of tryptophan, histidine, biotin, arginine and proline, as well as purines. In addition, a certain proportion of the proteins were enriched in the adipocytokine signaling pathway, p53 signaling pathway, Hedgehog signaling pathway, Notch signaling pathway, mTOR signaling pathway and Rap1 signaling pathway, as well as signaling pathways regulating stem cell pluripotency. In addition, certain N-glycoproteins were classified into type 1 T-helper (Th1) and Th2 cell differentiation, osteoblast differentiation, bladder cancer, malaria, extracellular matrix (ECM)-receptor interactions, axon guidance and cell adhesion molecules (CAMs).

*Subcellular localization of glycoproteins*. The subcellular localization of the 169 differential glycoproteins was analyzed (Fig. 5). The results indicated that 40.94% of the differential glycoproteins were classified as extracellular proteins, 38.01% as plasma membrane proteins, 7.02% as cytoplasmic proteins, 5.85% as lysosomal proteins, 4.68% as endoplasmic reticulum proteins and 3.51% as nuclear proteins.

*Interaction analysis of IgAN-related differentially expressed glycoproteins*. The interaction analysis of IgAN-related differentially expressed glycoproteins was performed using the String database for visualization (http://string-db.org/). Nodes of different colors indicate individual proteins and lines of different thicknesses indicate the strength of the interaction between two different proteins. Among 169 different glycoproteins, most of the N-glycoproteins were interconnected (Fig. 6A). Among the 11 upregulated specific differential

