NAZARBAYEV UNIVERSITY

SCHOOL OF MEDICINE

URINARY PROTEIN PROFILING USING MASS SPECTROMETRY FOR DETECTION OF CKD BIOMARKERS

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Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the author's original work. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Signed

Zhalaliddin

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Abbreviations	Definition
A1AG2	Alpha-1-acid glycoprotein 2
A1BG	Alpha-1B-glycoprotein
A2GL	Leucine-rich alpha-2-glycoprotein
AMBP	Protein AMBP (alpha-1-microglobulin/bikunin precursor)
ATRN	Attractin
B2MG	Beta-2-microglobulin
CADH2	Cadherin-2
CD44	CD44 antigen
CD59	CD59 glycoprotein
CERU	Ceruloplasmin
CG	Control group
CKD	Chronic kidney disease
CLUS	Clusterin
CVD	Cardiovascular disease
CRNN	Cornulin
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
emPAI	Exponentially modified protein abundance index
EPI	Epidemiology Collaboration
ESKD	End-stage kidney disease
FBN1	Fibrillin-1
FDR	False discovery rate
FETUA	Alpha-2-HS-glycoprotein (fetuin-A)
ESR	Erythrocyte sedimentation rate
HBB	Hemoglobin subunit beta
GO	Gene Ontology
HGB	Hemoglobin
IC1	Plasma protease C1 inhibitor
IGHA1	Immunoglobulin heavy constant alpha 1
IGK	Immunoglobulin kappa light chain
IGKC	Immunoglobulin kappa constant
IGL1	Immunoglobulin lambda-1 light chain
IQR	Interquartile range
KDIGO	Kidney Disease: Outcomes Quality Initiative
KNG1	Kininogen-1
KVD20	Immunoglobulin kappa variable 3D-20
LC	Liquid chromatography
LG3BP	Galectin-3-binding protein
LMAN2	Vesicular integral-membrane protein VIP36
LV39	Immunoglobulin lambda variable 3–9

LYVE1	Lymphatic vessel endothelial hyaluronic acid receptor 1
MDRD	Modification of Diet in Renal Disease
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NSMC	National Scientific Medical Center
OSTP	Osteopontin
P3IP1	Phosphoinositide-3-kinase-interacting protein 1
PGRP1	Peptidoglycan recognition protein 1
PLT	Platelet
PTECs	Proximal tubular epithelial cells
PTGDS	Prostaglandin-H2 D-isomerase
RBC	Red blood cell
REG1A	Lithostathine-1-alpha
RNAS1	Ribonuclease pancreatic
SAP	Prosaposin
SGLT2	Sodium-glucose co-transporter 2
SH3L3	SH3 domain-binding glutamic acid-rich-like protein 3
THRB	Prothrombin
UROM	Uromodulin
VMO1	Vitelline membrane outer layer protein 1 homolog
VTDB	Vitamin D-binding protein
VTNC	Vitronectin
WBC	White blood cell

Abstract

Proteinuria is a significant risk factor for the progression of chronic kidney disease (CKD) and its complications. The kidneys excrete various individual proteins, some of which may affect CKD progression or serve as biomarkers for disease severity. However, limited data exist regarding specific urinary proteins and their relationship with CKD severity. This thesis aimed to investigate the urinary proteome in CKD patients and healthy controls, examining its connection to kidney function and proteinuria to identify potential diagnostic and prognostic proteins associated with CKD progression in two cohorts.

In the initial cross-sectional study, urine samples from 137 individuals (88 CKD patients and 49 healthy controls) were analyzed using mass spectrometry-based proteomics. Protein identification was conducted using Mascot software with the SwissProt database, and statistical analyses included both parametric and non-parametric methods. Peptide quantification was performed using the exponentially modified protein abundance index (emPAI), and regression analyses were conducted to assess associations between urinary proteins and estimated glomerular filtration rate (eGFR), adjusting for proteinuria. A total of 704 individual urinary proteins were detected across the entire cohort, with distinct differences observed between patients and healthy individuals. The emPAI demonstrated a significant variation in total protein levels between CKD patients and controls (p = .007). Notably, specific urinary proteins, including AMBP, VTDB, FETUA, and B2MG, exhibited negative associations with kidney function in CKD patients. In contrast, UROM, SH3L3, RNAS1, OSTP, LV39, KNG1, CERU, CD59, CD44, and A1AG2 were positively associated with kidney function across the entire cohort, while CERU, A1BG, and LV39 consistently demonstrated positive associations in CKD patients compared to controls. These findings suggest that specific urinary proteins can be markers of kidney dysfunction or preservation in CKD.

A follow-up study was conducted to further investigate urinary proteomics and its prognostic potential in the progression of CKD among a cohort of 18 CKD patients and 15 healthy individuals. The study revealed significant dynamic shifts in protein associations with eGFR over time. At baseline, urinary proteomic profiles showed distinct differences between CKD patients and controls. FBN1 exhibited a positive correlation with eGFR, while FETUA demonstrated a significant negative association. Over time, VTDB shifted from a negative to a positive correlation with eGFR, whereas FBN1 and CD44 transitioned from a positive to a negative association. These results emphasize CD44, FBN1, and VTDB as promising prognostic biomarkers, offering insights into disease progression.

This comprehensive analysis of urinary proteomics highlights the importance of specific proteins as diagnostic and prognostic indicators in CKD. The evolving relationships between urinary proteins and kidney function underscore their potential utility in CKD diagnosis, progression monitoring, and therapeutic interventions.
Keywords: chronic kidney disease, urinary proteomics, biomarker, proteinuria, VTDB

Chapter 1: Introduction

1.1. Background

1.1.1. Chronic Kidney Disease and Proteinuria

Chronic kidney disease (CKD) is a progressive disease characterized by a gradual decline in kidney function and structural deterioration (Levin et al., 2013; Zoccali et al., 2017). CKD is primarily diagnosed using kidney function or damage markers that persist for more than three months, such as serum creatinine-based estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73 m² or proteinuria > 150 mg/24 hours (albuminuria > 30 mg/24 hours), respectively (Levin et al., 2013). The eGFR level and the degree of proteinuria/albuminuria determine the severity of CKD.

Proteinuria refers to an increased total protein level in the urine, whereas albuminuria specifically denotes the abnormal excretion of albumin. However, it should be mentioned that in clinical practice, the presence of total protein that exceeds 150 mg/24 hours on a urinalysis test is referred to as microalbuminuria, while protein levels greater than 500 mg/24 hours are classified as proteinuria (see Table 1). Albumin is the most abundant large serum protein in normal urine; its normal concentration is below 30 mg/24 hours. Increased albumin levels serve as a marker of glomerular damage, whereas increased excretion of low-molecular-weight proteins indicates tubular dysfunction. A recent review discussed the individual markers in more detail (Mizdrak et al., 2022).

Table 1

Proteinuria Classification

	Units	Proteinuria categories Description and range		
		Normal range	Moderately increased	Severely increased
			Microalbuminuria	Proteinuria
Protein excretion rate	mg/24-h	< 150	150–499	> 500
Albumin excretion rate	mg/24-h	< 30	30–299	> 300

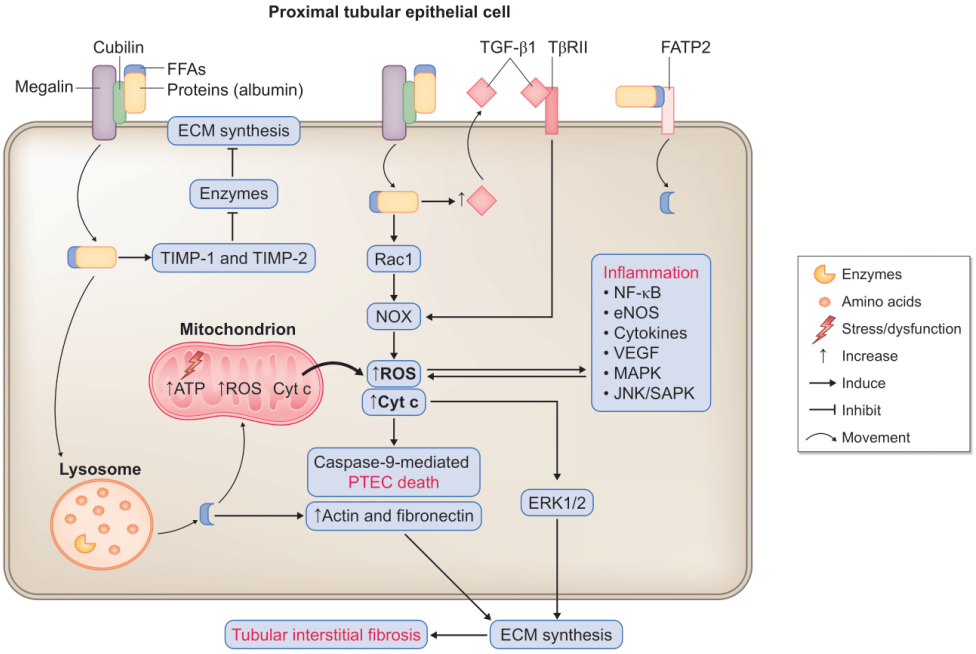
The pathogenesis of proteinuria involves two main renal mechanisms. The first is the dysfunction of the glomerular filtration barrier, which allows the abnormal passage of plasma proteins, thereby resulting in glomerular proteinuria (Haraldsson, Nyström, & Deen, 2008). Persistent glomerular protein leakage places pathological stress on PTECs, activating cytotoxic and inflammatory pathways that work together to promote chronic tubulointerstitial inflammation and fibrosis (Makhammajanov et al., 2024a). Subsequently,

the second mechanism is a reduced tubular reabsorptive capacity, leading to impaired retrieval of filtered proteins and causing tubular proteinuria (Dickson et al., 2014; Weisz, 2021). Despite advancements in nephrology, clinical diagnostics mainly depend on a limited panel of urinary proteins, such as κ/λ light chains and albumin (Katzmann et al., 2005; Kuritzky, Toto, & Van Buren, 2011). However, a wide variety of other urinary proteins either escape detection or lack standardized clinical application, limiting their potential diagnostic utility.

Proteinuria is a widely used clinical biomarker for CKD and its progression. It is also considered a risk factor for CKD progression and associated outcomes in both non-diabetic and diabetic individuals (Cravedi & Remuzzi, 2013). Abnormal filtration of plasma proteins through the glomerulus leads to cytotoxic effects on proximal tubular epithelial cells (PTECs) during reuptake, thereby causing tubulointerstitial injury, inflammation, fibrosis, and tubular atrophy (Ledingham, 1990; Perico, Benigni, & Remuzzi, 2019). Unfortunately, these proteinuria-induced pathological events irreversibly lead to end-stage kidney disease (ESKD) and nonfunctioning kidneys (Liu & Lv, 2019).

Because of the tubular toxicity of filtered plasma proteins, the rapid progression of CKD is often correlated and expected in individuals with higher proteinuria levels (Chen et al., 2016; Go et al., 2018; Krolewski et al., 2017). However, some patients with higher proteinuria levels experience gradual kidney function decline, whereas patients with low proteinuria levels may have rapid CKD progression (Krolewski et al., 2017). This variability may be due to differences in the toxic properties and concentrations of filtered proteins affecting PTECs, which may be influenced by factors such as their cell origin, triggers for secretion, biological functions, molecular mass, reabsorptive capacity, protein-bound molecular moieties (Makhammajanov et al., 2024a; Perico, Benigni, & Remuzzi, 2019). For instance, it has been suggested that filtered albumin can enhance cellular reactive oxygen species production, leading to tubulointerstitial inflammation, and stimulate endothelin-1, which contributes to tubulointerstitial fibrosis (Figure 1) (Liu et al., 2018; Makhammajanov et al., 2024a). We recently discussed the tubular toxicity of filtered proteins in detail in a review of the literature (Makhammajanov et al., 2024a).

Figure 1



Note. *Albuminuria (Proteinuria) Induced Reactive Oxygen Species Activation and its Effect on PTECs.* Reprinted from "Tubular toxicity of proteinuria and the progression of chronic kidney disease", by Z. Makhammajanov, A. Gaipov, A. Myngbay, R. Bukasov, M. Aljofan, & M. Kanbay, 2024, *Nephrology Dialysis Transplantation, 39*(4), 589-599 (https://doi.org/10.1093/ndt/gfad215). Copyright 2023 by the Authors.

1.1.2. Biomarkers and Their Importance

Biomarkers are objectively measurable biological markers that belong to a wide range of subcategories of medical signs and are quickly and accurately reproduced under appropriate medical conditions (Strimbu & Tavel, 2010). Biomarkers provide important insights into physiological and pathophysiological states and are instrumental for early diagnosis, prognosis, and treatment evaluation (Biomarkers Definitions Working Group, 2001; Strimbu & Tavel, 2010). For example, in hospitals, biomarkers help to diagnose diseases in different stages and assess the effects of treatments on the body, along with clinical outcomes. Common biomarkers that are effective in hospital practice include body temperature, blood pressure, body mass index, X-ray images, complete blood count results, and urinalysis results such as proteinuria. Besides, in clinical trials, they are used as surrogate endpoints to measure treatment efficacy (Biomarkers Definitions Working Group, 2001).

Identifying reliable biomarkers is crucial, given the importance of early diagnosis in CKD management. Biomarkers not only improve diagnostic accuracy but also enable timely interventions that can slow or reverse the disease progression. For example,

reducing proteinuria to 30% has been shown to decrease the risk of CKD progression to ESKD by 23.7%, regardless of the drug class used (Heerspink et al., 2015).

1.2. Problem Statement

1.2.1. The Global Burden of Chronic Kidney Disease

Recently, CKD was found to be present in approximately 10% of adults in developed countries (Sundström et al., 2022). In the general population, CKD is most common in individuals over 65 years old, primarily due to comorbidities and age-related declines in kidney function, with cumulative damage contributing to its multifactorial development (Hill et al., 2016). In the last three decades, the CKD-associated mortality rate has been observed with a yearly increase of up to nearly 1% (Kassebaum et al., 2016; Thomas et al., 2017; Wang et al., 2016). This disease is also among the leading causes of death worldwide, accounting for over 1.2 million deaths in 2017 (Carney, 2020). In the same year, CKD was ranked 12th among the leading causes of death worldwide (Carney, 2020). However, it is estimated that CKD may rise to the fifth rank by 2040 (Foreman et al., 2018).

