Open Access

*OBM Transplantation*

Original Research

# A Urinary Proteomics Approach to Predict Treatment Response in Acute Antibody-Mediated Rejection

Somaye-Sadat Heidari [1], Nooshin Dalili [1], Shiva Samavat [1], Shiva Kalantari [2], Leonard Foster [3], Kyung-Mee Moon (Jenny) [3], Mohsen Nafar [1,*]

1. Chronic Kidney Disease Research Center (CKDRC), Shahid Labbafinejad Hospital, Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran; E-Mails: s.heidary@sbmu.ac.ir; dr.nooshindalili@gmail.com; shsamavat@gmail.com; nafar@sbmu.ac.ir
2. Department of Cancer Biology, University of Cincinnati College of Medicine, Cincinnati, USA; E-Mail: shivakalantari_81@yahoo.com
3. Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada; E-Mails: foster@msl.ubc.ca; kyungmee@mail.ubc.ca

* **Correspondence:** Mohsen Nafar; E-Mail: nafar@sbmu.ac.ir

**Academic Editor:** Maurizio Salvadori

**Special Issue**: Kidney Transplantation - Clinical and Surgical Challenges

*OBM Transplantation*
2023, volume 7, issue 3
doi:10.21926/obm.transplant.2303196

**Received:** July 12, 2023
**Accepted:** September 18, 2023
**Published:** September 25, 2023

**Abstract**

Acute antibody-mediated rejection (AMR) is a severe complication affecting kidney allografts' long-term survival. Timely detection and appropriate treatment of AMR are crucial for improving graft outcomes. This study aimed to discover non-invasive urinary biomarkers that can predict the response to therapy in patients with AMR. Materials and Methods: In this case-control study, urine samples from 21 biopsy-proven AMR patients underwent proteomics using label-free quantification. The patients were divided into two groups: responders and non-responders to treatment based on their graft function. Urinary proteins were identified, and their expressions were compared between the two groups to identify potential candidate biomarkers. Out of the 1020 identified proteins, 257 proteins were found to be differentially expressed between the two groups. Among these, 153 proteins showed increased expression in non-responder patients, while 104 proteins showed decreased expression. Non-responder patients exhibited higher activation of complement pathway and extracellular matrix degradation than responders. Insulin-like growth factor binding protein 6 (IGFBP-6) emerged as the most sensitive and specific biomarker for predicting non-response to treatment in patients with AMR. Our study has successfully identified urinary proteome biomarkers that can distinguish and predict non-responder patients with AMR. These biomarkers are associated with various biological processes that reflect the pathogenesis and severity of AMR. Further research is necessary to validate these findings.

**Keywords**

Antibody-mediated rejection; kidney allograft; urinary biomarker; proteomics; IGFBP-6

## 1. Introduction

Kidney transplantation represents the optimal treatment for individuals with end-stage renal disease, providing significant improvements in both quality of life and overall survival. However, this procedure has challenges, particularly the risk of rejection by the recipient's immune system. Among the various forms of rejection, acute antibody-mediated rejection (AMR) emerges as a prominent complication following transplantation, significantly impacting kidney allografts' long-term function and viability [1].

The timely and accurate AMR diagnosis is crucial to enhance graft outcomes. Unfortunately, current diagnostic methods have certain limitations. Increased serum creatinine and proteinuria are commonly employed indicators of allograft dysfunction and injury. However, they manifest as late markers of rejection [2]. Renal biopsy, regarded as the gold standard for diagnosis, is an invasive procedure and not conducive to repeated monitoring [3]. Consequently, there is a pressing need for sensitive and minimally invasive biomarkers that can facilitate early and precise prediction of allograft rejection [3].

Non-invasive and reliable biomarkers hold the potential to aid clinicians in predicting and assessing transplant outcomes. By enabling early intervention and optimization of immunosuppression therapy, these biomarkers can serve as valuable guides in successfully treating acute rejections, reducing morbidity and enhancing long-term allograft survival [4]. Recent studies have focused on exploring novel methods, such as proteomics to identify non-invasive and reliable biomarkers for accurate detection and prediction of acute rejection. Proteomics is the study of the proteome, the complete set of proteins an organism can express. Proteomics can help us discover disease biomarkers, which are proteins that indicate the presence, severity, progression, or response to treatment of a disease. By comparing the proteome of healthy and sick people, we can identify the key proteins involved in the disease process and investigate their function about the disease pathology and treatment outcome. Utilizing omics technologies such as proteomics, researchers have discovered new biomarkers in both circulation and urine samples [5-8]. However, previous investigations have not agreed on a single biomarker for predicting the response to therapy in acute rejection.

Non-invasive and reliable biomarkers can also assist physicians in estimating the risk of transplant rejection and predicting treatment response. Ideally, biomarker-guided approaches would allow tailored treatments for individual patients, reducing the risks associated with immunosuppressive drugs and offering the possibility of early preventive interventions, thereby decreasing patient mortality rates and improving long-term survival [9]. The discovery of new, particular biomarkers for diagnosing acute rejection is imperative, as non-invasive biomarkers permit frequent patient monitoring and adjustment of immunosuppressive treatments based on biomarker changes [10].

Urine, as a readily accessible sample, offers a direct reflection of biological events occurring within the allograft tissue. This characteristic renders urine specimens suitable for proteomics studies [11]. The objective of this study was to analyze the urinary proteome of patients with AMR, to identify potential biomarkers that can predict the response to AMR treatment. We hypothesized that there would be differences in urinary proteins between responders and non-responders to treatment based on their graft function. It was anticipated that novel urinary biomarkers could be discovered, serving as non-invasive indicators of treatment response in AMR patients.

## 2. Patients and Methods

### *2.1 Patients*

A case-control study involving kidney recipients with biopsy-proven AMR was conducted at Labbafinejad hospital (Tehran, Iran). The study included patients between the ages of 16 and 65 who received the same immunosuppressive regimens (tacrolimus, mycophenolate mofetil, and prednisone). Exclusion criteria comprised urinary tract infection and obstruction. This study adhered to the guidelines outlined in the 2013 revision of the Declaration of Helsinki (1967) and was approved by the Ethics Committee of the National Institute of Medical Sciences Research Development (NIMAD), Tehran, Iran. All participants provided written informed consent.

### *2.2 Urine Sample Collection and Preparation*

Second-morning urine samples were collected from patients prior to the kidney biopsy procedure. Each sample was supplemented with protease inhibitor and sodium azide (100 µl/10 ml urine) to inhibit protease and bacterial activity. The proteome component of the urine samples was separated using Amicon Ultra-15 Centrifugal Filter Units with a 3 kDa cutoff (Millipore, Billerica, MA, USA) following the manufacturer’s instructions. The samples were subsequently stored at -80 °C for further analysis.