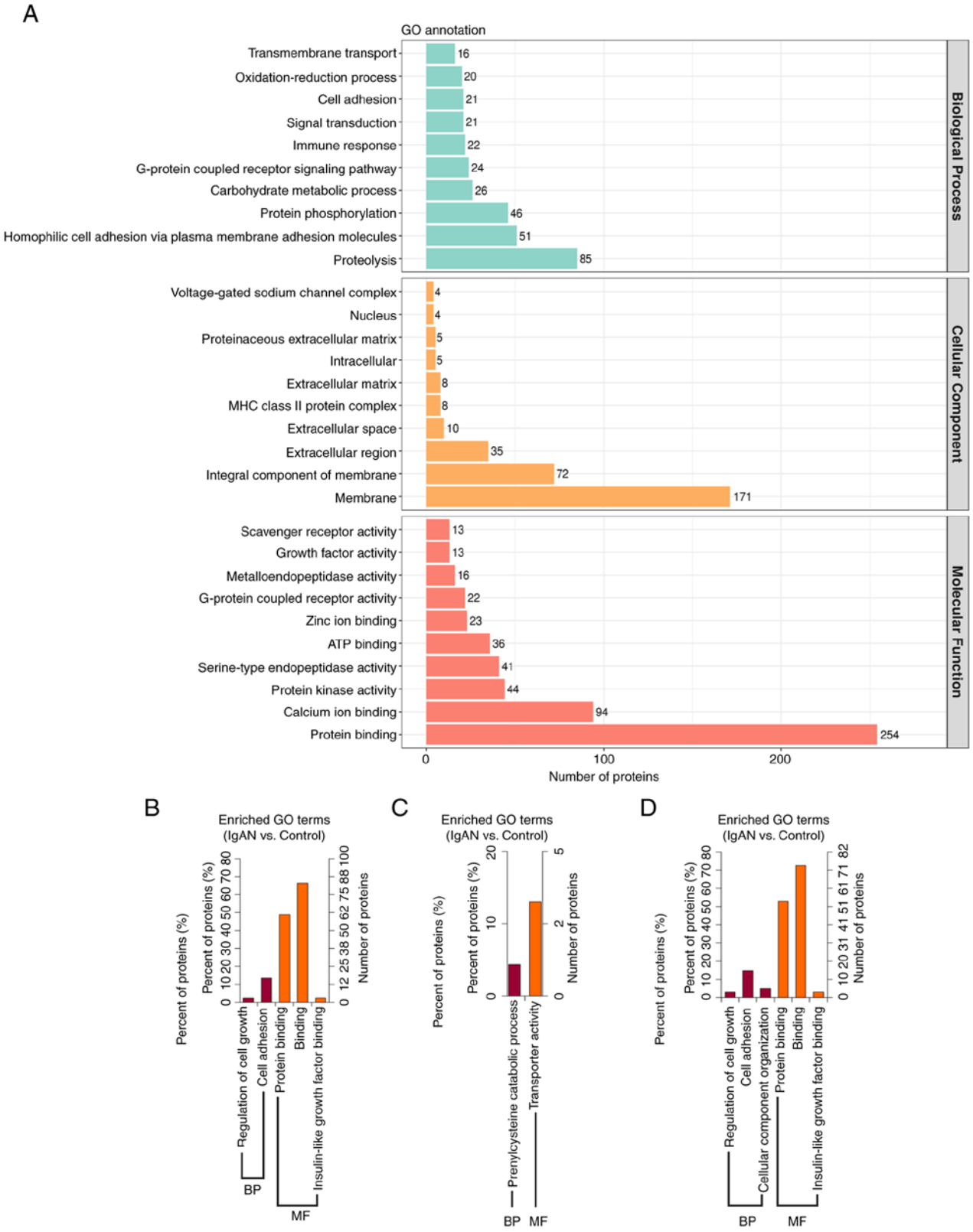


Figure 3. GO analysis of functional N-glycoproteins in IgAN vs. healthy controls. (A) GO annotation of functional N-glycoproteins detected in IgAN vs. healthy controls. (B) GO analysis of downregulated differential urine N-glycoproteins in IgAN. (C) GO analysis of upregulated differential urine N-glycoproteins in IgAN. (D) GO analysis of overall differential urine N-glycoproteins in IgAN (n=3 per group). IgAN, IgA nephropathy; GO, Gene Ontology; BP, biological process; MF, molecular function.

glycoproteins, CDK5 interacts with CABLES2 (Fig. 6B). Furthermore, among the 22 downregulated specific differential glycoproteins, 10 were interlinked (Fig. 6C).

*Urine concentration of AFM by ELISA in the validation cohort.* The expression levels of AFM in the urine of 30 healthy controls and 30 patients with IgAN were then measured by