One of the main reasons for the steady increase in CKD-associated mortality is the rising incidence of CKD, driven by an ageing population and the increasing prevalence of CKD-causing conditions, including diabetes mellitus and arterial hypertension (Gansevoort et al., 2013). Diabetes mellitus, cardiovascular disease (CVD), and arterial hypertension are the primary risk factors for CKD (Webster et al., 2017). Another key factor contributing to increased mortality is the late-stage diagnosis of CKD, mainly in stages 3–5 (Carpio et al., 2022).

1.2.2. Limitations of Clinical Markers

Serum creatinine-based eGFR is the most commonly used diagnostic marker for assessing kidney function in everyday clinical practice (Perrone, Madias, & Levey, 1992). The eGFR is calculated using formulas such as the CKD Epidemiology Collaboration (CKD-EPI) and the Modification of Diet in Renal Disease (MDRD). These formulas can be inaccurate, particularly in early-stage CKD patients with normal or near-normal kidney function, although the CKD-EPI equation is generally more accurate than the MDRD (Stevens et al., 2010). Still, the CKD-EPI equation has limitations due to the fact that creatinine levels are influenced by factors such as muscle mass, diet, medications, and variability in kidney tubular secretion, leading to inaccuracies in certain populations, including older adults, where age-related declines in kidney function can result in over diagnosis of CKD. Also,

individuals with low muscle mass (e.g., older adults and those with malnutrition or special diets) may have an underestimated eGFR, while those with higher muscle mass can experience an overestimation (Nankivell et al., 2020).

In addition to eGFR, proteinuria is another commonly used and established clinical marker for CKD. However, its effectiveness as an early diagnostic tool is limited due to variability in the types and concentrations of proteins excreted and sensitivity issues, particularly when protein levels are low (Lamb, MacKenzie, & Stevens, 2009; Sumida et al., 2020). Despite its use in clinical practice, the limitations of proteinuria highlight the need for more specific urinary protein biomarkers to detect CKD in its early stages. These limitations mean that relying solely on eGFR and proteinuria can delay the diagnosis of CKD, potentially missing the optimal window for treatment.

Unfortunately, initiating treatment during the later stages of CKD imposes a significant financial burden. At this stage, treatment may not significantly improve outcomes or prevent complications such as ESKD and CVD. Therefore, early intervention is essential to improving patient outcomes and reducing healthcare costs associated with advanced CKD.

1.3. Research Gap

Previous proteomic investigations have identified numerous potential biomarkers for CKD; however, translating these findings into clinical practice has remained challenging. Furthermore, these studies frequently focused on analyzing blood proteins (Ramírez Medina et al., 2023) or spot urine samples (Choi et al., 2017; Good et al., 2010;), which may limit their representativeness and may not fully capture the dynamic nature of CKD-related proteomic changes (Catanese et al., 2023).

1.4. Hypothesis

Urine is a noninvasive and easily accessible biological fluid containing over a thousand proteins. Exploring its proteomic composition through mass spectrometry offers significant potential to advance our understanding of kidney diseases. We hypothesize that specific urinary biomarkers can enable the early detection of CKD and reflect dynamic proteomic changes associated with kidney damage. By identifying these biomarkers, we aim to improve the diagnosis and prognosis of CKD and enhance our understanding of its progression.

1.5. Study Aims

The primary aim of this study was to identify specific urinary biomarkers that could be used in clinical practice for the early detection of CKD. By comparing the urinary protein profiles of CKD patients in stages 1–3 and healthy individuals, we aimed to uncover proteome patterns that could facilitate early diagnosis and intervention, ultimately improving CKD management.

The secondary aim was to follow up with participants to evaluate the association between urinary protein profiles and changes in eGFR over time. Through this analysis, we aimed to identify prognostic biomarkers that could reliably predict CKD progression and contribute to the development of improved diagnostic and monitoring strategies for CKD.

1.6. Research Objectives

1.6.1. *Main Study*

- Compare urinary protein profiles between CKD patients (stages 1–3) and healthy participants.
- Examine associations between urinary proteins and kidney function (eGFR) using linear regression and Spearman correlation.
- Identify specific urinary biomarkers that distinguish CKD patients from healthy individuals.
- Perform pathway analyses to identify biological and pathological processes related to the identified urinary biomarkers.

1.6.2. *Follow-up Study*

- Track changes in eGFR over time in CKD patients and healthy participants.
- Assess associations between changes in urinary protein profiles and changes in eGFR using linear regression and Spearman correlation.
- Identify urinary biomarkers that prognosis CKD progression and decline in kidney function.

1.7. Relevance and Significance

Early detection and intervention are crucial for preserving kidney function and improving health outcomes in CKD patients. Effective management strategies, including lifestyle modifications and targeted medications, can slow or even halt the progression of CKD (Kalantar-Zadeh et al., 2021; Yan & Cheng, 2021). Lifestyle changes such as adopting a plant-based, low-salt, and low-protein diet may prevent glomerular hyperfiltration and promote kidney health. Medications, such as sodium-glucose co-transporter 2 (SGLT2) inhibitors and renin-angiotensin-aldosterone system (RAAS) modulators, have been shown to reduce intraglomerular pressure and proteinuria, independently of blood glucose and

blood pressure levels. Additionally, newer drugs, such as nonsteroidal mineralocorticoid receptor antagonists, show potential for kidney protection through antifibrotic and anti-inflammatory actions, as well as for improving disease outcomes (Kalantar-Zadeh et al., 2021).

In advanced stages, especially ESKD, kidney replacement therapy—such as dialysis or transplantation—becomes necessary. Therefore, early screening and detection of CKD in its early stages are essential for slowing or halting its progression and preventing further kidney damage. Biomarkers play an important role in enabling early diagnosis and effective disease management. Identifying reliable urinary biomarkers could lead to more effective CKD management and reduce the global burden of this disease.

Chapter 2: Materials and Methods

In this study, we employed a cross-sectional design, beginning with an initial pilot analysis of 42 participants. Subsequently, an entire cohort of 137 participants was analyzed. Of these, 33 participants were followed up for an extended period to investigate longitudinal changes. Unless otherwise specified, the procedures, analyses, and results refer to the whole cohort as the main study. Specific distinctions are made when discussing the pilot or follow-up study methodologies and results.

2.1. Patients and Study Design

The study involved 49 healthy control participants and 88 CKD patients with proteinuria. The control and patient groups were age-matched, with a mean age of 37.2 years (*SD* = 7.9) and 38.6 years (*SD* = 12.3), respectively. The control group consisted of 31% males and 69% females, while the patient group consisted of 52% males and 48% females. All study participants were recruited from March 2020 until the end of 2022 by practicing physicians at the National Scientific Medical Center (NSMC, Astana, Kazakhstan). These physicians worked at the NSMC and recruited the study participants from their routine outpatient and inpatient consultations. Before enrollment, the participants were familiarized with study protocols and procedures by physicians and gave informed consent (refer to Appendix A for the informed consent form).

The inclusion criteria were any ethnic male and female individuals with ≥ 18 and ≤ 70 years and CKD patients with stages 1–3. Specifically, participants were Kazakhstani citizens from the Kazakh, Russian, and Uzbek ethnic groups, according to the government census categories. CKD was defined by physicians based on clinical evaluation, kidney function, and kidney damage markers, including proteinuria and hematuria. As a kidney function evaluation, serum creatinine-based estimated glomerular filtration rate by the CKD-EPI equation was calculated with an updated formula in 2021 (Delgado et al., 2022). Patients were categorized into CKD stages 1, 2, and 3 using eGFR according to Kidney Disease: Outcomes Quality Initiative (KDIGO) 2012 CKD guidelines (Levin et al., 2013). The patients included in the study were diagnosed with glomerular illnesses based on the eGFR, protein level in urine (proteinuria > 150 mg/24-hour), and clinical evaluation. However, a kidney biopsy was unavailable to confirm the diseases due to a technical issue. Besides, the control group was defined by selecting participants without clinical or laboratory indicators of chronic kidney disease.

In addition, the following criteria were applied to exclude participants from the analyses: < 18 and > 70 years old individuals, eGFR < 60 mL/min for healthy participants, and eGFR < 30 mL/min for patients. Also, individuals with acute infectious diseases, cancer, other life-threatening comorbid diseases, and pregnant females were excluded from the control and CKD groups.

2.2. Urine Sample Collection

All participants were given instructions for spot and 24-hour urine collection (refer to Appendix B).

2.3. Laboratory Tests and Data Collection

The participants were instructed to collect mid-stream early morning and 24-hour urine samples. In addition, hospital nurses collected blood samples from all participants during hospital admission or visits of participants to the outpatient clinic of the NSMC. The sample collection was performed without regard to patient outcomes. Participants were informed about the urine and blood sample collection procedures by physicians during the enrollment.

All hospital laboratory investigators and researchers involved in the project were blinded to the clinical outcome data of the patients. The laboratory investigators conducted urinalysis, urine biochemical analysis, and blood tests for metabolic and complete blood count parameters. Obtained blood metabolic parameters for all participants were total protein, creatinine, uric acid, urea, lipids, and glucose. Early morning urine samples were used for complete urinalysis but only results from the 24-hour urine samples, including biochemical (e.g., proteinuria) and proteomics analyses, were used in the thesis. All clinical laboratory analyses were carried out on a COBAS Integra 400 plus instrument (Roche Diagnostics, Indianapolis, Indiana, United States) using a colorimetric method.

Hospital physicians recorded medical history, demographic data, and comorbid diseases from medical records and patients directly during outpatient and inpatient medical check-ups after participant interviews. After removing the personally identifiable information, the hospital transferred the recorded data to the project's principal investigator.

2.4. Sample Preparation

After the clinical laboratory tests, the participants' remaining 24-hour urine, spot urine, and blood samples were delivered to NUSOM and stored at -80°C in 1 mL, 5 mL, and 50 mL

tubes. Next, mass spectrometry analysis was conducted using 10 mL aliquots of 24-hour urine samples at the National Center for Biotechnology (NCB) in Astana. During the sample preparation for mass spectrometry, an acetone precipitation method was used to extract proteins from 10 mL of 24-urine samples using the protocol described by Sun and Gao (2009). In brief, samples were mixed with a protease inhibitor (1 mM phenylmethanesulfonyl fluoride), centrifuged at 3000g for 1 hour at 4°C, and supernatants were precipitated with cold acetone overnight at 4°C. Extracted final protein pellets were resuspended in 25 mM ammonium bicarbonate and stored at -80°C until further use. Next, following the manufacturer's instructions, each sample's total urine protein concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, United States). Then, a concentration of proteins between 30 and 50 μg was used for in-solution protein digestion.

2.5. In Solution Protein Digestion

The proteins were reduced using 5 mM dithiothreitol at 60°C for 20 minutes to break disulfide bonds, followed by alkylation with 100 mM iodoacetamide at 37°C for 20 minutes to prevent reformation of these bonds. Total urinary protein was digested with 20 ng/μL trypsin at 37°C overnight. The resulting peptides were purified and concentrated using 0.6 μL C18 ZipTip reverse-phase tips (Millipore, Burlington, Massachusetts, United States) to ensure the effective removal of contaminants and optimal peptide recovery. The eluted peptides were then concentrated using a SpeedVac concentrator (Eppendorf, Hamburg, Germany) and subsequently resuspended in 16 μL of 0.1% trifluoroacetic acid to prepare them for analysis.

2.6. Mass Spectrometry Analysis

Fifteen and a half microliters of the resuspended sample was transferred to a liquid chromatography vial and loaded into the autosampler of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. A nanoflow reversed-phase C18 LC-MS/MS was used to analyze the study's samples. A trapping column, specifically an Acclaim PepMap100 C18 pre-column, was used for additional peptide clean-up and concentration prior to liquid chromatography separation. A Dionex high-performance liquid chromatography pump was used for chromatography. The peptides were separated over a 75-minute multistep acetonitrile gradient at a flow rate of 0.3 mL/min, using an Acclaim PepMap100 C18 Rapid Separation Liquid Chromatography (RSLC) column (Thermo Scientific, Waltham, Massachusetts, United States). After separation, ionization was

achieved using a whole captive spray ion source (capillary voltage: 1500 V, dry temperature: 150°C, dry gas flow rate: 3.0 L/min), coupled with the Impact II Electrospray Ionization Quadrupole Time-of-Flight mass spectrometer (Bruker Daltonics, Bremen, Germany). Full-scan mass spectra were acquired at a rate of 2.0 Hz, followed by a single MS/MS spectrum. The MS/MS data were then analyzed using Data Analysis 3.4 (Bruker Daltonics, Bremen, Germany) and saved in Mascot generic format (*.mgf).

Next, proteins and peptides were identified using saved .mgf data, which was searched via the Mascot 2.6.1 software (Matrix Science, London, United Kingdom) against the Swiss-Prot database (UniProt Consortium, 2021). The Swiss-Prot protein database was taxonomically restricted to *Homo sapiens* (human) which at release time contained 20,396 sequences. The Mascot software search parameters included carbamidomethylation of cysteine as a fixed modification (due to the introduction of alkylation during the sample preparation) and methionine oxidation as a variable modification. A maximum of two missed tryptic cleavages of proteins was allowed. Peptide and protein identification thresholds were set at $p < .05$ and $p < .001$. The false discovery rate (FDR) was estimated using a decoy database and controlled at 1%. Mass error tolerances were set to 100 ppm for MS and 0.05 Da for MS/MS. Peptide quantification was performed using the Exponentially Modified Protein Abundance Index (emPAI) method (Ishihama et al., 2005). Thus, the emPAI was calculated for the detected proteins in the Mascot software.

The proteomic dataset was processed using log2 transformation followed by normalization based on average and slope adjustments to ensure data consistency and reliability for further analysis.

2.7. Gene Ontology Enrichment Analysis

To gain functional insights into excreted urinary proteins, Gene Ontology (GO) enrichment analysis was performed using the clusterProfiler (Wu et al., 2021) and org.Hs.eg.db (Carlson, 2022) packages in R to identify significant GO terms for proteins positively and negatively correlated with eGFR, adjusting for proteinuria. The enrichGO function was applied to categorize proteins based on their enrichment in molecular functions, cellular components, and biological processes. Proteins from both correlation and regression analyses were included. An FDR threshold of .05 was applied to determine significant enrichment.