### *2.3 Treatment and Outcome Assessment*

AMR patients received treatments, including intravenous immune globulin (IVIG), plasmapheresis, rituximab, switching from cyclosporine to tacrolimus, and increasing mycophenolate dosage. Follow-up of patients occurred over a two-week period, with individuals being categorized as non-responders if they experienced graft loss, no decrease in creatinine levels by at least 30%, or continued presence of anti-human leukocyte antigen (anti-HLA) antibodies. Patients who demonstrated a positive response to the treatments were classified as responders.

### *2.4 Urine Protein Measurement and Separation*

Urine protein levels were measured using the Bicinchoninic acid protein assay kit (Thermo Scientific, USA). To separate the proteins, 50 µg of each sample was loaded onto 10% SDS-PAGE gels and subjected to electrophoresis. The gel lane for each piece was divided into three fractions: fraction 1 containing low- abundant proteins, fraction 2 containing the thick albumin band, and fraction 3 containing the remaining proteins.

### *2.5 Protein Digestion and Peptide Extraction*

Protein digestion was carried out using trypsin (Promega, USA) overnight at 37°C in 50 mM ammonium bicarbonate. Peptides were subsequently extracted using C18- StageTips columns (3 M Empire, USA). The samples were eluted with 80% acetonitrile in 0.1% formic acid and then concentrated using a vacuum concentrator (Eppendorf, Germany).

### *2.6 Liquid Chromatography Tandem Mass Spectrometry Analysis (LC-MS/MS)*

LC-MS/MS analysis was performed using as EASY-nLC 1000 HPLC (Thermo Fisher, USA) coupled with a Quadrupole Time-of-Flight Mass Spectrometry Impact II (Bruker Daltonics, Germany). The setup included a nanoelectrospray ionization source, a trap column, and an analytical column. Peptides were resuspended in 5% acetonitrile and 0.5% formic acid and loaded onto the trap column at a pressure of 850 bar. The analysis was conducted at a flow rate of 0.25 µl/min.

### *2.7 Data Analysis and Biomarker Identification*

MS/MS Data were analyzed using MaxQuant software (version 1.5.3.30). The MS/MS spectra were searched against the human UniProt database. Proteins that had at least two unique peptides and a false discovery rate (FDR) of less than 1% were considered identified. The fold changes of each protein were calculated and compared between the two groups to determine the direction of their changes. The abundance of each protein in the urine samples of responders and non-responders was quantified, and their abundance ratio was calculated.

Statistical Package for Social Sciences Version 16 (SPSS Inc., Chicago-USA) was used to calculate performance metrics for each protein as potential biomarkers: including the area under the curve (AUC), sensitivity and specificity. Candidate biomarkers were selected based on the AUC values with proteins having the highest AUC values considered the most promising biomarkers for predicting the response to treatment in AMR patients.

### *2.8 Pathway Analysis and Functional Evaluation*

Signaling pathways and gene involvement of the candidate biomarkers were investigated using the Database for Annotation, Visualization, and Integrated Discovery (DAVID: http://www.david.niaid.nih.gov) and Kyoto Encyclopedia of Genes and Genomes (KEGG: https://www. genome.jp). Only pathways and ontologies with p-values and FDR less than 0.05 were considered.

## 3. Results

### *3.1 Demographic and Clinical Data*

A total of 21 patients with biopsy-proven AMR were included in this study. Among them, 5 patients (23.8%) did not respond to treatment (non-responders), while 16 patients (76.2%) responded to treatment (responders) based on their graft function assessment after two weeks of treatment. Table 1 presents the demographic and clinical characteristics of the patients. The mean age of the patients at the time of diagnosis was 40 ± 12 years, with an age range of 19 to 65 years. The majority of patients (80%) were recipients of their first kidney transplant. Most patients in both groups tended to have allograft biopsies within the first year post-transplantation. No significant differences were observed between the two groups regarding estimated glomerular filtration rate (eGFR), serum creatinine, and blood urea nitrogen. However, the non-responder group exhibited significantly higher urinary protein levels than the responder group ($p < 0.05$). Lipid profiles, hemoglobin, and hematocrit were similar between the groups.

**Table 1** Demographic and clinical characteristics of AMR patients according to treatment response.

| **Parameters** | **Groups** | |
|---|---|---|
| | Responder (n = 16) | Non-responder (n = 5) |
| Male (%) | 62.5 | 20 |
| Donor type (%) | | |
| Deceased donor | 7 | 2 |
| Living donor | 9 | 3 |
| First transplantation | 12 | 5 |
| Age at transplant (year) | 42.2 ± 13.3$^{\#}$ | 29.6 ± 16.6 |
| Age at biopsy (year) | 42.5 ± 13.5$^{\#}$ | 32.6 ± 13.6 |
| Biopsy time after transplant (%) | | |
| >1 year | 13 | 3 |
| <1 year | 3 | 2 |
| Lab data | | |
| eGFR (mL/min/1.73 $m^2$) | 31.9 ± 12.2 | 23.4 ± 21.8 |
| Urine protein ((mg/ml) | 11.8 ± 9.6 | 25.5 ± 16.9* |
| Creatinine (mg/dl) | 2.4 ± 1.1 | 4 ± 2.7 |
| BUN (mg/dl) | 61.5 ± 28 | 73 ± 20.5 |
| Hb (g/dl) | 10.8 ± 1.5 | 10.4 ± 2 |
| HcT (%) | 33 ± 4.6 | 31.7 ± 6.6 |
| Chol (mg/dl) | 163.7 ± 53.4 | 148.5 ± 47.3 |
| TG (mg/dl) | 148.5 ± 47.3 | 148.2 ± 45 |
| LDL-C (mg/dl) | 148.2 ± 45 | 94.3 ± 15.7 |
| HDL-C (mg/dl) | 84.3 ± 15 | 64.6 ± 12.7 |
| VLDL-C (mg/dl) | 54.1 ± 12.7 | 39.6 ± 9.1 |

*. Significance level was set as <0.05. #. Results are expressed as mean ±2SD.

### *3.2 Differential Proteins*

The urinary proteome of the patients was analyzed using LC-MS/MS. A total of 1020 proteins and peptides were identified in the urine samples. To identify differential proteins between the two groups, the main primary data excluded proteins that were not detected by mass spectrometry in both groups by 75% or more. Consequently, 257 proteins were considered as differential proteins between the two groups. Among these, 153 proteins exhibited increased expression, while 104 proteins displayed decreased expression in the urine samples of non-responders compared to responders. The differential proteins were ranked based on their fold change (FC) values, representing the ratio of peak intensities between the two groups. Figure 1 illustrates the top 20 differential proteins with the highest FC values in both directions.