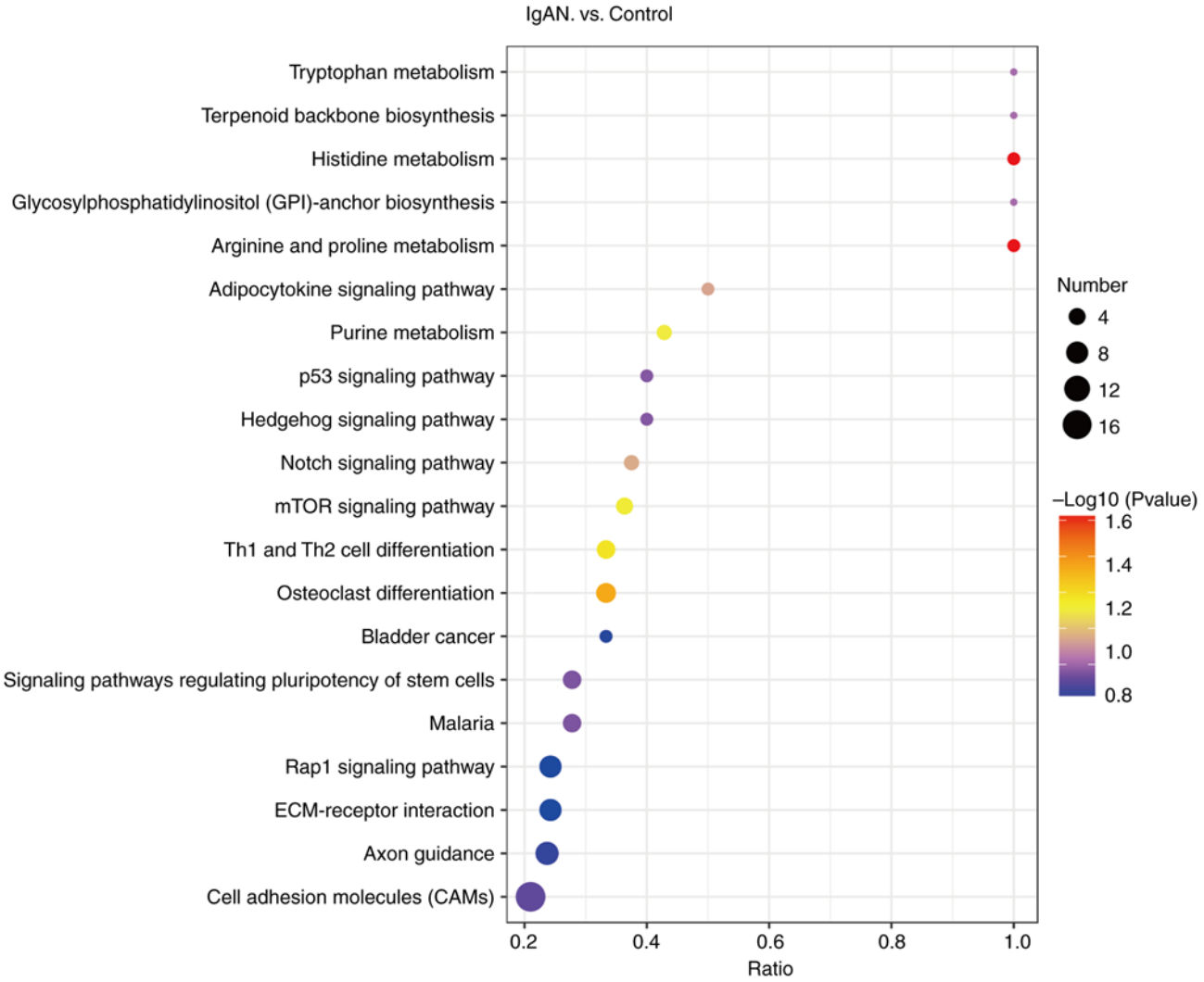


Figure 4. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis in the IgAN vs. healthy control group (n=3 per group). IgAN, IgA nephropathy.

ELISA (Table VI). The results indicated that AFM levels were significantly higher in the patients with IgAN compared to the healthy controls (Fig. 7A). To predict the potential impact of AFM as a biomarker candidate in differentiating IgAN from healthy individuals, ELISA results from these participants were further analyzed to generate ROC curves, even though the number of enrolled participants was small (Fig. 7B). The AUC was 0.720 (95% CI: 0.587 to 0.853). The corresponding sensitivity was 0.667 and specificity was 0.700. These results suggest that the expression level of the AFM indicator in urine is moderately diagnostic for IgAN.

## Discussion

The present study aimed to identify potential urinary proteomic biomarkers for the diagnosis of patients with IgAN using glycoprotein MS analysis. It was found that the expression levels of certain N-glycoproteins were significantly altered by IgAN. Most of the altered N-glycoproteins had roles in the regulation of cell growth, cell adhesion and cellular component organization, as well as protein binding, insulin-like growth factor binding and other kinds of binding. The altered N-glycoproteins were significantly associated with a variety of metabolic functions, including tryptophan, histidine and purines, signaling pathways of various pathways, such as p53, Notch and mTOR signaling pathways, Th1 and Th2 cell differentiation, osteoclast differentiation, bladder cancer, malaria, ECM-receptor interactions, axon guidance and CAMs. These results suggest that N-glycoproteins are involved in the pathological progression of IgAN through various mechanisms. It was further found that IgAN significantly altered the expression levels of numerous N-glycoproteins in urine. Most of them are extracellular and membrane proteins, indicating the organization and regulation of the immune response, and cellular components, such as nucleus, intracellular, extracellular matrix and membrane, due to their high viscosity. To our knowledge, this is the first exploration of using glycoproteomics to study the integrated picture of IgAN.

In the present study, 37 N-glycoproteins were upregulated and 132 N-glycoproteins were downregulated in patients with IgAN compared to healthy controls. Of these, 11 N-glycoproteins were upregulated and 22 N-glycoproteins were downregulated and specifically expressed in patients with IgAN compared to healthy controls. Some of the genes and corresponding proteins