2.8. Reactome Pathway Analysis

Pathway enrichment analysis was conducted using the ReactomePA package (Yu & He, 2016) in R to identify biological pathways associated with proteins exhibiting positive or negative eGFR correlations after adjusting for proteinuria. The enrichPathway function was utilized to detect significantly enriched pathways, applying *p* and *q* values with a statistical threshold of .05. The analysis included proteins identified through both correlation and regression tests. This approach allowed for identifying molecular pathways that might be involved in kidney function and disease progression.

2.9. Protein-Protein Interaction Networks

Protein-protein interaction networks were analyzed using STRING-DB (version 12) (Szklarczyk et al., 2019). Gene identifiers for the proteins that positively and negatively correlated with eGFR (adjusted for proteinuria) were used for input into the web platform of the STRING. This facilitated the identification of predicted and known interactions, incorporating data from different sources, such as text mining, co-expression patterns, and experimental studies from scientific literature. This analysis contributed to a comprehensive understanding of how these proteins interact in biological systems associated with kidney function.

2.10. Statistical Analyses

All statistical analyses were conducted using different versions of Stata MP2 and R software. For the pilot study, Stata MP2 version 16.1 was used. For the main study, Stata MP2 version 18.0 and R version 4.3.0 were used, while the follow-up study was performed using Stata MP2 version 18.0 and R version 4.4.0. Descriptive statistics for continuous variables were reported as mean ± standard deviation (*SD*) for normally distributed data and as the median with interquartile range (IQR) for non-normally distributed data.

Two-sided *t*-tests were applied to compare continuous variables between CKD patients and healthy control participants for parametric data, and the Wilcoxon rank-sum test was used for non-parametric data. For comparisons across multiple CKD stage groups and the control group, one-way ANOVA was used for normally distributed data, and the Kruskal-Wallis test was used for non-normally distributed data. Categorical variables were analyzed using the chi-square test and reported as frequencies and percentages.

Correlations between kidney function (measured by eGFR) and the urinary proteome (quantified as emPAI values) were evaluated using Spearman's rank correlation test. Additionally, we performed linear regression analysis between eGFR and urinary proteome with and without adjustment for the effect of 24-hour proteinuria to identify

kidney function-associated urinary proteins.

2.10.1 Follow-up study

The relationship between proteomic profiles and eGFR was examined using Spearman's rank correlation. To further investigate the association between protein levels and eGFR, linear regression models were constructed. In each model, eGFR served as the dependent variable, while the emPAI value of each protein was the independent variable. The models were adjusted for proteinuria as a covariate. To assess changes over time, an interaction term between protein levels and the follow-up period was included. Regression results were presented as regression coefficients, *p* values, standard errors, and *t*-statistics. A *p* value of $< .05$ was considered statistically significant. Additionally, a power analysis based on the study sample size of 33 participants (18 CKD patients and 15 controls) yielded a power estimate of 0.99 for detecting effects on the primary outcome measure, eGFR.

2.11. Data visualization

Data visualizations were performed using the ggplot2 package (Wickham & Wickham, 2016) in R. For the main study, a heatmap was generated to illustrate the presence and distribution of proteins in control and CKD stage 1–3 groups. Volcano plots were constructed to display the relationship between eGFR and urinary proteins, showing effect sizes and significance levels. Also, the most enriched Reactome pathways and GO terms were visualized to highlight key biological pathways and processes. Bar plots were created to illustrate the top significantly enriched pathways for proteins negatively and positively associated with kidney function. For improved visual clarity, results were presented on a -log10 scale.

In the follow-up study, a heatmap was generated to compare the distribution of the urinary proteome between CKD and control groups at baseline and follow-up time points. To illustrate longitudinal changes, line plots were created to depict changes in KDIGO CKD stages, eGFR, and 24-hour total protein levels across these two time points. Additionally, interaction plots were generated to examine the relationship between emPAI values of urinary proteome and eGFR by highlighting their significant associations and corresponding trends.

2.12. Ethical Considerations

Written informed consent was obtained from all participants before they were enrolled in the study. The study protocol was thoroughly reviewed and received ethical approval from

the Nazarbayev University Institutional Review Ethics Committee (approval number: NU-IREC 208/06122019, approval date: February 25, 2020). In addition, this study is registered on ClinicalTrials.gov under the identifier NCT04311684 and has a registration date of March 17, 2020. All research activities were conducted in full compliance with the ethical principles of the Declaration of Helsinki for research involving human participants. All laboratory procedures strictly adhered to good clinical and laboratory practices to ensure that participant rights, safety, and well-being were prioritized. All procedures, including patient recruitment, data collection, and sample analysis, were conducted carefully to maintain confidentiality and data integrity.

Chapter 3: Results

We initially analyzed 42 participants, comprising 21 individuals with early-stage CKD and 21 healthy controls, as reported by Gaipov et al. (2022). This preliminary analysis provided valuable insights, and its findings were largely consistent with those of the main study. Therefore, to maintain conciseness, this thesis presents results from the full cohort of 137 participants, which included 88 CKD patients and 49 healthy controls. The findings of the main study were published by Makhammajanov et al. (2024b).

In addition, a subset of 33 participants (18 CKD patients and 15 controls) was followed over an extended period to assess longitudinal changes in kidney function and urinary proteome profiles. The findings from this follow-up study were recently published by Makhammajanov et al. (2025) and are presented separately in this thesis to highlight changes over time and provide insights into disease progression.

3.1. Clinical and Biochemical Profiles of the Study Cohort

3.1.1. Main Study

Table 2 presents an overview of all participants classified into CKD risk levels based on eGFR and proteinuria levels, following the KDIGO 2012 CKD guidelines (Levin et al., 2013). Specifically, 45.3% of the total participants were identified as having a low to moderately increased risk of progressing to kidney failure, the majority of whom were healthy controls. Conversely, the remaining 54.7% of participants (i.e., patients) were identified as having a high to very high risk of progressing to ESKD. For further analysis, CKD patients were classified into CKD stages 1, 2, and 3 based on eGFR.

Table 2

KDIGO CKD Classification of Participants

Predicting the prognosis of CKD by eGFR and proteinuria categories: KDIGO 2012				Persistent proteinuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				< 150 mg/24-h	150–499 mg/24-h	≥ 500 mg/24-h
eGFR categories (mL/min/1.73m²) Description and range	G1	Normal or high	≥ 90	33 (24.1%)	21 (15.3%)	31 (22.6%)
	G2	Mildly decreased	60–89	6 (4.4%)	2 (1.5%)	20 (14.6%)
	G3a	Mildly to moderately decreased	45–59	0	1 (0.7%)	12 (8.7%)
	G3b	Moderately to severely decreased	30–44	11 (8%)	0	0
	G4	Severely decreased	15–29			
	G5	Kidney failure	< 15			

Note. Green color represents a low risk; yellow represents a moderately increased risk; orange represents a high risk; and red represents a very high risk. A = albuminuria (in our study, it is proteinuria); CKD = chronic kidney disease; eGFR = estimated glomerular filtration rate; G = glomerular filtration rate (estimated); KDIGO = Kidney Disease: Improving Global Outcomes. Adapted from "Candidate protein biomarkers in chronic kidney disease: a proteomics study", by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P. Kovesdy, S. H. Abidi, & A. Gaipov, 2024, *Scientific Reports*, *14*(1), Article 14014 (https://doi.org/10.1038/s41598-024-64833-8). Copyright 2024 by the Authors.

Table 3 presents the overall characteristics of the study participants. The control and patient groups were matched for age, with mean ages of 37.2 years (*SD* = 7.9) for the controls and 38.6 years (*SD* = 12.3) for the patients. However, the gender distribution differed substantially, with females comprising 69% of the control group compared to 48% of the patient group.

Key clinical and biochemical parameters showed notable differences among the groups (Table 3). Kidney function and damage markers deteriorated significantly with advancing CKD stages. Specifically, the eGFR was markedly reduced ($p < .001$), and proteinuria levels increased dramatically, rising from micrograms to several grams as CKD progressed ($p < .001$). Meanwhile, serum creatinine and serum urea levels were elevated ($p < .001$ for both), indicating impaired renal filtration.

Metabolic dysregulation was also evident in CKD patients. Significant changes were observed in metabolic blood markers, including serum total protein, total cholesterol, and serum uric acid ($p < .001$ for all), highlighting the extensive impact of CKD on metabolic profiles. Furthermore, inflammatory activity was heightened in CKD patients. White blood cell (WBC) counts and platelet (PLT) levels were significantly higher compared to controls ($p < .001$), and the erythrocyte sedimentation rate (ESR) was markedly elevated ($p < .001$), consistent with systemic inflammation.

Other parameters, such as hemoglobin (HGB) and red blood cell (RBC) counts, did not show significant differences between groups ($p = .239$ and $p = .205$, respectively). However, the prevalence of anemia increased substantially in advanced CKD stages, affecting 33% of stage 3 patients compared to 12% in stage 1. Hypertension was also more prevalent in the later stage, occurring in 37% of stage 1 patients and rising to 42% in stage 3, highlighting the growing cardiovascular burden associated with CKD progression.

Table 3

Clinical and Biochemical Characteristics of Participants

Parameters	CG, *n* = 49	CKD stage 1, *n* = 40	CKD stage 2, *n* = 24	CKD stage 3, *n* = 24	*p*
Demographics					
Age, year	36 (31–42)	33 (25–44)	39 (32–50)	41 (36–49)	.065
Gender, female, n (%)	34 (69)	24 (60)	7 (29)	7 (29)	**.001**
eGFR, mL/min/1.73 m^2	110 ± 13	116 ± 14	72 ± 9	45 ± 10	**< .001**
CKD etiology					
Glomerular, *n* (%)		32 (80)	16 (67)	17 (71)	
Transplant, *n* (%)		1 (3)		3 (12)	
Diabetic, *n* (%)		1 (3)	1 (4)	1 (4)	
Lupus, *n* (%)		2 (5)	1 (4)		
CKD of unknown etiology, *n* (%)		4 (10)	6 (25)	3 (12)	
Comorbidities					
Hypertension, *n* (%)	4 (8)	15 (37)	9 (37)	10 (42)	
Anemia, *n* (%)	2 (4)	5 (12)	2 (8)	8 (33)	
Laboratory data					
WBC 10 × 10^9/L	5.8 (5.1–6.4)	7.4 (5.6–11.6)	7 (5.7–9.8)	7.2 (6.2–8.9)	**< .001**
PLT 10 × 10^9/L	240.9 ± 58.4	306.6 ± 70.8	266.6 ± 71.1	265.4 ± 60.9	**< .001**

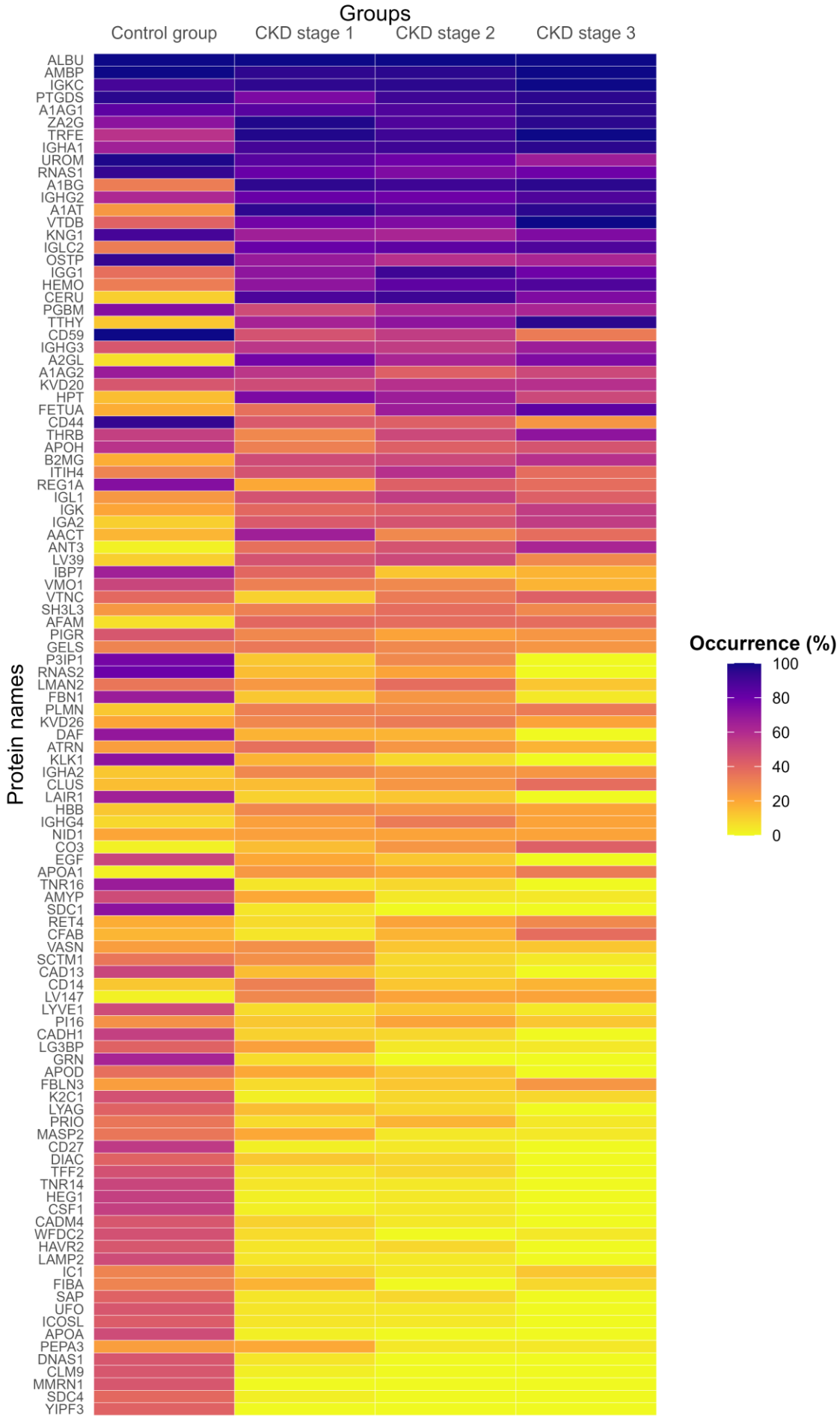
Parameters	CG, *n* = 49	CKD stage 1, *n* = 40	CKD stage 2, *n* = 24	CKD stage 3, *n* = 24	*p*
RBC 10 × 10^{12}/L	4.7 ± 0.5	4.5 ± 0.5	4.7 ± 0.8	4.5 ± 0.8	.205
HGB g/L	137 ± 17.9	130.9 ± 19.6	138 ± 24.2	128.3 ± 24.6	.239
ESR, mm/h	8 (5–12)	24 (14–35)	19 (7–30)	18.5 (11–41)	**< .001**
Serum total protein, g/L	69.8 ± 3.4	58.9 ± 10.1	60.7 ± 11.2	57.9 ± 15.1	**< .001**
Total cholesterol, mmol/L	4.7 (4.2–5.1)	6.3 (4.1–7.2)	6 (4.8–7.1)	5.7 (5–6.5)	**< .001**
Serum creatinine, µmol/L	63.9 (55.3–71.8)	61.5 (49.3–69.9)	106.6 (94.9–124.4)	155.3 (139.7–169.7)	**< .001**
Serum uric acid, µmol/L	308.3 ± 93.8	314.8 ± 86.4	414.5 ± 86.4	475.9 ± 122.4	**< .001**
Total bilirubin, µmol/L	7.8 (6.2–12.3)	6.6 (4.6–11.1)	8.2 (3.4–12.6)	5.3 (3.2–7.8)	**.037**
Serum urea, mmol/L	4.3 (3.7–4.9)	4.2 (3.5–5.4)	7 (5.7–9.3)	9.9 (8.5–11.5)	**<.001**
Serum glucose, mmol/L	5.1 (4.9–5.6)	4.8 (4.4–5.3)	4.9 (4.5–5.4)	5.4 (5.1–5.7)	**.001**
Biochemical analysis of 24-hour urine samples					
Proteinuria, g/24-h	0.1 (0.1–0.1)	1.3 (0.5–3.1)	2 (0.8–3.2)	3.3 (0.9–6.4)	**< .001**
Proteomic data of 24-hour urine samples					
Detected proteins, *n*	446	360	251	202	
emPAI for total protein	41.7 (18.8–54.8)	61.4 (35.6–80.7)	49.8 (30.3–101.7)	63.6 (39.5–89.8)	**.007**