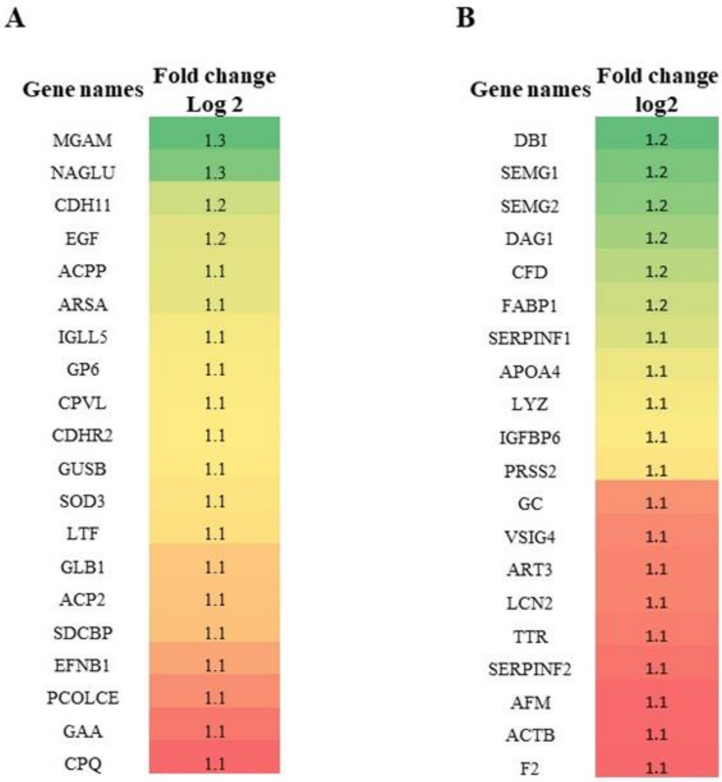


**Figure 1** Color graphics of increased proteins in responder group (A) and non-responder group (B). Maltase-glucoamylase and Alpha-N-acetylglucosaminidase proteins in the responder group and Acyl-CoA-binding protein and Semenogelin-1 proteins in the non-responders group increased.

### *3.3 Candidate Biomarkers*

To identify candidate biomarkers for predicting AMR treatment response, the differential proteins were further filtered based on their FC, and 10 proteins (5 up-regulated and 5 down-regulated) were selected for further analysis. The specificity and sensitivity of these proteins were evaluated by performing receiver operating characteristic (ROC) curve analysis and calculating the area under the curve (AUC) values for each protein. Table 2 presents the ten selected candidate biomarkers demonstrating promising discriminatory potential between non-responders and responders.

**Table 2** Candidate biomarkers with highest AUCs in the differentiation of non-responders from responders.

| accession number# | Gene name | Protein name | AUC* | fold ** change | direction change |
|---|---|---|---|---|---|
| P24592 | Insulin-like growth factor-binding protein 6 | IGFBP6 | 0.94 | 4.4 | up |
| Q9H3G5 | Probable serine carboxypeptidase CPVL | CPVL | 0.9 | 3.6 | down |
| P07108 | Acyl-CoA-binding protein | DBI | 0.89 | 10.5 | up |
| P06727 | Apolipoprotein A-IV | APOA4 | 0.88 | 6.6 | up |
| P07148 | Fatty acid-binding protein | FABP1 | 0.82 | 5.7 | up |
| Q9BYE9 | Cadherin-related family member 2 | CDHR2 | 0.8 | 3.2 | down |
| P36955 | Pigment epithelium-derived factor | SERPINF1 | 0.8 | 6.9 | up |
| Q14118 | Dystroglycan | DAG1 | 0.8 | 8.4 | up |
| Q9HCN6 | Platelet glycoprotein VI | GP6 | 0.79 | 3.8 | down |
| P54802 | Alpha-N-acetylglucosaminidase | NAGLU | 0.76 | 9.2 | down |

#. Uniqe number of proteins in Uniprot database. *. AUC: area under curve. **. Fold changes based on peak intensity from MS/MS.

### *3.4 Gene Ontology and Pathways Analysis*

Gene ontology (GO) and pathway analysis were conducted on the 257 differential proteins using the online DAVID database to gain insight into the biological processes and pathways involved in AMR treatment response.

Gene ontology was obtained in a categorized and clustered form. Data with a Benjamini index of less than 0.05 for each obtained cluster were accepted, while other data were excluded. The gene ontology analysis was presented in three classifications: biological process, molecular function, and cellular components (Figure 2). Proteolysis and complement activation emerged as the main biological processes involving 51 and 34 differential proteins, respectively. Molecular function analysis revealed that 39 proteins were associated with serine endopeptidase, and 23 proteins displayed antigen-binding properties. The differential proteins were primarily distributed across various cell membrane components (24 proteins) and cell junctions (14 proteins).