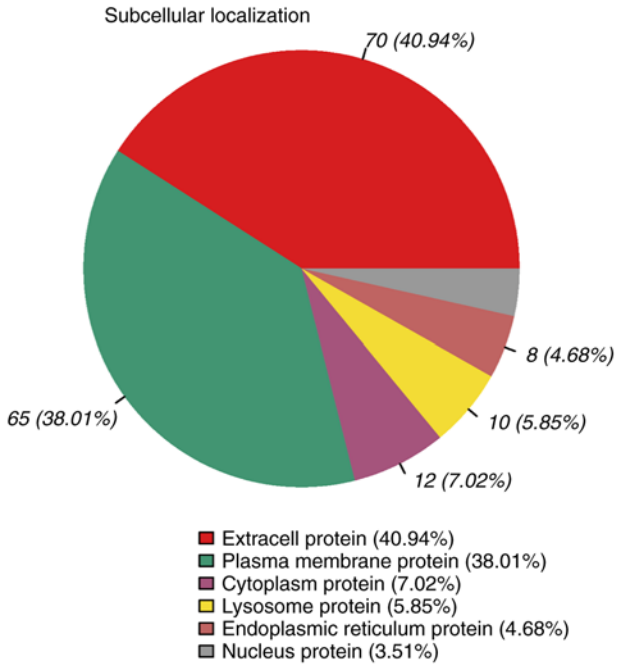


Figure 5. Subcellular localization of differentially expressed N-glycoproteins in patients with IgAN and healthy controls (n=3 per group). IgAN, IgA nephropathy.

identified in the present study have been shown to be associated with the diagnosis, pathological process and prognosis of kidney disease, particularly in IgAN. Lin *et al* (34) found that the methylated CpG corresponding to the WDR82 gene was associated with IgAN in a Chinese population. Buren *et al* (35) noted that the unique expression of CCL25 in small intestinal cells is a form of defective adaptive humoral immune response to mucosal immunogens in patients with IgAN. CD274 (PD-L1), a ligand for PD-1, has been shown to be associated with the development of glomerular injury and inflammation in the peripheral blood of patients with primary glomerulonephritis (36). In addition, increased collagen levels were observed in kidney biopsy specimens from patients with anti-phospholipase A2 receptor autoantibodies associated with membranous nephropathy (37). In the inflammatory injury of glomerulonephritis, DCN expression levels in thylakoid cells are reduced by upregulation of OTU deubiquitinase, ubiquitin aldehyde binding 1, a member of deubiquitinating enzymes (38). In *et al* (39) recently noted that the HLA-DRB1 allele was associated with the progression of IgAN in Korean patients. Jiyun *et al* (40) revealed the association of HLA-DRB1 gene and the primary IgAN in Han Chinese. Zhan *et al* (41) further found that HLA-DRB1 protein expression levels in peripheral blood lymphocytes of patients with IgAN were significantly lower than those of healthy controls and the severity of IgAN was also correlated with peripheral blood lymphocytes in Chinese individuals. Additional studies have further demonstrated the effect of HLA-DRB1 on genetic susceptibility, disease progression, severity, ethnic heterogeneity and geospatial risk of IgAN (39,40,42-47). Hahn *et al* (48) indicated that IL7R gene polymorphism was associated with susceptibility to IgAN in Korean children. The above available evidence further supports the feasibility of clinical application of urinary glycoprotein in kidney disease.

The two most common types of glycosylation are O-glycosylation and N-glycosylation. Eukaryotic O-glycosylation indicates that enzymes of the endoplasmic reticulum and Golgi apparatus append sugars to Ser or Thr residues. N-glycosylation in eukaryotes indicates that N-glycans are attached to N-X-S/T/C sequences. High-throughput site- and structure-specific characterization of different N-glycosylations under pathological conditions using MS-based N-glycoproteomics has become one of the common approaches for the discovery of putative disease biomarkers. Therefore, in the present study, the option to test only N-glycoproteins was pursued, rather than two glycosylations. A significant difference in up- and downregulation of N-glycoprotein expression was found by comparing IgAN and healthy controls based on $P<0.05$ and $|logFC|>2$. Moon *et al* (49) found that CP was uniquely detected and upregulated in patients with IgAN. Therefore, CP is considered one of the candidate biomarkers for differentiating early IgAN by a proteomic approach of urinary exosomes (49-51). Urinary AFM is also a potential prognostic biomarker and classifier of IgAN and kidney injury (29,52,53). The results of the present study indicated that urinary AFM was significantly elevated in adult patients with IgAN compared to levels in healthy controls, which is similar to experimental results from LC-MS/MS analysis of urine from children with IgAN (29). These N-glycoproteins may be considered diagnostic biomarkers for patients with IgAN. Furthermore, interaction analysis of differentially expressed N-glycoproteins associated with IgAN indicated that 22 downregulated specific differential glycoproteins were associated with each other and among 11 upregulated specific differential glycoproteins, CDK5 was associated with CABLES2. This may shed light on the various functions of N-glycoproteins in the pathological process of IgAN compared to healthy volunteers. The fact that similar data could not be found in published articles made it difficult to assess the sample size using the PASS software. To ensure reproducibility of the experiment and normality of the data distribution, three patients with IgAN and three healthy volunteers were included. Significant differences between the two groups for the five top N-glycoproteins mentioned above were obtained. A statistical power analysis was then performed based on the expression levels of CP, CNDP1, BCHE, GPLD1, AFM, PTPRK, NRXN1, LAMA2, EFNA4 and PCDH19 and it was found that the value of 1-β exceeded 0.8.