Note. Numeric variables with a normal distribution are reported as mean ± *SD*, and those with a non-normal distribution are reported as median (IQR). Significant values are indicated in bold. CG = control group; CKD = chronic kidney disease group; eGFR = estimated glomerular filtration rate; emPAI = exponentially modified protein abundance index; ESR = erythrocyte sedimentation rate; HGB = hemoglobin; PLT = platelet; RBC = red blood cells; WBC = white blood cells. Adapted from "Candidate protein biomarkers in chronic kidney disease: a proteomics study," by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P. Kovesdy, S. H. Abidi, & A. Gaipov, 2024, *Scientific Reports*, *14*(1), Article 14014 (https://doi.org/10.1038/s41598-024-64833-8). Copyright 2024 by the Authors.

Urine proteomic profiling showed significant differences in the number of identified proteins and the emPAI values between the groups (p = .007). A total of 714 individual proteins were detected from urine samples across 137 participants. Healthy controls exhibited a higher number of detected proteins (446) compared to CKD patients, with a progressive reduction observed in more advanced CKD stages (360 in stage 1, 251

in stage 2, and 202 in stage 3). The distribution and incidence rate of the detected urinary proteins across the control and patient groups were illustrated in Figure 2 and highlighted apparent differences in proteome composition. Specifically, there was a marked decrease in the overall presence of urinary proteins in the patient groups compared to controls. However, a small subset of proteins was found to be more prevalent in the patient groups. The distribution of protein types also varied markedly between groups.

Figure 2


Groups
Control group
CKD stage 1
CKD stage 2
CKD stage 3
Protein names
ALBU
AMBP
IGKC
PTGDS
A1AG1
ZA2G
TRFE
IGHA1
UROM
RNAS1
A1BG
IGHG2
A1AT
VTDB
KNG1
IGLC2
OSTP
IGG1
HEMO
CERU
PGBM
TTHY
CD59
IGHG3
A2GL
A1AG2
KVD20
HPT
FETUA
CD44
THRB
APOH
B2MG
ITIH4
REG1A
IGL1
IGK
IGA2
AACT
ANT3
LV39
IBP7
VMO1
VTNC
SH3L3
AFAM
PIGR
GELS
P3IP1
RNAS2
LMAN2
FBN1
PLMN
KVD26
DAF
ATRN
KLK1
IGHA2
CLUS
LAIR1
HBB
IGHG4
NID1
CO3
EGF
APOA1
TNR16
AMYP
SDC1
RET4
CFAB
VASN
SCTM1
CAD13
CD14
LV147
LYVE1
PI16
CADH1
LG3BP
GRN
APOD
FBLN3
K2C1
LYAG
PRIO
MASP2
CD27
DIAC
TFF2
TNR14
HEG1
CSF1
CADM4
WFDC2
HAVR2
LAMP2
IC1
FIBA
SAP
UFO
ICOSL
APOA
PEPA3
DNAS1
CLM9
MMRN1
SDC4
YIPF3
Occurrence (%)
100
80
60
40
20
0

Note. *Heatmap of Distribution Rate of Proteins in the Control and Patient Groups.* Heatmap illustrating protein distribution rates. The X-axis of the heatmap illustrates different sample groups, while individual proteins are shown on the Y-axis. The color gradient (0–100%) represents the detection frequency of proteins, with darker shades indicating higher occurrence. CKD = chronic kidney disease. Adapted from "Candidate protein biomarkers in chronic kidney disease: a proteomics study," by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P. Kovesdy, S. H. Abidi, & A. Gaipov, 2024, *Scientific Reports, 14*(1), Article 14014 (https://doi.org/10.1038/s41598-024-64833-8). Copyright 2024 by the Authors.

3.1.2. Follow-up Study

The general and biochemical characteristics of the follow-up study participants are detailed in Table 4. The follow-up study included 33 participants (18 patients and 15 controls) with a mean age of 37.8 years (*SD* = 9.9) and a median follow-up duration of 368 days (IQR: 250–472). The patient group comprised 39% female participants, while the control group comprised 73% female participants.

At baseline, CKD patients had significantly reduced eGFR ($p = .001$) and elevated proteinuria ($p < .001$) compared to healthy controls. Additional significant differences included higher serum creatinine ($p = .008$), serum urea ($p = .019$), and serum uric acid levels ($p = .044$), as well as hypoproteinemia ($p < .001$), hyperlipidemia ($p = .01$), and elevated ESR ($p < .001$) in CKD patients.

During the follow-up period, CKD patients showed overall improvement in most parameters, including an increase in eGFR and a reduction in proteinuria levels. However, these values remained abnormal when compared to the control group.

Proteomic analysis of 24-hour urine samples revealed dynamic changes in urinary protein composition over time. At baseline, the number of detected proteins was lower in CKD patients (171 vs. 271 in controls). At follow-up, CKD patients exhibited an increase in the number of detected proteins (285 vs. 252 in controls). The emPAI for total protein substantially decreased over time in the patient group.

Table 4

Clinical and Biochemical Characteristics of Followed-up Participants

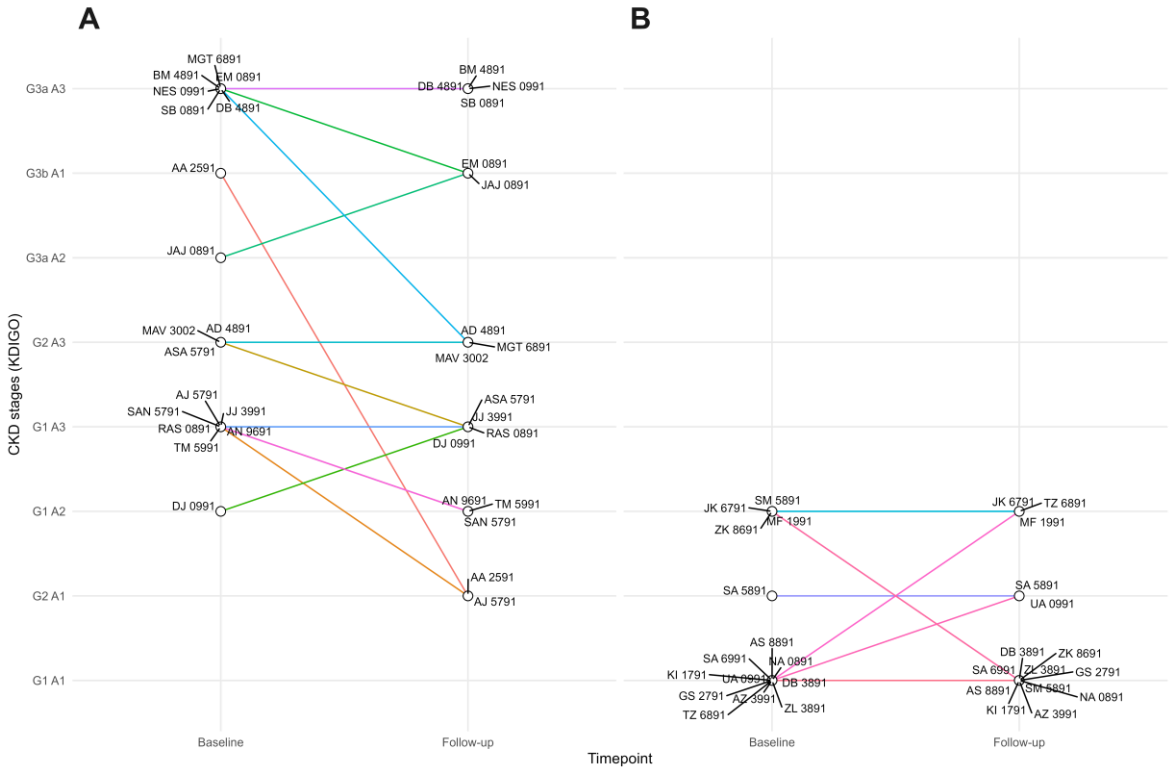
Parameters	Baseline		*p*	Follow-up		*p*
	CG, *n* = 15	CKD, *n* = 18		CG, *n* = 15	CKD, *n* = 18	
Demographics						
Age, year	37 ± 8	39 ± 11	.563	38 ± 8	39 ± 11	.612

Gender, female, *n* (%)	11 (73)	7 (39)		11 (73)	7 (39)	
eGFR, mL/min/1.73 m^2	112.9 (105.6–122.9)	66.7 (53.7–110.4)	**.001**	110 (106.9–118)	76 (55–100.4)	**.002**
CKD etiology						
Glomerular, *n* (%)		14 (77.8)			14 (77.8)	
Transplant, *n* (%)		1 (5.6)			1 (5.6)	
Diabetic, *n* (%)		1 (5.6)			1 (5.6)	
Lupus, *n* (%)		1 (5.6)			1 (5.6)	
CKD of unknown etiology, *n* (%)		1 (5.6)			1 (5.6)	
Comorbidities						
Hypertension, *n* (%)	1 (7)	12 (67)		1 (7)	12 (67)	
Anemia, *n* (%)	1 (7)	5 (28)			3 (16.7)	
Laboratory data						
WBC 10 × 10^9/L	6.1 (5.2– 6.2)	7.5 (5.7–8.9)	.06	6.2 (5.5–7)	7.6 (5.3–9.4)	.2
PLT 10 × 10^9/L	251 ± 42	273.7 ± 42	.082	242.6 ± 50.4	299.9 ± 75.8	**.027**
RBC 10 × 10^{12}/L	4.8 ± 0.6	4.4 ± 0.6	.041	4.7 ± 0.6	4.5 ± 0.5	.61
HGB g/L	134.2 ± 18	125.8 ± 21	.241	133.8 ± 18.2	131.9 ± 18.3	.783
ESR, mm/h	9.6 (6–12)	21 (14–30)	**< .001**	11.3 (6–15)	17.3 (7.5–28)	.202
Serum total protein, g/L	69.8 (67.5–72.6)	54.6 (45.8–65.2)	**< .001**	70.5 ± 3.1	62.3 ± 6.9	**< .001**
Total cholesterol, mmol/L	4.6 ± 0.6	6 ± 1.7	**.01**	4.7 ± 0.6	5.5 ± 1.8	.23
Serum creatinine, μmol/L	63.7 (53.2–71.8)	106.4(66.7–148.5)	**.008**	65 (54.5–69.8)	111.1 (71–141)	**.003**
Serum uric acid, μmol/L	295.4 (245.9–313.5)	376.9 (297–406)	**.044**	328.9 (252.4–483.5)	408.7(339.7–502.2)	**.045**
Total bilirubin, μmol/L	8.9 (5.23–11.9)	8.3 (4–9.1)	.244	8.1 (6.3–10.1)	9.4 (4.8–14.5)	.83
Serum urea, mmol/L	4.2 (3.6–4.7)	6.5 (3.9–8.7)	**.019**	4.5 (3.4–4.9)	7.4 (4.9–9.9)	**.003**
Serum glucose, mmol/L	5.2 (4.9–5.63)	6.4 (4.7–5.4)	.715	5.4 ± 0.74	5.1 ± 1.6	.086
Biochemical analysis of 24-hour urine samples						
Proteinuria, g/24-h	0.1 (0.07–0.15)	3.1 (1–4)	**< .001**	0.12 (0.08–0.14)	2.1 (0.5–3.51)	**< .001**
Proteomic data of 24-hour urine samples						
Detected proteins, *n*	271	171		252	285	
emPAI for total protein	47 (29.3–56.3)	86 (60.6–105.5)	**< .001**	34.2 (9.2–46.5)	44.8 (25.7–54.6)	.135

Note. Numeric variables with a normal distribution are reported as mean ± *SD*, and those with a non-normal distribution are reported as median (IQR). Significant values are indicated in bold. CG = control group; CKD = chronic kidney disease group; eGFR = estimated glomerular filtration rate; emPAI = exponentially modified protein abundance index; ESR = erythrocyte sedimentation rate; HGB = hemoglobin; PLT = platelet; RBC = red blood cells; WBC = white blood cells. Adapted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules*, *15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