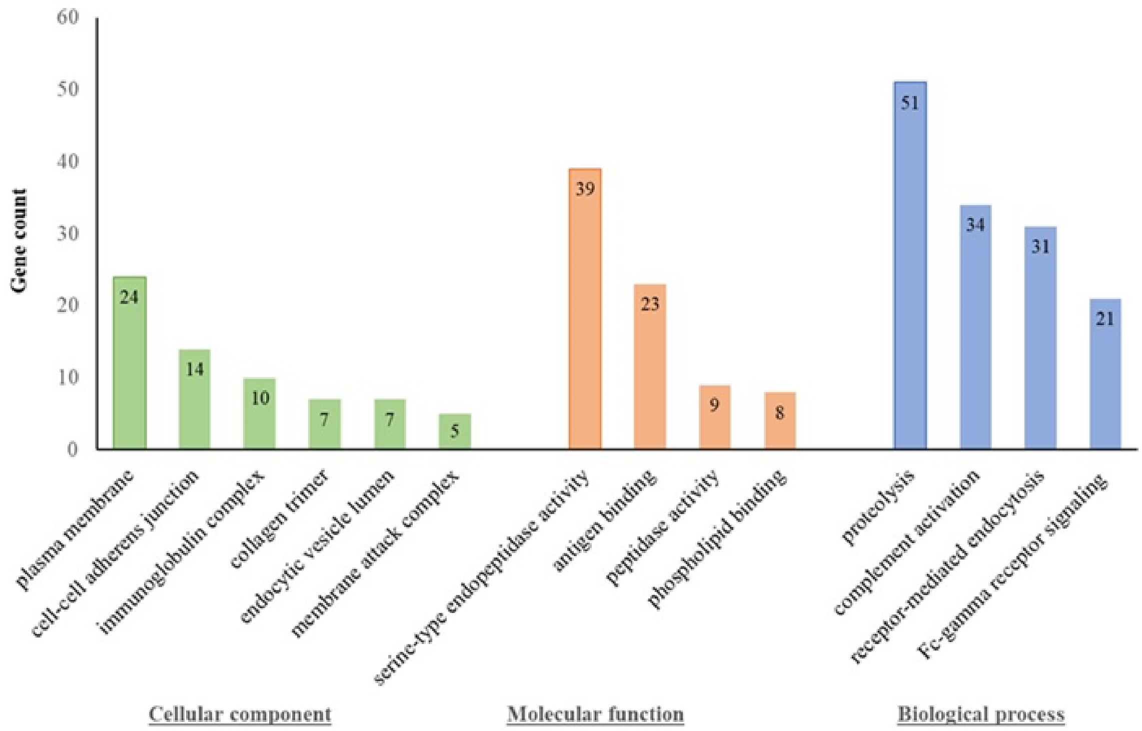


**Figure 2** Gene ontology analysis diagram for differential proteins between two groups of study. The differential proteins were analyzed in the three classifications of biological processes, molecular function and participation in cellular components in DAVID bioinformatics online resource. Differential proteins with 51 proteins were mainly involved in proteolysis. The most important molecular function of these proteins was serine endopeptidase activity and antigen binding. Most of the proteins were distributed in cell membranes and junctions.

Differential proteins were investigated using the DAVID bioinformatics resource, specifically searching BIOCARTA, KEGG, and BBID databases to identify active biological pathways in the studied groups. The analysis of differential protein pathways from the KEGG database revealed that the complement pathway and coagulation cascade were prominent, involving 18 proteins. These proteins included alpha-2-macroglobulin(A2M), coagulation factor II, thrombin(F2), complement C3(C3), complement C4B (Chido blood group) (C4B), complement C5(C5), complement C6(C6), complement C7(C7), complement C9(C9), complement factor B(CFB), complement factor D(CFD), complement factor H(CFH), fibrinogen beta chain(FGB), kininogen 1(KNG1), plasminogen(PLG), serpin family A member 1(SERPINA1), serpin family C member 1(SERPINC1), serpin family F member 2 (SERPINF2), serpin family G member 1(SERPING1) . These proteins were found to be increased in non-responders and were associated with the complement pathway and coagulation cascade (p-value = 0.000000000017) (Figure 3).

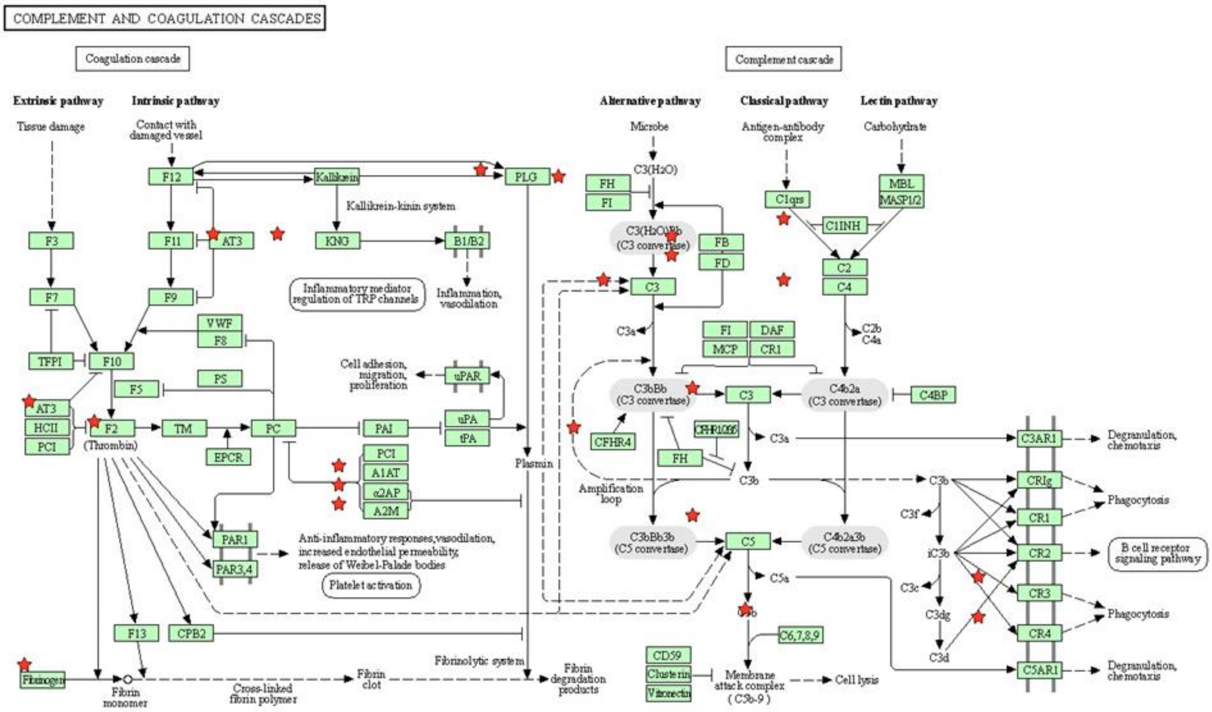


**Figure 3** Pathway analysis of differentially expressed and increased proteins from KEGG online database. This analysis shows that complement pathway and coagulation cascade with 18 proteins is the main pathway that is activated in non-responders. The increased proteins are marked with a red star. Complement activation starts from three classical, lectin, and secondary pathways, and these three pathways activate C3 in a final joint pathway and assemble the membrane attack complex. This complex induces apoptosis and necrosis. Complement activation and the coagulation cascade have mutual effects in activating each other.

The analysis of decreased protein pathways in non-responders indicated that many of these proteins are involved in receptor-extracellular matrix interactions. These proteins include (agrin (AGRN), collagen type VI alpha 1 chain (COL6A1), collagen type VI alpha 3 chains (COL6A3), fibronectin 1 (FN1), platelet glycoprotein VI (GP6), heparan sulfate proteoglycan 2 (HSPG2), secreted phosphoprotein 1 (SPP1), tenascin XB (TNXB) and vitronectin (VTN). As shown in Figure 4, these proteins exhibited decreased activity in non-responders and were associated with receptor-extracellular matrix interactions (p-value = 0.00004)).