To validate the results of the proteomics analysis, AFM proteins upregulated in patients with IgAN were selected in the validation cohort and further examined by ELISA. The validation results indicated that the expression levels of AFM in the urine of patients with IgAN were significantly higher compared to the healthy controls. This result indicates that the expression levels of AFM indicators in urine are moderately effective in the diagnosis of IgAN. AFM was chosen as a representative discriminatory glycoprotein for the following reasons: i) AFM was one of the upregulated significantly different N-glycoproteins by LC-MS/MS comparing IgAN and healthy controls with $P<0.05$ and $|\log_2 FC|>2$. ii) The expression level of AFM in urine was previously examined in pediatric patients with IgAN (29), while it has not been investigated in adult patients with IgAN. iii) A human AFM ELISA kit was available from Novus Biologicals to easily, simply

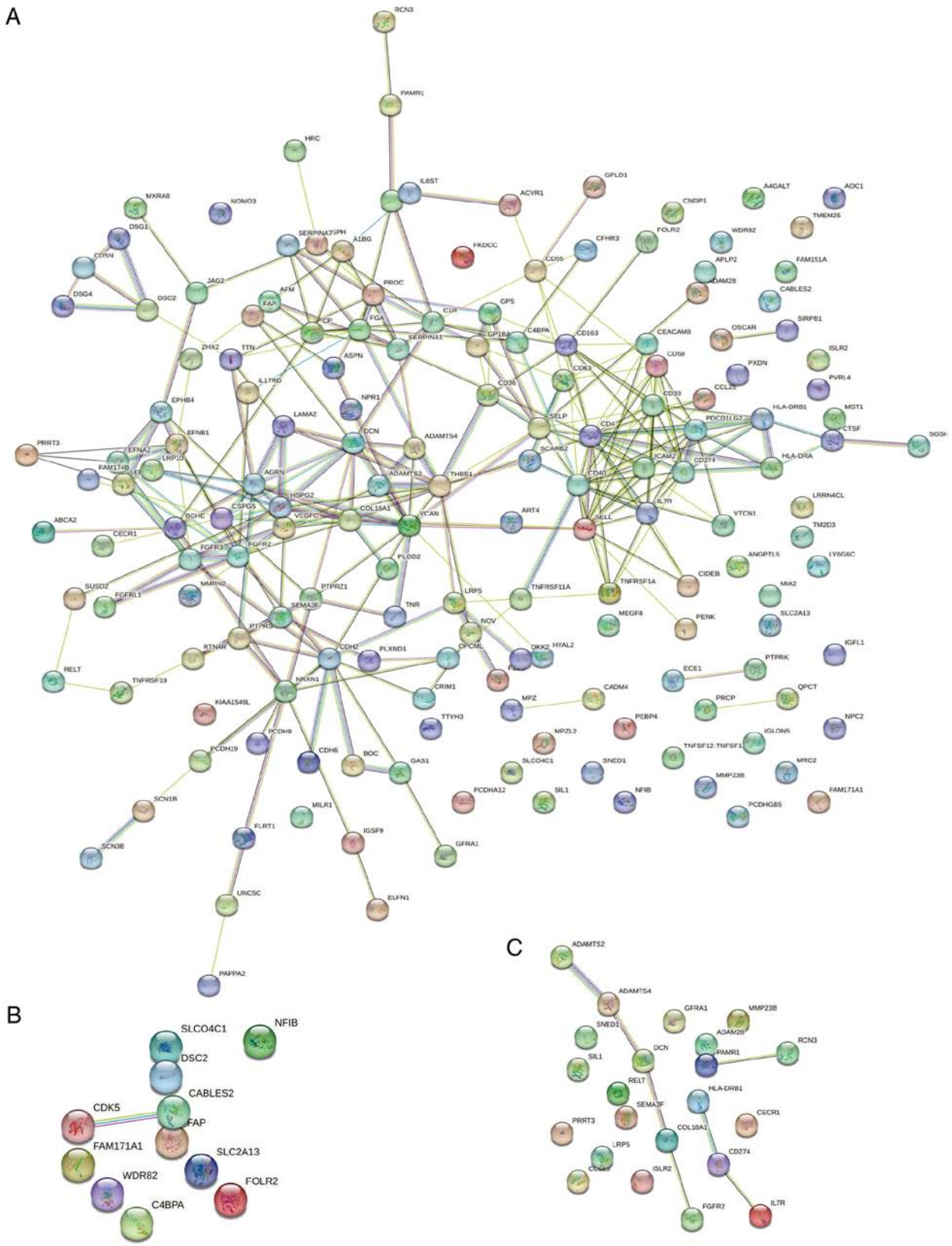