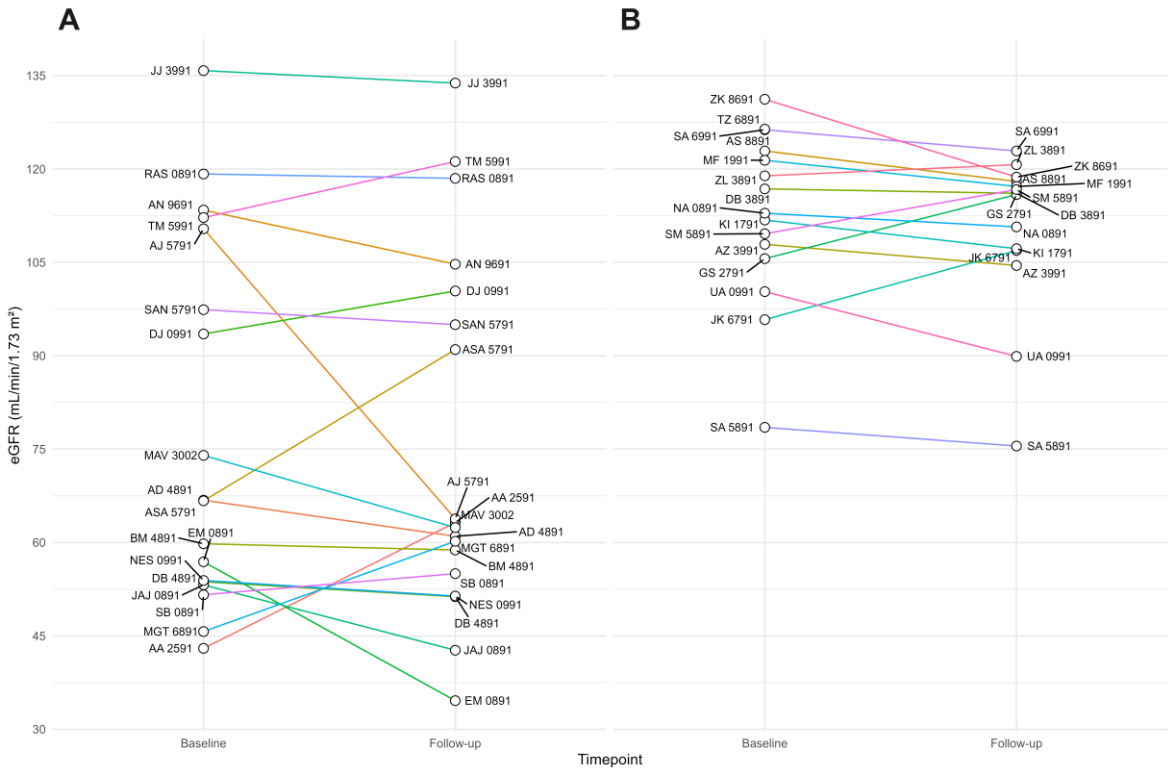
The progression of CKD stages over time was evaluated using eGFR and proteinuria according to the KDIGO classification (Levin et al., 2013). As illustrated in Figure 3, the progression of CKD was observed in only two patients during the follow-up period. Specifically, one patient progressed from stage G1 A2 to G1 A3, reflecting an increase in proteinuria severity, while another transitioned from stage G3a A2 to G3b A1, indicating a decline in eGFR but an improvement in proteinuria. For most patients, their CKD risk categories either remained stable or showed improvement throughout the study period. However, it is important to note that many of these individuals experienced declines in their eGFR (Figure 4) or increases in proteinuria levels (Figure 5).

Figure 3



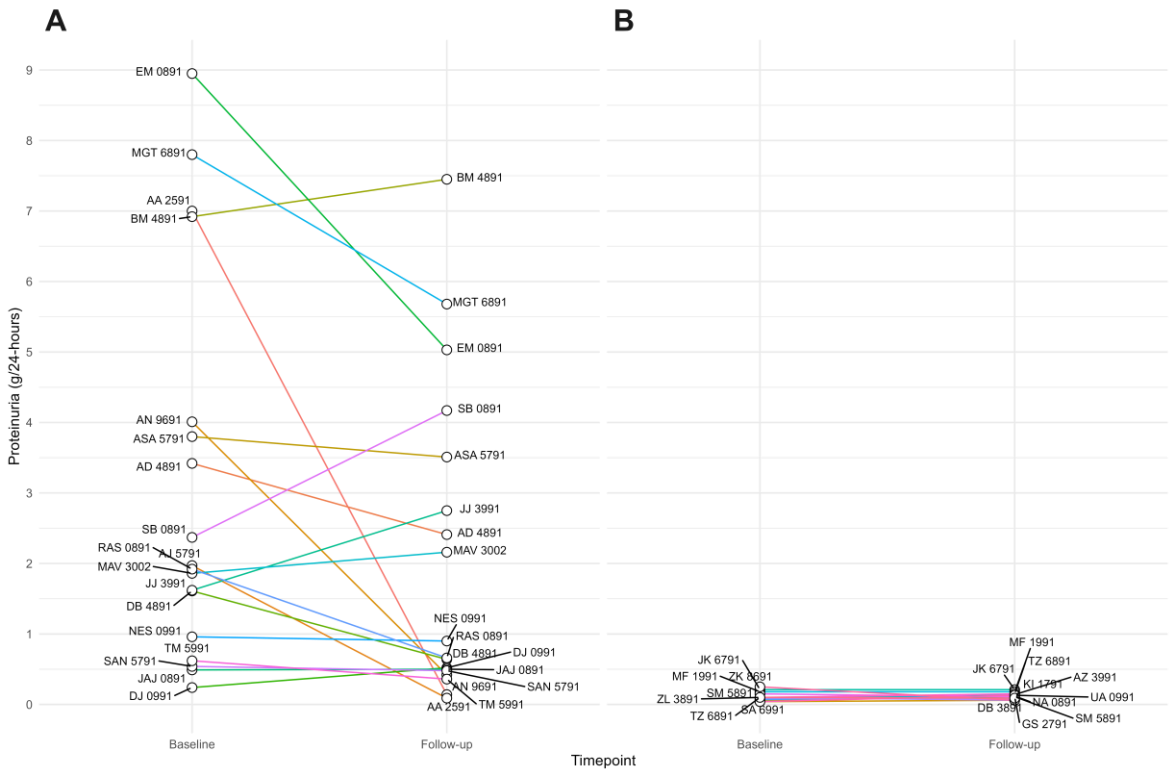
Note. Line Graph Illustrating Changes in CKD Stages throughout the Follow-up Period. The CKD stage changes over time for the patient group (A) and the control group (B) are categorized based on eGFR and proteinuria levels following the KDIGO 2012 guidelines. The X-axis displays individual participant IDs to track changes from baseline timepoint to follow-up timepoint, while the Y-axis represents CKD stages, reflecting varying levels of risk. Reprinted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules, 15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

Figure 4



Note. Line Graph Illustrating Changes in eGFR throughout the Follow-up Period. The eGFR changes over time for the patient group (A) and the control group (B), following the KDIGO 2012 guidelines. The X-axis displays individual participant IDs to track changes from baseline timepoint to follow-up timepoint, while the Y-axis represents eGFR levels. Reprinted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules, 15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

Figure 5



Note. Line Graph Illustrating Changes in Proteinuria throughout the Follow-up Period. The proteinuria changes over time for the patient group (A) and the control group (B), following the KDIGO 2012 guidelines. The X-axis displays individual participant IDs to track changes from baseline timepoint to follow-up timepoint, while the Y-axis represents proteinuria levels. Reprinted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules, 15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

3.2. Correlation between Urinary Proteins and Kidney Function

3.2.1. Main Study

The correlation analysis between kidney function and the urinary proteome identified associations between specific urinary proteins and eGFR across all study participants (Table 5). Positive correlations with eGFR were observed for proteins such as UROM, RNAS1, CERU, A1AG2, OSTP, SH3L3, CD44, LV39, KNG1, REG1A, CD59, VTNC, VMO1, CLUS, PTGDS, and IC1. Conversely, proteins, including VTDB, IGL1, KVD20, IGK, B2MG, IGHA1, and FETUA, were negatively correlated with eGFR. Although these correlations were statistically significant, their strength was generally moderate.

Table 5

Correlation between Protein emPAI Values and eGFR across the Entire Cohort

Protein entries	rho	*p*	N	Molecular weight (Da)
FETUA	-.441	**< .001**	59	39
B2MG	-.357	**.008**	54	14
IGK	-.307	**.032**	49	23
KVD20	-.286	**.018**	68	12
IGL1	-.27	**.048**	54	23
VTDB	-.268	**.01**	93	53
IGHA1	-.192	**.042**	112	43
IC1	.535	**.009**	23	55
CLUS	.498	**.008**	28	52
VMO1	.485	**< .001**	48	21
VTNC	.431	**.005**	41	54
CD59	.425	**< .001**	87	14
REG1A	.42	**< .001**	61	19
KNG1	.406	**< .001**	102	72
LV39	.401	**.009**	42	12
CD44	.362	**.001**	78	81
SH3L3	.359	**.022**	41	10
OSTP	.325	**.001**	101	35
A1AG2	.282	**.014**	76	24
CERU	.266	**.017**	80	122
RNAS1	.241	**.01**	114	18
UROM	.234	**.011**	116	70
PTGDS	.191	**.036**	121	21

Note. Significant values are indicated in bold. N = number of observations; rho = Spearman's rank correlation coefficient. Adapted from "Candidate protein biomarkers in chronic kidney disease: a proteomics study," by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P. Kovesdy, S. H. Abidi, & A. Gaipov, 2024, *Scientific Reports, 14*(1), Article 14014 (https://doi.org/10.1038/s41598-024-64833-8). Copyright 2024 by the Authors.

To assess group-specific differences, separate correlation analyses were performed for CKD patients and control participants (Table 6). Among CKD patients, proteins such as CERU, A1BG, CD59, and LV39 showed positive correlations with eGFR, whereas VTDB, AMBP, B2MG, and FETUA displayed negative correlations. In contrast, proteins CRNN and PGRP1 exhibited a positive correlation with eGFR in control participants, while LG3BP, SAP, and ATRN displayed a negative correlation.

Table 6

Correlation between Protein emPAI Values and eGFR by Two Groups

Protein entries	CKD group			Control group			Molecular weight (Da)
	rho	*p*	N	rho	*p*	N	
FETUA	-.501	< **.001**	50	-.417	.26	9	39
B2MG	-.445	**.002**	45	-.483	.185	9	14
AMBP	-.244	**.026**	84	.131	.374	48	39
VTDB	-.230	**.049**	73	-.024	.919	20	53
LV39	.402	**.014**	37	-.6	.278	5	12
CD59	.396	**.013**	39	.199	.174	48	14
A1BG	.328	**.003**	83	-.198	.474	15	54
CERU	.32	**.005**	75	-.5	.382	5	122
ATRN	.222	.295	24	-.691	**.021**	11	158
SAP	1.0	.024	4	-.63	**.005**	19	58
LG3BP	-.173	.606	11	-.572	**.009**	20	65
PGRP1	.1	.869	5	.566	**.045**	13	22
CRNN	-1.0	.317	2	.56	**.048**	13	53

Note. Significant values are indicated in bold. CKD = chronic kidney disease group; N = number of observations; rho = Spearman's rank correlation coefficient. Adapted from "Candidate protein biomarkers in chronic kidney disease: a proteomics study," by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P. Kovesdy, S. H. Abidi, & A. Gaipov, 2024, *Scientific Reports, 14*(1), Article 14014 (https://doi.org/10.1038/s41598-024-64833-8). Copyright 2024 by the Authors.

3.2.2. Follow-up Study

Assessment of urinary protein correlations with eGFR over time revealed distinct patterns across the cohort (Table 7). At baseline, IGHA1 and VTDB showed negative correlations with eGFR; however, only IGHA1 remained significantly associated at follow-up. Several proteins, including CD59, VTNC, A1AG2, CD44, PTGDS, and RNAS1, displayed significant positive correlations at baseline, though only CD59 and RNAS1 retained these associations over time.

Table 7

Correlation between Protein emPAI Values and eGFR across the Entire Cohort over Time

Protein entries	Baseline			Follow-up		
	rho	*p*	N	rho	*p*	N
VTDB	-.47	**.024**	23	.099	.67	21
IGHA1	-.11	.57	27	-.44	**.033**	24
VTNC	.66	**.04**	10	.067	.86	9
A1AG2	.57	**.008**	21	.017	.94	19
RNAS1	.56	< **.001**	33	.6	**.002**	26
PTGDS	.52	**.002**	32	.21	.26	29
CD44	.49	**.013**	25	-.13	.56	21
CD59	.48	**.021**	23	.49	**.015**	24

Note. Significant values are indicated in bold. N = number of observations; rho = Spearman's rank correlation coefficient. Adapted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules, 15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

Analysis of protein correlations with eGFR within specific groups revealed notable changes over time in CKD patients (Table 8). For example, VTDB, which exhibited a negative correlation at baseline, transitioned to a significant positive correlation over time, suggesting an evolving relationship with kidney function as CKD progresses. In contrast, CERU initially showed a positive correlation but did not retain this relationship with eGFR during follow-up in the CKD group. Also, CD59 exhibited a significant positive correlation only at follow-up in the patient group. At baseline, ATRN displayed a negative correlation with eGFR in the control group, while A1BG showed a positive correlation; however, neither protein maintained these associations during the follow-up.