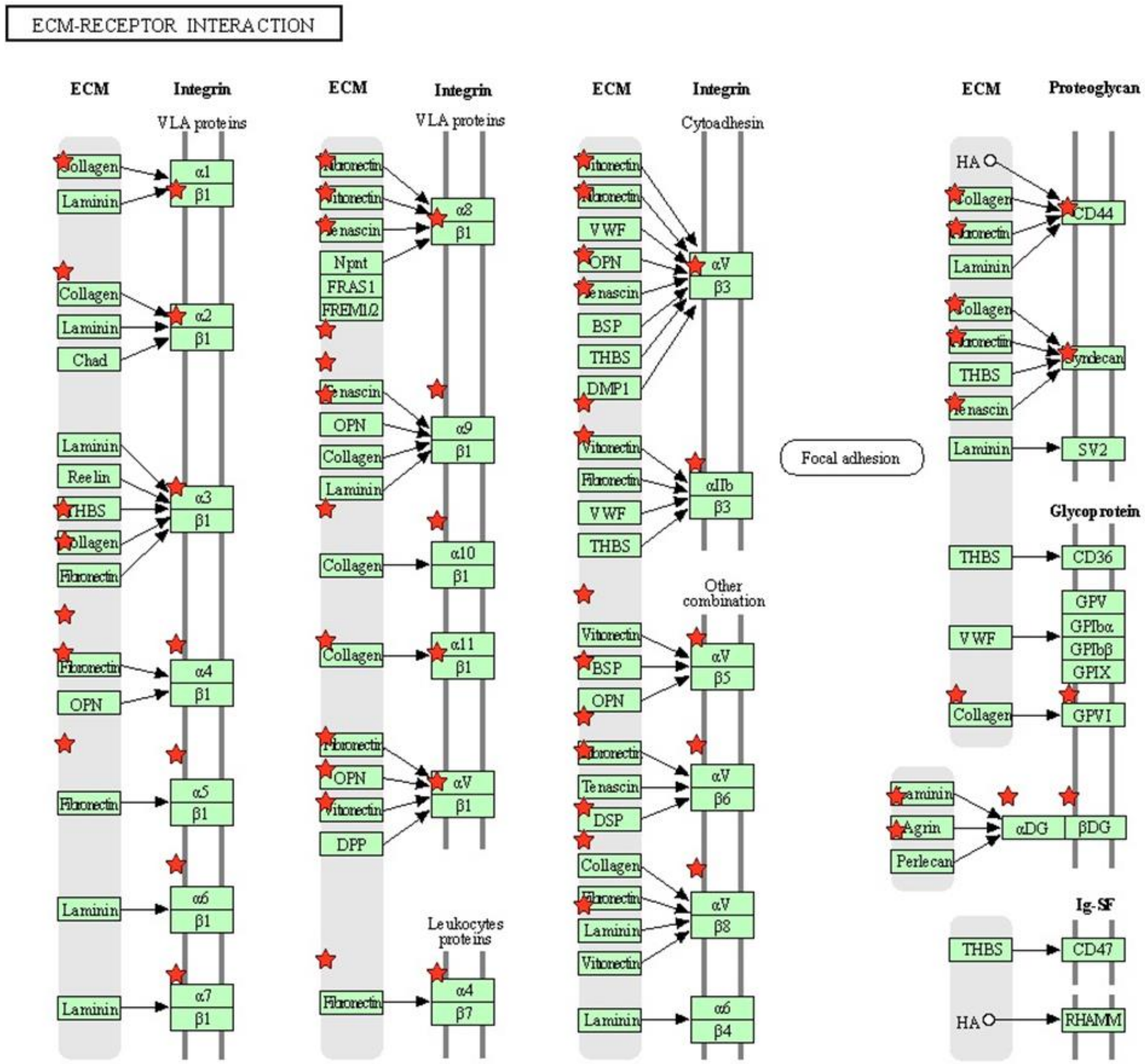


**Figure 4** Pathway analysis of reduced proteins from KEGG online database. The result of the analysis shows that the reduced proteins are involved in receptor-extracellular matrix interactions. Nine related proteins (AGRN, COL6A1, COL6A3, FN1, GP6, HSPG2, SPP1, TNXB, VTN) were decreased in these interactions. Downregulated proteins are marked with a red star.

### *3.5 Protein Network Analysis*

To investigate potential interactions and relationships among the candidate biomarkers, protein network analysis was conducted using the online functional analysis database of the protein-protein interaction network (STRING: https://string-db.org). The study revealed that three biomarker proteins, Acyl-CoA-binding protein, Apolipoprotein A-IV, and Fatty acid-binding protein, were directly or indirectly connected through nodes and edges (Figure 5). However, no protein associations were found for the remaining submitted proteins in the database.

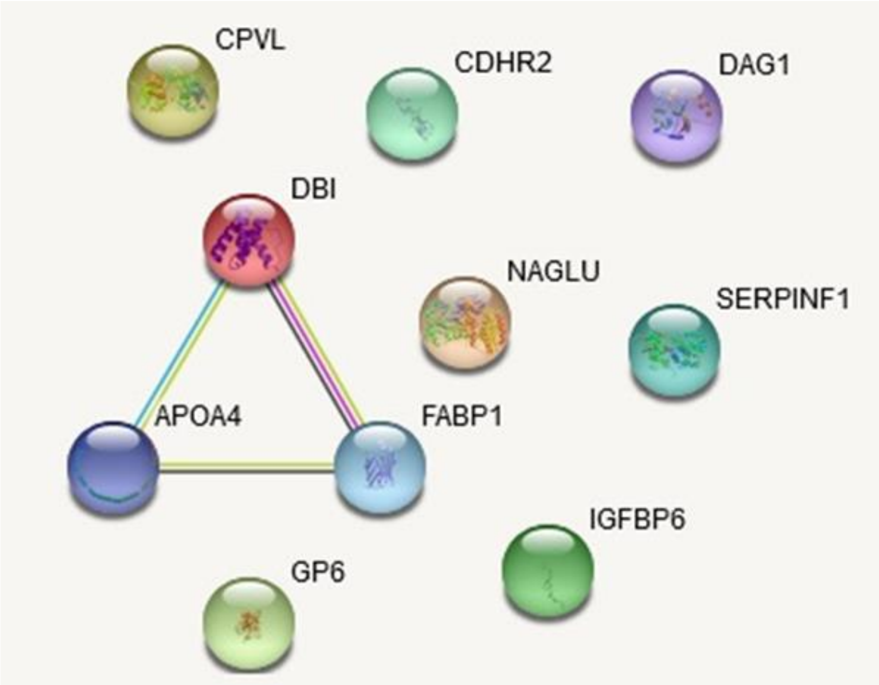


**Figure 5** Protein network analysis of candidate biomarkers using online functional analysis of protein-protein interaction network (STRING) database. The analysis showed that there are protein connections in the biomarker proteins. A network of 3 proteins including DBI, FABP1 and APOA4 proteins were related to each other. No interaction was found for other proteins. Proteins are displayed as nodes, and connection lines between nodes are shown in five colors for each type of protein connection. The signs of connection lines are as follows: black line: co-expressed genes, green line: neighboring genes, pale blue line: protein homology, purple line: experimental evidence. The more the number of lines between the nodes, the greater the connection between them.