Figure 6. Interaction analysis of differentially expressed glycoproteins associated with IgAN. (A) Interactions of 165 differential N-glycoproteins. (B) Interactions of 11 upregulated specific differential N-glycoproteins. (C) Interactions of 22 downregulated specific differential N-glycoproteins (n=3 per group). IgAN, IgA nephropathy.

and effectively detect its expression level in urine. The results indicated an AUC of 0.720 (95% CI: 0.587-0.853) with a corresponding sensitivity of 0.667 and specificity of 0.700. These results suggest that the expression level of the indicator AFM in urine has moderate diagnostic efficacy for IgAN. Further key differential glycoproteins identified by LC-MS/MS should

Table VI. Characteristics of all participants enrolled for AFM ELISA analysis.

| Characteristic | Healthy controls (n=30) | IgAN patients (n=30) | P-value |
|---|---|---|---|
| Age, years | 45.367±10.240 | 45.100±10.330 | 0.920 |
| Sex, M/F | 15/15 | 15/15 | >0.999 |
| Creatinine, μmol/l | 114.900±47.416 | 72.067±10.748 | <0.001 |
| Urea nitrogen, μmol/l | 9.441±3.148 | 5.593±0.502 | <0.001 |
| Hemoglobin, g/l | 127.733±7.575 | 125.267±5.848 | 0.163 |
| CRP, mg/l | 0.452±0.117 | 0 | <0.001 |
| AFM, pg/ml | 25.162±7.462 | 20.197±6.271 | 0.007 |

IgAN, IgA nephropathy; CRP, C-reactive protein; M, male; F, female.