Table 8

Correlation between Protein emPAI Values and eGFR by Two Groups over Time

Protein entries	Baseline						Follow-up					
	CKD group			Control group			CKD group			Control group		
	rho	*p*	N	rho	*p*	N	rho	*p*	N	rho	*p*	N
VTDB	-.48	.051	17	-.54	.26	6	.59	**.029**	14	.68	.095	7
CERU	.52	**.032**	17	-1	.16	3	.24	.36	16	NA	NA	NA
A1BG	.43	.077	18	1	**.024**	4	.39	.12	17	.4	.59	4
ATRN	.19	.64	8	-1	**.024**	4	-.89	**.026**	6	-.4	.59	4
CD59	.19	.64	8	.18	.52	15	.64	**.05**	10	.25	.39	14

Note. Significant values are indicated in bold. CKD = chronic kidney disease group; N = number of observations; rho = Spearman's rank correlation coefficient. Adapted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules, 15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

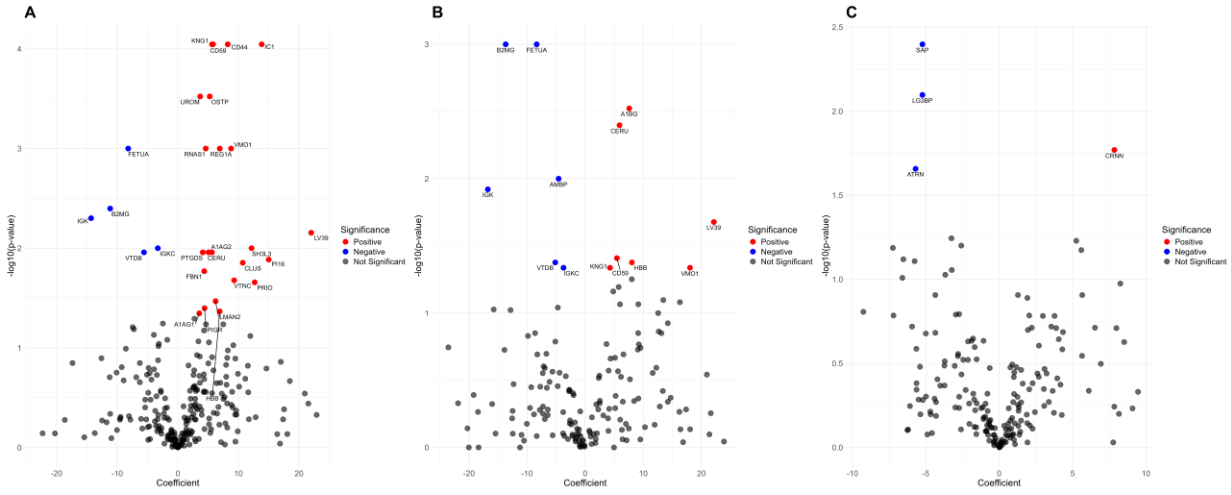
3.3. Associations between Urinary Proteome and Kidney Function

3.3.1. Main Study

After identifying proteins correlated with eGFR, regression analysis was conducted to explore their specific relationships with kidney function. We generated volcano plots to

visualize the regression test results, as shown in Figure 6. These plots display the magnitude and significance of the relationship between individual proteins and eGFR. The analysis revealed that among the correlated proteins, VTDB, IGK, FETUA, and B2MG were inversely associated with eGFR across the entire cohort. In contrast, proteins such as VMO1, UROM, RNAS1, REG1A, PTGDS, OSTP, LV39, KNG1, CERU, CD59, CD44, VTNC, IC1, CLUS, SH3L3, and A1AG2 exhibited a direct positive association with eGFR (Figure 6A). Also, to better understand the relationship between proteins and kidney function, regression analysis was conducted separately for the patient and control groups. In the patient group, correlated proteins, including VTDB, AMBP, FETUA, and B2MG, were negatively associated with eGFR. At the same time, CERU, A1BG, CD59, and LV39 were positively associated (Figure 6B) (see Appendix C (Table C1) for the full list of urinary proteins identified as potential biomarkers and their characteristics). In the control group, LG3BP, SAP, and ATRN were negatively associated with eGFR, whereas CRNN was the only protein demonstrating a positive association (Figure 6C).

Figure 6



Note. *Volcano Plot Illustrating an Association between Protein emPAI Values and eGFR.* The plot presents regression analysis results for the entire cohort (A), CKD patients (B), and control groups (C). The X-axis represents coefficient values, indicating the direction and strength of association for individual proteins with eGFR, while the Y-axis displays the -log10-transformed *p* values for proteins with adjusted *p* values of < .05. Proteins positively associated with eGFR ($p < .05$) are marked in red, while proteins negatively associated with eGFR ($p < .05$) are marked in blue. Proteins without statistically significant associations are shown as gray dots. Reprinted from "Candidate protein biomarkers in chronic kidney disease: a proteomics study," by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P.

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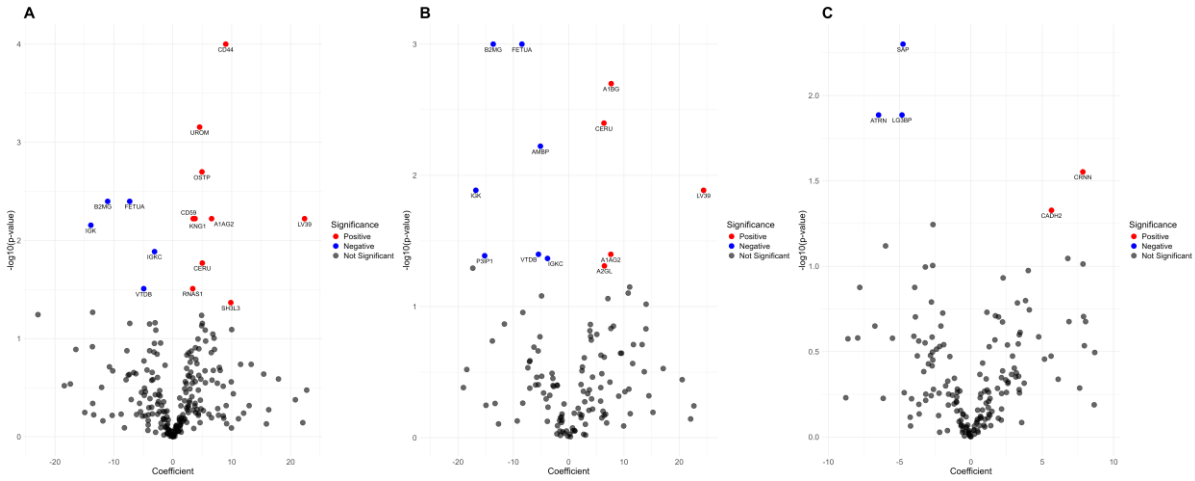
Furthermore, to account for the potential confounding effects of 24-hour proteinuria, a regression analysis was conducted to assess the relationship between the correlated urinary proteins and eGFR while adjusting for this variable (Figure 7). The plot shows that the coefficients and significance levels of the protein-eGFR associations were largely unchanged after adjusting for proteinuria. Specifically, proteins such as VTDB, IGK, FETUA, and B2MG maintained negative associations with eGFR in the entire cohort. Meanwhile, UROM, SH3L3, RNAS1, OSTP, LV39, KNG1, CERU, CD59, CD44, and A1AG2 displayed positive associations (Figure 7A). In a group-specific regression analysis, the associations between proteins and eGFR were examined separately for the CKD and control groups, adjusting for proteinuria. In the CKD group, VTDB, AMBP, FETUA, and B2MG consistently displayed negative associations with eGFR, while CERU, A1BG, and LV39 demonstrated consistent positive associations (Figure 7B). In contrast, LG3BP, SAP, and ATRN were consistently inversely associated with eGFR within the control group, whereas CRNN was the only protein consistently demonstrating a positive association (Figure 7C).

Figure 7



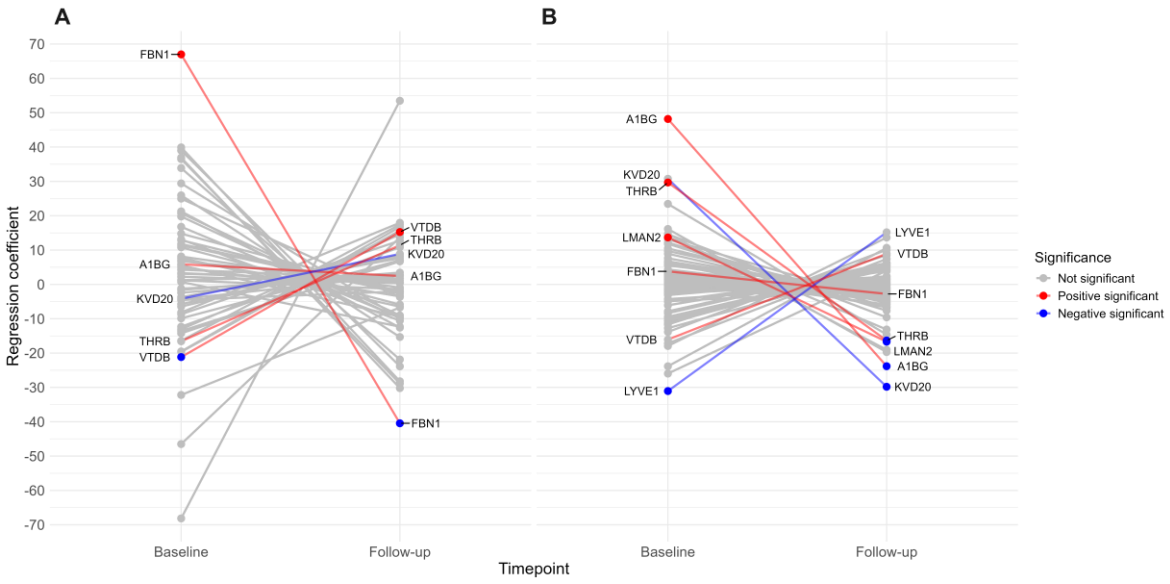
Note. *Volcano Plot Illustrating an Association between Protein emPAI Values and eGFR Adjusting for Proteinuria*. The plot presents regression analysis results for the entire cohort (A), CKD patients (B), and control groups (C). The X-axis represents coefficient values, indicating the direction and strength of association for individual proteins with eGFR, while the Y-axis displays the -log10-transformed *p* values for proteins with adjusted *p* values of < .05. Proteins positively associated with eGFR ($p < .05$) are marked in red, while proteins negatively associated with eGFR ($p < .05$) are marked in blue. Proteins without statistically significant associations are shown as gray dots. Reprinted from

"Candidate protein biomarkers in chronic kidney disease: a proteomics study," by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P. Kovesdy, S. H. Abidi, & A. Gaipov, 2024, *Scientific Reports*, *14*(1), Article 14014 (https://doi.org/10.1038/s41598-024-64833-8). Copyright 2024 by the Authors.

3.3.2. Follow-up Study

To further explore the relationships between eGFR and proteins in the follow-up study, regression analyses were performed within control and patient participants, evaluating baseline and follow-up values (Figure 8). VTDB exhibited an opposite trend among the patients: it started with a negative baseline interaction coefficient with eGFR; however, it revealed a positive association during follow-up (Figure 8A). FBN1 was strongly positively associated with baseline eGFR, although it exhibited an inverse interaction coefficient during follow-up. In the control group, A1BG, THRB, and LMAN2 were positively associated with baseline eGFR, whereas LYVE1 showed a negative baseline coefficient (Figure 8B). KVD20 also showed a positive association with eGFR without statistical significance at baseline. However, the associations of these KVD20, A1BG, THRB, and LMAN2 proteins shifted to negative at follow-up. At the same time, LYVE1 moved to positive during the follow-up, although the positive coefficient of LYVE1 did not achieve statistical significance at follow-up.

Figure 8

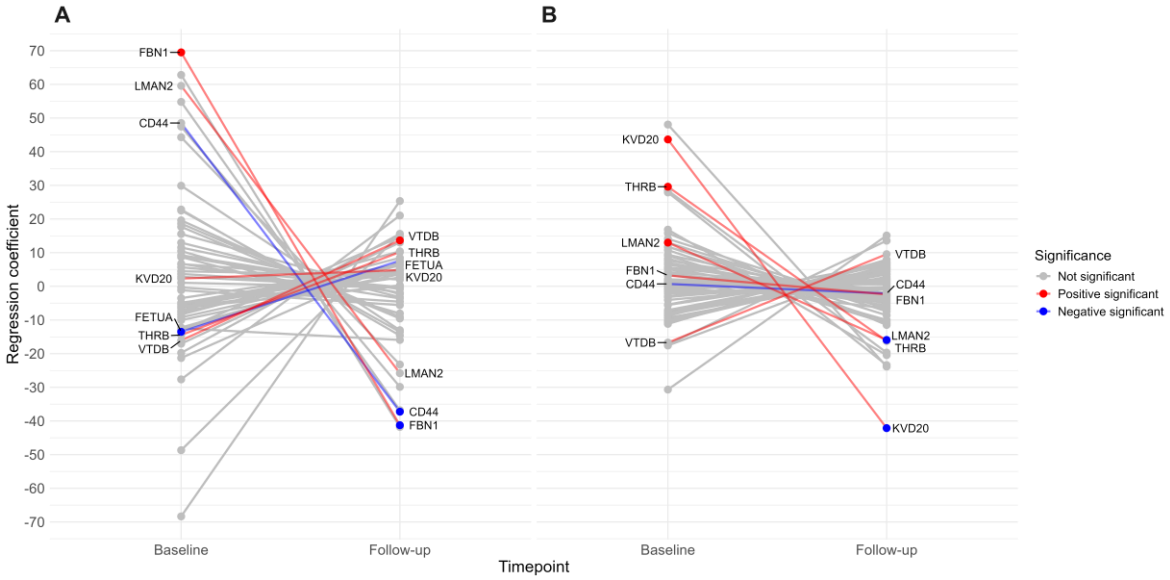


Note. Interaction Plot Illustrating the Association between Protein emPAI Values and eGFR over Time. Regression analysis was conducted separately for the CKD (A) and control (B) groups, focusing on proteins with statistically significant coefficients. For

clarity, significant interactions are visually highlighted with color coding in both groups, although not all reached statistical significance. Reprinted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules, 15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

Regression analyses were also conducted to account for the impact of proteinuria between the two groups in the follow-up study (Figure 9). In CKD patients, CD44 showed a positive baseline coefficient with eGFR, though this did not reach statistical significance, and its follow-up coefficient became negative (Figure 9A). FBN1 was positively associated with eGFR at baseline, but this shifted to an inverse association during follow-up. Similarly, FETUA was negatively associated with baseline eGFR but moved to a positive follow-up association, although it lacked statistical significance. VTDB was negatively associated with baseline eGFR, which was not statistically significant but demonstrated a consistent positive follow-up interaction coefficient in patients.