## 4. Discussion

The LFQ method was employed to explore the urinary proteome profile of AMR patients and identify predictive biomarkers for treatment response. Proteomics analysis revealed 257 differentially expressed proteins between responders and non-responders, most upregulated in the non-responder group and associated with the "complement and coagulation pathway". GO analysis further highlighted the main biological functions of these proteins, emphasizing proteolysis and complement activation. These findings align with the proposed pathogenesis of AMR, which involves the interaction between complement and coagulation activation [12]. The increased activity of these pathways in non-responder patients may contribute to resistance to treatment.

The downregulated proteins in the non-responder group were primarily involved in ECM-matrix interactions. The color graphic representation of proteins indicates that specific ECM-related proteins exhibited decreased levels in non-responder patients, distinguishing them from the responder group. Changes in proteins such as cadherin-2 and dystroglycan among the candidate biomarkers suggest altered binding and interactions between cells and ECM, indicating remodeling

[13]. These alterations were more prevalent in the non-responder patients, potentially indicating a higher severity of AMR within this group.

Antibody-antigen binding in the immune response has been suggested with mitochondrial damage and the production of reactive oxygen species [14]. This process can inhibit glycolysis enzymes such as glyceraldehyde-3 phosphate dehydrogenase, phosphofructokinase-1, and pyruvate kinase. Consequently, the pentose phosphate pathway is activated to compensate for the reduced glycolysis cycle and to provide more NADPH for the antioxidant system [15]. The lower levels of glycolysis enzymes such as maltase-glucoamylase and alpha-N-acetylglucosaminidase in the non-responder patients may reflect a metabolic shift towards the pentose phosphate pathway and indicate increased severity of oxidative stress and activation of the antioxidant system in this group.

Further analysis of the differential proteins led to the identification of 10 proteins with the highest sensitivity and specificity as candidate biomarkers for discriminating and predicting non-responder patients. Among these biomarkers, IGFBP-6 exhibited the most heightened sensitivity and increased expression in the urine proteome of non-responder patients. IGFBP-6 is a glycoprotein secreted from various tissues that regulate cell proliferation, differentiation, and survival by binding to IGF or inducing apoptosis independently [16]. Increased expression of IGFBP-6 has been observed in the kidney in certain renal diseases such as FSGS, IgA nephropathy, and lupus nephritis [17]. IGFBP-6 levels in the serum may also increase in kidney diseases, potentially due to upregulation of its gene and reduced renal clearance, and decrease after transplantation [18]. Glucocorticoid therapy post-transplantation may lower growth hormone levels and increase IGFBPs in the plasma [19]. The role of IGFBP-6 in renal acute rejection is not fully understood. Still, its elevation in the urine proteome of non-responder patients may be associated with increased apoptosis and tubular injuries, leading to the leakage of this low-molecular-weight protein into the urine. Given the high sensitivity and specificity of IGFBP-6 as a biomarker, it appears that this protein can predict the response to rejection therapy in AMR and could serve as a potential therapeutic target.

CPVL is a serine carboxypeptidase that is expressed in immune cells such as macrophages and monocytes and plays a role in phagocytic fragments digestion and antigen presentation [20]. It is also expressed in kidney tissue [21], and its presence in the urine of patients with acute rejection has been reported [22]. The specific role of this protein in acute graft rejection is still unknown. Still, it may be involved in homeostasis as its gene region is mutated in Wilms's renal tumor, which is associated with high blood pressure and impaired homeostasis [23]. In our study, we observed lower urinary CPVL in non-responder patients. This suggests reduced tissue expression of this protein in this patient group, and further exploration is warranted to investigate its potential as a biomarker for predicting the response to acute rejection therapy.

APOA4 is a glycoprotein involved in the reverse transport of cholesterol. Previous studies have reported elevated levels of APOA4 in the plasma and urine proteomes of patients with acute rejection [5, 24]. Our study found higher urinary levels of APOA4 in the non-responder group compared to the responder group. APOA4 is filtered and reabsorbed by the kidneys, and its increase in urine reflects impaired renal tubular function in proteinuric kidney diseases. Therefore, it has been proposed as a predictive biomarker for chronic kidney disease [25]. The high urinary levels of APOA4 in patients with AMR may indicate more severe glomerular damage and a lack of response to treatment in these individuals.

FABP1 is a protein produced in the epithelium of the proximal tubules and the liver, and it plays a role in the transport and metabolism of fatty acids. Elevated urinary levels of FABP1 are indicative of severe tubulointerstitial damage and impaired protein reabsorption in the proximal tubules [26] and have been identified as a biomarker for the progression of chronic kidney disease and chronic glomerulonephritis [27, 28]. High urinary FABP1 levels also predict adverse outcomes such as transplant rejection in transplant patients [29, 30]. FABP1 is filtered and reabsorbed in the proximal tubules, and its increase in urine is not accompanied by a change in serum levels [27]. FABP1 expression also increases in animal models of IgA nephropathy [31]. Therefore, hadthe non-responder patients may have reduced FABP1 expression in the kidney tissue and reduced FABP1 reabsorption in the renal tubules. Furthermore, network analysis in the STRING database showed protein-protein interactions among three proteins: DBI, FABP1, and APOA4. DBI protein facilitates beta-oxidation of fatty acids after they are taken up by FABP1 [32]. FABP1 also binds to lipid oxidation products to counteract the oxidative stress caused by lipid catabolism [32]. These two proteins may increase in response to increased tissue energy demands in acute inflammatory conditions in allograft rejection. Non-responder patients may express more of these proteins to meet their needs in response to inflammation and oxidative stress.

SERPINF1 (pigment epithelium-derived factor; PEDF) is a serine protease inhibitor with anti-angiogenic, anti-fibrotic, and anti-inflammatory effects and is highly expressed in the renal tubules and glomeruli. High serum levels of PEDF are a compensatory response to glomerular injury and a predictor of diabetic nephropathy progression [33]. Kidney expression and urinary PEDF levels are also elevated in these patients [34]. PEDF levels decrease in patients undergoing dialysis and normalize after transplantation [35, 36]. In our study, we found higher urinary PEDF levels in non-responders. This is consistent with previous reports of increased urinary PEDF levels in acute and chronic renal rejection [37, 38]. The exact mechanism of urinary PEDF elevation in acute rejection is unclear. Still, it may be part of the rejection process because PEDF can affect podocytes, induce apoptosis, increase proteinuria, and worsen kidney function through actin remodeling [39]. Thus, high urinary PEDF levels in non-responders in our study may indicate more severe disease progression and inflammation.