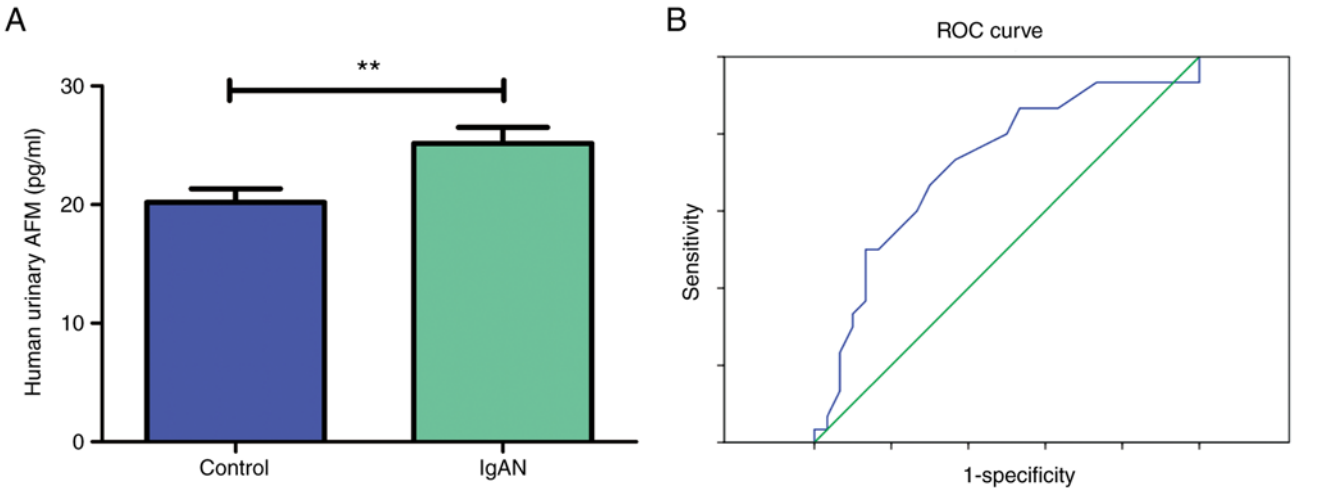


Figure 7. Urine concentration of AFM by ELISA in the validation cohort. (A) Expression level of AFM in urine from healthy controls and patients with IgAN by ELISA. **P<0.01. (B) ROC curve of AFM for the differentiation between the two groups (n=30 per group). IgAN, IgA nephropathy; ROC, receiver operating characteristic.

be assessed and combined to improve the diagnostic efficacy of IgAN and establish a multiparametric model for diagnostic performance.

IgAN was first described histologically as intercapillary deposition of IgA-IgG in 1968 by Berger and Hinglais (54). Worldwide, the prevalence of IgA nephropathy varies widely and is highest in East Asian individuals (55). IgAN accounts for ~40% of all native-kidney biopsies in Japan, 25% in Europe and 12% in the United States (56,57). Currently, the gold standard for the diagnosis of IgAN remains renal biopsy, which, as an invasive diagnostic method, has numerous limitations and may lead to multiple complications (58,59). Traditional clinicopathological indicators remain inadequate to predict the diagnosis, progression and prognosis of IgAN, such as blood pressure, albuminuria and hematuria. Urine glycoproteomics technology has been indicated to be feasible as a non-invasive approach to explore and develop diagnostic biomarkers in patients with IgAN (33,52,53). Furthermore, the use of urine has several advantages over blood or kidney tissue: i) Urine samples are easily accessible and noninvasive. ii) Urine does not contain any other substances that may interfere with the analysis, such as enzymes and immunoproteins in the blood. iii) The proteins and composition of urine may change during kidney injury and related diseases. iv) Urine samples are feasible for patients with contraindications to biopsy. Therefore, urine may be considered a better option to reflect the severity of the kidney and as a source of biomarkers for diagnosis and prognosis.

The limitations of the present study were as follows. First, the sample size was small both in terms of enrolled patients with IgAN and healthy volunteers, and in future studies, a larger sample will be used to reduce the variability of the analysis by further validation (29). In addition, due to the small sample size, the progression and severity of IgAN were not taken into account. Furthermore, due to the lack of follow-up, it was not possible to assess the relationship between glycoprotein expression levels and the prognosis of patients with IgAN. As another limitation, the effect of drug use on the expression levels of relevant glycoproteins still requires to be further explored. Finally, more key differential glycoproteins through LC-MS/MS should be identified and combined to increase the diagnostic efficacy of IgAN and establish a multi-parameter model with improved diagnostic performance. Therefore, more samples and more influencing factors should be considered to obtain more accurate and reliable results to reveal the clinical

diagnosis and prognosis of urine proteomics in patients with IgAN.

In conclusion, the present study demonstrated the importance of glycoproteins in urine as basic specific diagnostic biomarkers for IgAN. Further investigation is necessary to expand their clinical application.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (http://www.ebi.ac.uk/pride/archive/projects/PXD041151).

**Authors' contributions**

JL, LW, HG and ML collected and analyzed the patient data. JL, JZ and HZ conceived and designed the experiments. JZ and HZ drafted and revised the paper. All authors have read and approved the final version of the manuscript. JL and HZ confirm the authenticity of all the raw data.

**Ethics approval and consent to participate**

This study was approved by the Ethics Committee of the Guangan People's Hospital (Guangan, China; approval no. 2021009). All participants provided written informed consent before enrollment.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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