Figure 9



Note. Interaction Plot Illustrating the Association between Protein emPAI Values and eGFR, Adjusted for Proteinuria over Time. Regression analysis was conducted separately for the CKD (A) and control (B) groups, focusing on proteins with statistically significant coefficients. For clarity, significant interactions are visually highlighted with color coding in both groups, although not all reached statistical significance. Reprinted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R.

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Proteins LMAN2 and KVD20 were positively associated with baseline eGFR but shifted to an inverse association during follow-up in the control group (Figure 9B). Similarly, THRB transitioned from a positive association at baseline to a negative association with eGFR at follow-up; however, this shift was not statistically significant.

3.4. Changes in Biomarker Levels from Baseline to Follow-up in the Follow-up Study

After consistently associated proteins with eGFR were identified, emPAI values were used to compare these protein levels at baseline and follow-up to evaluate changes over time (Table 9). Among the four proteins as candidate biomarkers assessed, FETUA and VTDB exhibited substantial increases, whereas FBN1 and CD44 showed notable declines.

Table 9

Changes in Biomarker Levels as a Percentage from Baseline to Follow-up

Candidate biomarker	Baseline (mean emPAI)	Follow-up (mean emPAI)	Percent change
VTDB	0.34	0.81	138
FETUA	-0.58	-0.98	68.97
CD44	-3.63	-2.39	-34.15
FBN1	-5.22	-4.52	-13.4

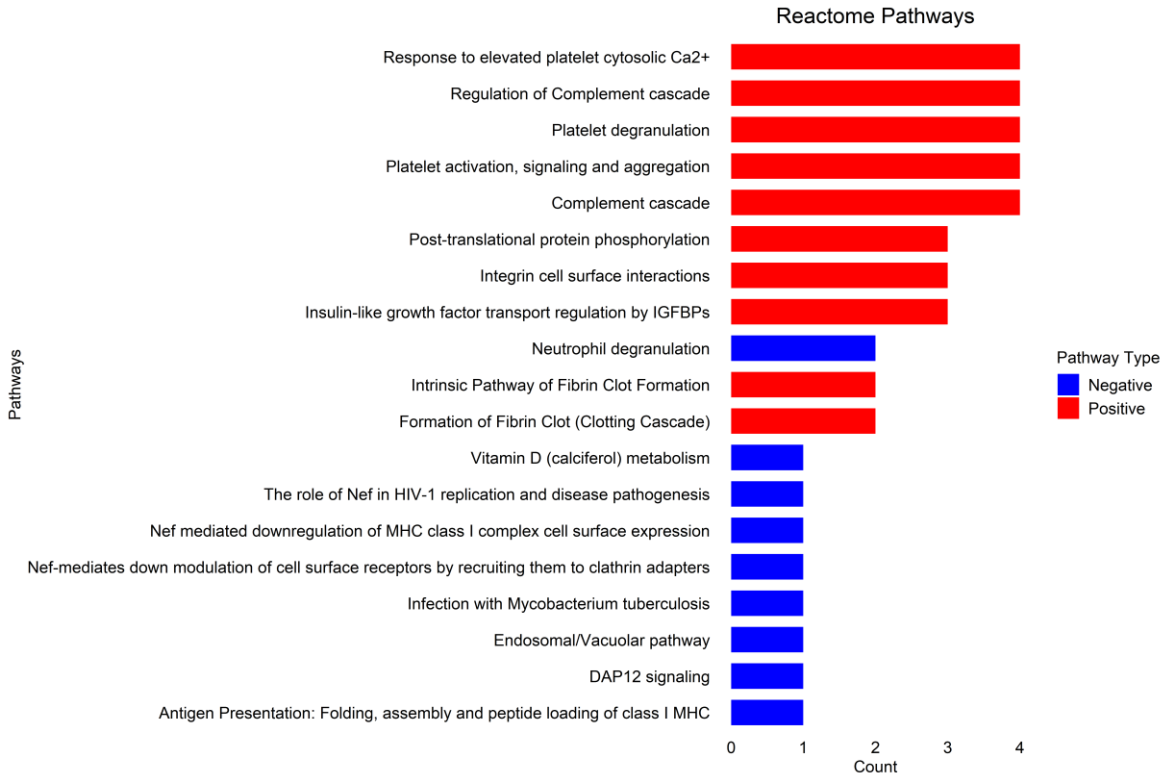
Note. emPAI = exponentially modified protein abundance index. Adapted from "Urinary proteomic shifts over time and their associations with eGFR decline in chronic kidney disease", by Z. Makhammajanov, K. Nurlybayeva, Z. Artikov, P. Tarlykov, M. Aljofan, R. Bukasov, D. Turebekov, S. H. Abidi, M. Kanbay, & A. Gaipov, 2025, *Biomolecules*, *15*(1), Article 45 (https://doi.org/10.3390/biom15010045). Copyright 2025 by the Authors.

3.5. Pathway Analysis of Associated Urinary Proteins

Pathway and Gene Ontology (GO) enrichment analyses were performed in the main study to gain functional insights into the proteins associated with kidney function. Pathway enrichment analysis of urinary proteins associated with eGFR, after adjusting for proteinuria, revealed distinct patterns (Figure 10). In the patient group, positively associated proteins were enriched in pathways related to immune regulation, including the complement system regulation, complement cascade, and homeostasis processes, including the platelet activation, signaling and aggregation, responses to elevated platelet cytosolic Ca2+, and clotting cascades. Additionally, positively associated proteins were linked to pathways involving integrin-mediated cell surface interactions and cellular adhesion. On

the other hand, in the CKD group, proteins negatively associated with eGFR were also predominantly enriched in immune-related pathways, including neutrophil degranulation.

Figure 10



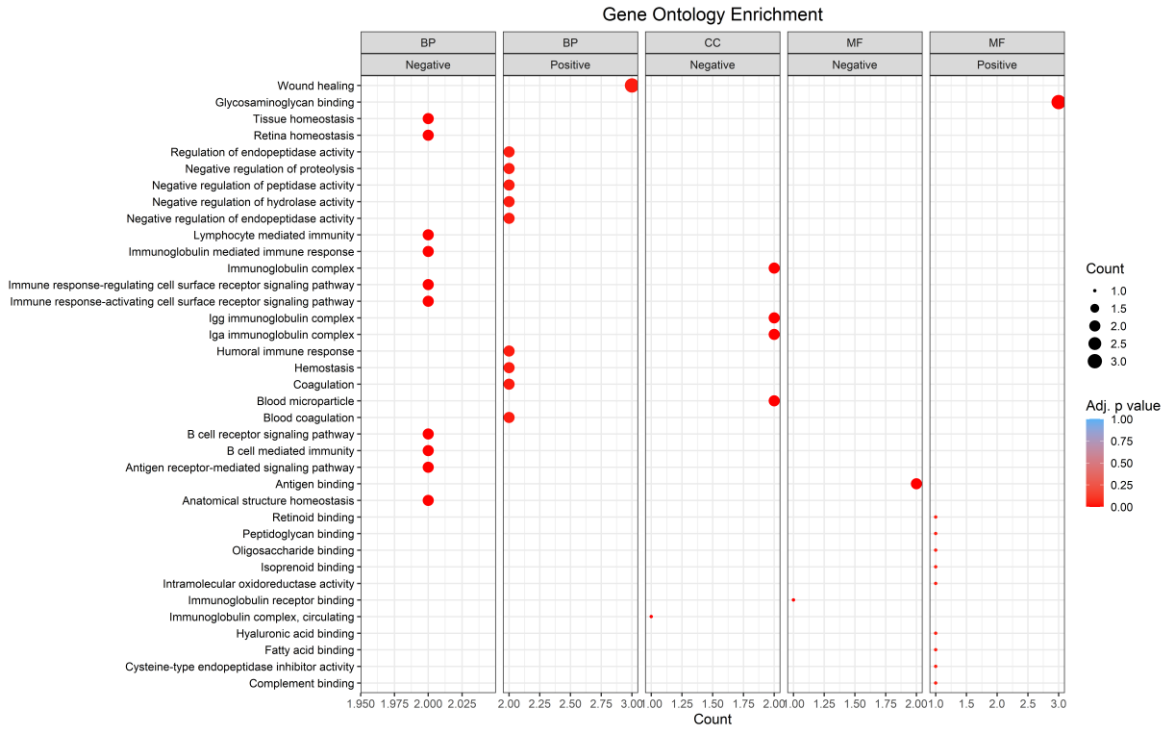
Note. Top Ten Pathways of the Negatively and Positively Associated Proteins with Kidney Function. Each bar represents a pathway, and the length corresponds to the count of proteins enriched in that pathway. The pathways are sorted based on their statistical significance (an adjusted *p* value of < .05 was used). Blue represents pathways associated with negatively associated proteins with eGFR adjusted for proteinuria, while red represents pathways related to positively associated proteins.

3.6. Gene Ontology Analysis of Associated Urinary Proteins

Figure 11 compares the significantly involved GO terms (adjusted *p* value < .05) between negatively and positively associated proteins with eGFR in the patient group. In the patient group, GO analysis focusing on biological processes indicated that proteins negatively correlated with eGFR were significantly enriched in anatomical structure homeostasis, tissue homeostasis, retina homeostasis, and immune response-related biological activities (Figure 11). In contrast, proteins positively correlated with eGFR were strongly enriched in pathways associated with the regulation and inhibition of endopeptidase activity, peptidase activity, hydrolase activity, and proteolysis. Furthermore, these proteins were also enriched

in processes related to the regulation of coagulation, hemostasis, and humoral immune response in patients.

Figure 11



Note. Top Ten Enriched GO Terms by Kidney Function-Associated Proteins. The plot highlights enriched terms across three ontology categories: biological process (BP), cellular component (CC), and molecular function (MF). Points on the plot represent a specific GO term, displayed on the Y-axis. The point size on the X-axis reflects the number of proteins associated with the term, while the color indicates the level of statistical significance (adjusted *p* value). The analysis highlights the top 10 enriched terms in each category and clarifies the differences between proteins that have negative and positive associations with kidney function. Reprinted from "Candidate protein biomarkers in chronic kidney disease: a proteomics study," by Z. Makhammajanov, A. Kabayeva, D. Auganova, P. Tarlykov, R. Bukasov, D. Turebekov, M. Kanbay, M. Z. Molnar, C. P. Kovesdy, S. H. Abidi, & A. Gaipov, 2024, *Scientific Reports*, *14*(1), Article 14014 (https://doi.org/10.1038/s41598-024-64833-8). Copyright 2024 by the Authors.

For cellular components, The GO analysis revealed a significant enrichment of proteins in blood microparticles and immunoglobulin complexes (IgA and IgG) specific to CKD patients (Figure 11).

Regarding molecular functions, negatively correlated proteins showed predominant enrichment in antigen-binding activities (Figure 11). In contrast, positively correlated

proteins were significantly enriched in functions related to glycosaminoglycan-binding and extracellular matrix structural components.

Chapter 4: Discussion

In this thesis, we used label-free quantitative untargeted proteomics to comprehensively characterize proteins in 24-hour urine collections from individuals with CKD stages 1–3 and healthy controls across two cohorts. In the first cohort, as the main study, we used a cross-sectional design to analyze urine samples from 88 CKD patients and 49 controls, while in the second cohort, we used a longitudinal follow-up design to analyze samples from 18 patients and 15 controls. In contrast to prior research that predominantly focused on blood biomarkers or spot urine samples in CKD studies, in our methodology, we relied on 24-hour urine collections to comprehensively evaluate proteomic changes reflective of early-stage CKD pathophysiology. While conventional proteomic studies often use differential expression analysis to identify upregulated or downregulated proteins, we implemented correlation and regression analyses adjusted for proteinuria, enabling a more precise exploration of the relationships between kidney function (i.e., eGFR) and urinary protein dynamics. These analyses revealed bidirectional associations (positive and negative) between eGFR and urinary protein abundance, with distinct proteomic signatures distinguishing CKD patients from controls in both cohorts. Also, the follow-up cohort demonstrated progressive shifts in protein profiles associated with CKD progression. Functional enrichment analysis further showed that proteins negatively correlated with eGFR were enriched in pathways linked to immune system activation, as well as tissue and structural homeostasis, whereas positively associated proteins were enriched in processes such as extracellular matrix organization, coagulation, cellular adhesion, and the regulation of immune responses and enzymatic activity.

Previous studies have highlighted proteinuria as a crucial marker and a risk factor for CKD progression and its associated complications (Barzilay, Farag, & Durthaler, 2024; Cravedi & Remuzzi, 2013; Liu & Lv, 2019). It is important to note that persistent proteinuria exceeding 150 mg/24 hours for more than three months is considered abnormal and serves as a defining criterion for CKD. In our study, the median (IQR) of proteinuria was 1.3 (0.5–3.1) g/24-hour in stage 1 CKD, 2 (0.8–3.2) g/24-hour in stage 2 CKD, and 3.3 (0.9–6.4) g/24-hour in stage 3 CKD, while it was only 0.1 (0.07–0.15) g/24-hour in control participants. This indicates that proteinuria levels increased progressively with advancing CKD stages, reflecting worsening kidney damage. In contrast, control participants exhibited significantly lower proteinuria levels, within the normal range, confirming the absence of kidney damage. Similarly, the eGFR, another essential criterion for CKD, demonstrated a consistent decline as CKD stages progressed to the second and third stages, indicating reduced kidney function, while the control group maintained high (i.e., normal)

eGFR levels (Table 3). Therefore, in the correlation analyses, we took into consideration the results of the entire cohort and evaluated patient and control groups separately, accounting for the impact of the control group's results within the whole cohort. Furthermore, we conducted regression analyses adjusted for proteinuria to strengthen the validity of our findings. Proteinuria is a potentially confounding variable, as it also serves as a key early indicator of kidney damage, particularly in stages 1 and 2 CKD, where eGFR remains compensatory preserved (Gorriz & Martinez-Castelao, 2012).