GP6 is a platelet membrane glycoprotein that acts as a receptor for collagen and triggers platelet aggregation [40]. GP6 level is elevated in the serum in microangiopathy lesions [41] and in patients with chronic kidney disease on dialysis but not in patients with successful transplantation [42]. Platelet factors are increased in various glomerulonephritis diseases and may affect the extracellular matrix and cause more glomerular inflammation [43]. GP6 is involved in recruiting platelets and monocytes to the vascular wall, regulating vascular permeability and activating leukocytes. It prevents bleeding due to inflammation, thereby preserving the integrity of the inflamed vascular wall [43]. GP6 deficiency or blockade has been shown to reduce platelet recruitment in glomerulonephritis models [44]. In our study, we observed lower urinary GP6 levels in non-responder patients, which may have resulted in increased inflammation and loss of basement membrane integrity, leading to a more acute disease condition than in responder patients.

### *4.1 Study Limitations*

The sample collection in this study was done on an accessible basis, and the sample size was small. Therefore, the urinary biomarkers of treatment response in our identified AMR patients might not be representative or generalizable. Moreover, these biomarkers are only candidates and need to be validated with a larger sample size and in an independent cohort to make our assessment of these biomarkers more precise.

## 5. Conclusions

In conclusion, we analyzed the urine proteome. We discovered highly sensitive and specific biomarkers for predicting the response to treatment in AMR patients. IGFBP-6 was identified as the most suitable biomarker with the highest score, and its increased urinary level might expect the response to treatment. However, further study is required to validate this biomarker as a predictive biomarker. Bioinformatics analysis showed that, in addition to enhanced complement activity, the extracellular matrix might undergo more changes in the non-responder patients. Moreover, alterations in lipid metabolism could be a compensatory response to the increased oxidative stress and inflammation in these patients. These findings may have clinical implications for AMR diagnosis, prognosis, and treatment.

## Author Contributions

Mohsen Nafar participated in performance of the research, research design and writing of the paper. Nooshin Dalili and Shiva Samavat participated in sample preparation and writing of the paper. Shiva Kalantari participated in sample preparation, data analysis and writing of the paper. Leonard Foster participated in performance of laboratory analysis and writing of the paper. Kyung-Mee Moon (Jenny) participated in performance laboratory analysis. Somaye-Sadat Heidari participated in sample preparation, data analysis and writing of the paper.

## Competing Interests

The authors have declared that no competing interests exist.

## References


1. Puttarajappa C, Shapiro R, Tan HP. Antibody-mediated rejection in kidney transplantation: A review. J Transplant. 2012; 2012: 193724.
2. Fonseca I, Reguengo H, Oliveira JC, Martins LS, Malheiro J, Almeida M, et al. A triple-biomarker approach for the detection of delayed graft function after kidney transplantation using serum creatinine, cystatin C, and malondialdehyde. Clin Biochem. 2015; 48: 1033-1038.
3. Ramalhete LM, Araújo R, Ferreira A, Calado CR. Proteomics for biomarker discovery for diagnosis and prognosis of kidney transplantation rejection. Proteomes. 2022; 10: 24.
4. Guzzi F, Cirillo L, Buti E, Becherucci F, Errichiello C, Roperto RM, et al. Urinary biomarkers for diagnosis and prediction of acute kidney allograft rejection: A systematic review. Int J Mol Sci. 2020; 21: 6889.
5. Mertens I, Willems H, Van Loon E, Schildermans K, Boonen K, Baggerman G, et al. Urinary protein biomarker panel for the diagnosis of antibody-mediated rejection in kidney transplant recipients. Kidney Int Rep. 2020; 5: 1448-1458.

6. Gudehithlu KP, Hart P, Joshi A, Garcia-Gomez I, Cimbaluk DJ, Dunea G, et al. Urine exosomal ceruloplasmin: A potential early biomarker of underlying kidney disease. Clin Exp Nephrol. 2019; 23: 1013-1021.
7. Heidari SS, Nafar M, Kalantari S, Tavilani H, Karimi J, Foster L, et al. Urinary epidermal growth factor is a novel biomarker for early diagnosis of antibody mediated kidney allograft rejection: A urinary proteomics analysis. J Proteomics. 2021; 240: 104208.
8. Jacquemont L, Soulillou JP, Degauque N. Blood biomarkers of kidney transplant rejection, an endless search? Expert Rev Mol Diagn. 2017; 17: 687-697.
9. Brunet M, Shipkova M, Van Gelder T, Wieland E, Sommerer C, Budde K, et al. Barcelona consensus on biomarker-based immunosuppressive drugs management in solid organ transplantation. Ther Drug Monit. 2016; 38: S1-S20.
10. Eikmans M, Gielis EM, Ledeganck KJ, Yang J, Abramowicz D, Claas FF. Non-invasive biomarkers of acute rejection in kidney transplantation: Novel targets and strategies. Front Med. 2019; 5: 358.
11. Nissaisorakarn V, Lee JR, Lubetzky M, Suthanthiran M. Urine biomarkers informative of human kidney allograft rejection and tolerance. Hum Immunol. 2018; 79: 343-355.
12. Nauser CL, Farrar CA, Sacks SH. Complement recognition pathways in renal transplantation. J Am Soc Nephrol. 2017; 28: 2571.
13. Theocharis AD, Manou D, Karamanos NK. The extracellular matrix as a multitasking player in disease. FEBS J. 2019; 286: 2830-2869.
14. Xie CB, Jane-Wit D, Pober JS. Complement membrane attack complex: New roles, mechanisms of action, and therapeutic targets. Am J Pathol. 2020; 190: 1138-1150.
15. Ramos-Martinez JI. The regulation of the pentose phosphate pathway: Remember Krebs. Arch Biochem Biophys. 2017; 614: 50-52.
16. Bach LA. Recent insights into the actions of IGFBP-6. J Cell Commun Signaling. 2015; 9: 189-200.
17. Brittain AL, Kopchick JJ. A review of renal GH/IGF1 family gene expression in chronic kidney diseases. Growth Horm IGF Res. 2019; 48: 1-4.
18. Liso A, Venuto S, Coda AR, Giallongo C, Palumbo GA, Tibullo D. IGFBP-6: At the crossroads of immunity, tissue repair and fibrosis. Int J Mol Sci. 2022; 23: 4358.
19. Hu H, Kwun J, Aizenstein BD, Knechtle SJ. Noninvasive detection of acute and chronic injuries in human renal transplant by elevation of multiple cytokines/chemokines in urine. Transplantation. 2009; 87: 1814-1820.
20. Mahoney JA, Ntolosi B, DaSilva RP, Gordon S, McKnight AJ. Cloning and characterization of CPVL, a novel serine carboxypeptidase, from human macrophages. Genomics. 2001; 72: 243-251.
21. Pshezhetsky AV, Hinek A. Serine carboxypeptidases in regulation of vasoconstriction and elastogenesis. Trends Cardiovasc Med. 2009; 19: 11-17.
22. Navarrete M, Korkmaz B, Guarino C, Lesner A, Lao Y, Ho J, et al. Activity-based protein profiling guided identification of urine proteinase 3 activity in subclinical rejection after renal transplantation. Clin Proteomics. 2020; 17: 1-4.
23. Grundy RG, Pritchard J, Scambler P, Cowell JK. Loss of heterozygosity for the short arm of chromosome 7 in sporadic Wilms tumour. Oncogene. 1998; 17: 395-400.
24. Perez JD, Sakata MM, Colucci JA, Spinelli GA, Felipe CR, Carvalho VM, et al. Plasma proteomics for the assessment of acute renal transplant rejection. Life Sci. 2016; 158: 111-120.

25. Lingenhel A, Lhotta K, Neyer U, Heid IM, Rantner B, Kronenberg MF, et al. Role of the kidney in the metabolism of apolipoprotein A-IV: Influence of the type of proteinuria. J Lipid Res. 2006; 47: 2071-2079.
26. Kawakami R, Matsui M, Konno A, Kaneko R, Shrestha S, Shrestha S, et al. Urinary FABP1 is a biomarker for impaired proximal tubular protein reabsorption and is synergistically enhanced by concurrent liver injury. J Pathol. 2021; 255: 362-373.
27. Kamijo A, Kimura K, Sugaya T, Yamanouchi M, Hikawa A, Hirano N, et al. Urinary fatty acid-binding protein as a new clinical marker of the progression of chronic renal disease. J Lab Clin Med. 2004; 143: 23-30.
28. Mou S, Wang Q, Li J, Shi B, Ni Z. Urinary excretion of liver-type fatty acid-binding protein as a marker of progressive kidney function deterioration in patients with chronic glomerulonephritis. Clin Chim Acta. 2012; 413: 187-191.
29. Yang J, Choi HM, Seo MY, Lee JY, Kim K, Jun H, et al. Urine liver-type fatty acid-binding protein predicts graft outcome up to 2 years after kidney transplantation. In: Transplantation proceedings. Amsterdam, Holland: Elsevier; 2014. pp. 376-380.
30. Yepes-Calderón M, Sotomayor CG, Pena M, Eisenga MF, Gans RO, Berger SP, et al. Urinary liver-type fatty acid-binding protein is independently associated with graft failure in outpatient kidney transplant recipients. Am J Transplant. 2021; 21: 1535-1544.
31. Zuo N, Suzuki Y, Sugaya T, Osaki K, Kanaguchi Y, Wang L, et al. Protective effects of tubular liver-type fatty acid-binding protein against glomerular damage in murine IgA nephropathy. Nephrol Dial Transplant. 2011; 26: 2127-2137.
32. Ferguson MA, Vaidya VS, Waikar SS, Collings FB, Sunderland KE, Gioules CJ, et al. Urinary liver-type fatty acid-binding protein predicts adverse outcomes in acute kidney injury. Kidney Int. 2010; 77: 708-714.
33. Hui E, Yeung CY, Lee PC, Woo YC, Fong CH, Chow WS, et al. Elevated circulating pigment epithelium-derived factor predicts the progression of diabetic nephropathy in patients with type 2 diabetes. J Clin Endocrinol Metab. 2014; 99: E2169-E2177.
34. Chen H, Zheng Z, Li R, Lu J, Bao Y, Ying X, et al. Urinary pigment epithelium-derived factor as a marker of diabetic nephropathy. Am J Nephrol. 2010; 32: 47-56.
35. Seres I, Szentimrei R, Szentpéteri A, Varga VE, Harangi M, Fülöp P, et al. Pigment epithelium-derived factor (PEDF) and lipid parameters in patients with end stage renal failure before and after kidney allotrasplantation. Atherosclerosis. 2018; 275: e252.
36. Terawaki H, Yamagishi SI, Funakoshi Y, Matsuyama Y, Terada T, Nakayama K, et al. Pigment epithelium-derived factor as a new predictor of mortality among chronic kidney disease patients treated with hemodialysis. Ther Apheresis Dial. 2013; 17: 625-630.
37. Moskowitz Kassai E, Mackelaite L, Chen J, Patel K, Dadhania DM, Gross SS, et al. Excretion of anti-angiogenic proteins in patients with chronic allograft dysfunction. Nephrol Dial Transplant. 2012; 27: 494-497.
38. Sigdel TK, Kaushal A, Gritsenko M, Norbeck AD, Qian WJ, Xiao W, et al. Shotgun proteomics identifies proteins specific for acute renal transplant rejection. Proteomics Clin Appl. 2010; 4: 32-47.
39. Huang N, Zhang X, Jiang Y, Mei H, Zhang L, Zhang Q, et al. Increased levels of serum pigment epithelium-derived factor aggravate proteinuria via induction of podocyte actin rearrangement. Int Urol Nephrol. 2019; 51: 359-367.

40. Moroi M, Jung SM. Platelet glycoprotein VI: Its structure and function. Thromb Res. 2004; 114: 221-233.
41. Yamashita Y, Naitoh K, Wada H, Ikejiri M, Mastumoto T, Ohishi K, et al. Elevated plasma levels of soluble platelet glycoprotein VI (GPVI) in patients with thrombotic microangiopathy. Thromb Res. 2014; 133: 440-444.
42. Ballow A, Gader AM, Huraib S, Al-Husaini K, Mutwalli A, Al Wakeel J. Platelet surface receptor activation in patients with chronic renal failure on hemodialysis, peritoneal dialysis and those with successful kidney transplantation. Platelets. 2005; 16: 19-24.
43. Boulaftali Y, Mawhin MA, Jandrot Perrus M, Ho Tin Noé B. Glycoprotein VI in securing vascular integrity in inflamed vessels. Res Pract Thromb Haemostasis. 2018; 2: 228-239.
44. Devi S, Kuligowski MP, Kwan RY, Westein E, Jackson SP, Kitching AR, et al. Platelet recruitment to the inflamed glomerulus occurs via an αIIbβ3/GPVI-dependent pathway. Am J Pathol. 2010; 177: 1131-1142.