4.1. Main Study

Comparative analysis revealed a marked reduction in urinary protein numbers in the patient group compared to the control group (Table 3). Proteomic profiling of complex biological specimens poses analytical challenges, primarily due to the broad dynamic range of protein abundances. For example, high-abundance proteins with concentrations exceeding ten orders of magnitude in plasma, including immunoglobulins and albumin, can hinder the detection of trace proteins, thereby limiting the sensitivity of mass spectrometry (Borberg et al., 2021). Likewise, during proteinuria, the urinary proteome shows increased levels of albumin and other high-abundance proteins, which can hinder the detection of low-abundance species. This interference is also referred to as the masking effect and complicates the accurate identification and quantification of low-abundance urinary proteins (Filip et al., 2015; Magagnotti et al., 2010), as demonstrated in Figure 2 and Table 3 for the patient groups with fewer proteins detected.

4.1.1. Correlation Analysis

Our findings were consistent with previous studies examining correlations between kidney function markers and protein molecular weight stratification. High-molecular-weight proteins (> 60 kDa), such as UROM, CERU, CD44, and KNG1, showed significant positive correlations with eGFR (Table 5), supporting the notion that larger proteins may reflect kidney function (Kalantari et al., 2013; Prikryl et al., 2017; Schaeffer, Devuyst, & Rampoldi, 2021). Conversely, low-molecular-weight proteins (< 60 kDa), including IGHA1, VTDB, IGL1, KVD20, IGK, B2MG, and FETUA, were negatively correlated with eGFR, consistent with previous research (Argyropoulos et al., 2017; Bassey et al., 2022; Shoukry, Bdeer, & El-Sokkary, 2015). We also observed positive correlations between eGFR and lower molecular weight proteins (e.g., PTGDS, RNAS1, and VMO1) across the cohort. These findings highlight the relationship between protein size, renal function, and disease progression.

In addition to molecular size, the charge of urinary proteins also affects the protein filtration through the glomerular barrier. The glomerular basement membrane (GBM) is negatively charged, generally restricting the passage of negatively charged proteins (Naylor, Morais, & Lennon, 2021). This may partially explain the increased excretion of specific low-molecular-weight proteins (e.g., A1BG, B2MG, FETUA, and VTDB), which have acidic isoelectric points (Kozlowski et al., 2022), indicating a potential role for charge-based filtration. However, in CKD, the loss of charge-selectivity in proteinuric conditions and/or due to GBM injury can also lead to increased filtration of negatively charged small proteins (Garsen et al., 2014). Although charge profiling was beyond the scope of this study, the interaction between size and charge-based filtration likely contributes to the observed proteomic patterns and warrants further investigation.

Correlation analysis within the CKD group further highlighted specific proteins that exhibited distinct correlations with eGFR (Table 6). CERU, A1BG, CD59, and LV39 showed positive correlations with eGFR, suggesting their potential as markers of relatively preserved kidney function. Conversely, VTDB, B2MG, FETUA, and AMBP emerged as proteins with negative correlations, although AMBP was not correlated in the entire cohort, suggesting their relevance to impaired kidney function.

4.1.2. Proteins Negatively Associated With Kidney Function

After the correlation analyses, the regression analyses further elucidated the intricate relationship between renal function and urinary proteins. In particular, the correlated proteins exhibiting negative coefficients, including VTDB, IGK, FETUA, and B2MG, showed significant associations with eGFR across the whole cohort, even after accounting for proteinuria. A more detailed subgroup analysis within the patient group further highlighted that AMBP, VTDB, FETUA, and B2MG proteins maintained consistent inverse associations with eGFR despite adjusting for the effects of proteinuria (Figure 6 and Figure 7). These consistent associations suggest that these proteins could serve as biomarkers of underlying renal dysfunction (Appendix C (Table C1)).

The rise in low-molecular-weight urinary proteins that were negatively associated with eGFR mainly results from proximal tubular dysfunction. Subsequently, this leads to impaired tubular reabsorption (Christensen et al., 2012). Therefore, these proteins can serve as potential biomarkers for kidney tubular damage and dysfunction. In this context, studies have shown that urinary B2MG can function as a diagnostic marker for tubular injury caused by nephrotoxic agents or systemic infections (Argyropoulos et al., 2017). Additionally, longitudinal investigations indicated a persistent elevation of urinary B2MG

in patients recovering from acute kidney injury due to snake bites, even after eGFR normalization, suggesting unresolved tubular dysfunction with implications for long-term renal outcomes (Jaswanth et al., 2019). Also, high urinary B2MG/creatinine levels were associated with lower eGFR one year after an episode of acute kidney disease (Puthiyottil et al., 2021). Moreover, B2MG has been reported to promote oxidative stress in PTECs through protein-ligand interactions in proteinuric kidney diseases (Argyropoulos et al., 2017; Fels et al., 2019). The oxidative damage pathway was also found to be enriched in patients with proteinuria, though this was not directly linked to B2MG (Hao et al., 2020).

Fetuin-A (FETUA) is a glycoprotein derived from hepatocytes. It has multiple roles and has emerged as another potential biomarker. FETUA has been reported to exhibit renoprotective properties by alleviating hypoxia-induced injury, inflammatory cascades, and fibrotic remodeling (Chekol et al., 2022). In recent investigations, urinary FETUA levels were inversely correlated with eGFR, highlighting its potential for monitoring the progression of CKD (Bassey et al., 2022; Piazzon et al., 2015).

Similarly, alpha-1-microglobulin/bikunin precursor (AMBP), a liver protein with antioxidant and cytoprotective functions, protects PTECs by maintaining mitochondrial integrity and neutralizing reactive oxygen species (Kristiansson et al., 2020). Elevated excretion of urinary AMBP has been observed in tubular injury linked to diabetic nephropathy and glomerulopathies (Amatruda et al., 2022; Kalantari et al., 2013). Vitamin D-binding protein (VTDB) has demonstrated a significant correlation with renal functional decline in diabetic and IgA nephropathy cohorts (Kalantari et al., 2013; Shoukry, Bdeer, & El-Sokkary, 2015). In addition, functional enrichment analyses further support the involvement of immune dysregulation and inflammatory pathways in early CKD pathogenesis, which were predominantly enriched by proteins negatively associated with eGFR. Overall, the consistent findings from multiple studies emphasize the clinical importance of these negatively associated proteins as potential markers of renal dysfunction, warranting further research to investigate their diagnostic and prognostic utility.

4.1.3. Proteins Positively Associated With Kidney Function

The correlated proteins with positive coefficients, such as UROM, SH3L3, RNAS1, OSTP, LV39, KNG1, CERU, CD59, CD44, and A1AG2, showed significant associations with eGFR across the whole cohort, even after accounting for proteinuria. Nevertheless, a subgroup analysis within the patient group revealed that only three proteins, CERU, A1BG, and LV39, maintained consistent positive associations with eGFR after adjusting

for proteinuria (Figure 6 and Figure 7). These consistent associations demonstrate the potential of these proteins as biomarkers for reflecting normal or high eGFR levels, suggesting preserved kidney function (Appendix C (Table C1)).

Proteins demonstrating positive correlations with eGFR in CKD include ceruloplasmin (CERU), a high-molecular-weight glycoprotein responsible for systemic copper transport (Lopez, Royer, & Shah, 2023). While the glomerular filtration barrier typically restricts the filtration of CERU, its presence in the tubular lumen during proteinuric states has been implicated in tubular toxicity of proteinuria under acidic tubular lumen microenvironments, potentially exacerbating renal injury (Ito et al., 2001). Besides, another research in IgA nephropathy patients supports CERU's positive association with eGFR (Kalantari et al., 2013), though its pathological role in tubular compartments warrants further exploration. Other proteins, such as alpha-1B-glycoprotein (A1BG) and immunoglobulin lambda variable 3–9 (LV39), remain underexplored in CKD contexts. A1BG, a plasma glycoprotein, has shown diagnostic relevance in differentiating pediatric steroid-resistant nephrotic syndrome from non-steroid-resistant types (Piyaphanee et al., 2011). Also, a urinary increase in acute kidney injury following snake bites has been demonstrated (Brasileiro-Martins et al., 2024). Nevertheless, the underlying causes, mechanisms, and progression of acute and chronic kidney diseases differ, though some biomarkers may overlap (Van Nynatten et al., 2023).

The functional analysis of eGFR-associated proteins highlights their role in maintaining extracellular matrix (ECM) stability, modulating immune responses, and regulating coagulation pathways in CKD. Proper ECM regulation is essential for preventing excessive accumulation, minimizing fibrosis, and preserving structural integrity. Proteinuria significantly contributes to kidney inflammation and fibrosis. It disrupts ECM breakdown and promotes ECM accumulation, which may eventually lead to ESKD (Stephan et al., 2004). Furthermore, the notable enrichment of platelet activation pathways suggests an adaptive response to glomerular endothelial injury aimed at mitigating blood loss following vascular damage. However, activated platelets may interact with leukocytes, intensifying renal inflammation (Finsterbusch et al., 2018; Gros, Ollivier, & Ho-Tin-Noé, 2015). These platelet-leukocyte aggregates have been proposed as potential biomarkers for kidney disease as well as the prognosis of patients (Finsterbusch et al., 2018). Thus, a decline in these urinary proteins in patients with CKD may further worsen disease progression by altering immune regulation, coagulation processes, and ECM homeostasis.

4.2. Follow-up Study

The control group had more detectable proteins at baseline than the patient group. While the number of detected proteins in the controls slightly declined over time (from 271 to 252), their kidney function and damage parameters, including eGFR and proteinuria, remained within normal ranges. This indicates a stable clinical and proteomic profile in the controls. However, in the patient group, detected proteins increased from 171 at baseline to 285 at follow-up. This increase is attributed to decreased proteinuria levels, which fell from a median of 3.1 g/24 hours to 2.1 g/24 hours throughout the study period. The stable profiles in the controls contrast with the dynamic proteomic changes observed in CKD patients, highlighting the specificity of protein shifts, such as those observed in VTDB, CD44, and FBN1, as markers of underlying CKD. Interestingly, although the controls did not show clinical deterioration, regression analyses revealed shifts in the associations between eGFR and several proteins, including LMAN2 and KVD20, from positive to negative over time. However, the dynamic proteomic changes in the controls are less likely to reflect pathological kidney changes and are more likely to represent baseline physiological variability.

Baseline analysis revealed that VTDB was negatively correlated with kidney function across the entire cohort, as well as within the CKD group. However, this association was statistically significant only in the subgroup analysis. Furthermore, VTDB was the only protein that consistently exhibited an inverse association with eGFR in regression models in the patient group. Nevertheless, this statistical significance was lost after adjusting for proteinuria. Several other proteins also showed associations in regression models at baseline, including Fibrillin-1 (FBN1), which exhibited a positive association, and FETUA, which was strongly negatively associated with eGFR in patients. Importantly, FETUA and VTDB were also negatively associated with eGFR in our main study, suggesting that FETUA and VTDB could be the earliest markers of kidney dysfunction.

During the follow-up, several proteins showed significant correlations with kidney function, with VTDB showing consistent positive associations with eGFR, even after adjusting for proteinuria. This contrasts with the negative relationship observed at baseline, suggesting a potential shift in its functional role as CKD progresses (Appendix C (Table C1)). The transition from an inverse to a positive association between VTDB and eGFR highlights its potential utility as a biomarker for monitoring improvement in kidney function among patients with CKD, although this does not necessarily reflect structural recovery in renal tissue. Additionally, FBN1 and CD44, which were positively associated with eGFR at baseline, exhibited negative correlations at follow-up, potentially reflecting

disease progression and declining kidney function (Appendix C (Table C1)). These findings highlight the prognostic significance of CD44, FBN1, and VTDB as markers, providing novel insights into their roles in disease progression and their relevance for clinical risk evaluation.

While overt CKD progression was observed in a few patients (Figure 3), a substantial number of patients exhibited declines in eGFR (Figure 4) or increases in proteinuria (Figure 5) over the follow-up period. Furthermore, even among those with improved proteinuria, levels remained pathologically elevated in most patients compared to controls, indicating ongoing subclinical disease activity. These findings support the continued relevance of CD44, FBN1, and VTDB as markers of disease dynamics, even in the absence of dramatic progression in all patients.

Comparing protein levels between baseline and follow-up further highlights the dynamic roles of these candidate biomarkers in disease progression. Notably, VTDB levels increased by 138% in emPAI values over time, coinciding with a shift to a positive association with eGFR. This dynamic change aligns with growing evidence of VTDB's multifaceted role in kidney pathophysiology. VTDB is not only a carrier of vitamin D metabolites but also participates in the actin scavenging system and modulates inflammatory responses via macrophage activation (White & Cooke, 2000). As a low-molecular-weight protein, VTDB is freely filtered through the glomerulus and normally reabsorbed by PTECs (Christensen et al., 2012). Thus, increased urinary VTDB in early CKD reflects impaired tubular reabsorption due to proximal tubular injury, consistent with its negative baseline association with eGFR (Denburg & Bhan, 2015; Mirković et al., 2013). However, the shift to a positive correlation with eGFR in our study and increased VTDB levels may suggest a complex interplay involving altered tubular handling, potentially including compensatory mechanisms. For instance, elevated VTDB levels could reflect increased synthesis to counteract vitamin D deficiency or mitigate inflammation and fibrosis in progressing CKD (Adamantidi et al., 2024; Agarwal, 2009; Delanghe et al., 2024). Alternatively, with reduced glomerular protein leakage, the remaining urinary VTDB might originate from increased tubular secretion or shedding. This could reflect either an adaptive response or ongoing tubular damage, despite overall improvements in kidney function. To the best of our knowledge, no previous longitudinal studies have reported dual associations of VTDB with eGFR. A study by Kalantari et al. (2013) identified VTDB as a predictive biomarker for IgA nephropathy severity; however, their analysis was based on kidney biopsy findings and lacked follow-up validation of association dynamics. Another longitudinal study in microalbuminuric patients found

VTDB to be a marker of tubulointerstitial damage, yet reported no significant correlation with eGFR (Mirković et al., 2013). These findings highlight the need for further investigation to fully understand the dynamic shifts in VTDB associations with kidney function and the implications of increased urinary VTDB in the context of improving proteinuria.

While our findings consistently highlight VTDB as a potential marker of kidney dysfunction and disease progression in both the main and follow-up studies, the absence of recorded medication data limits the complete clinical interpretation of VTDB dynamics. Specifically, treatments such as RAAS inhibitors and SGLT2 inhibitors may affect proteinuria levels, urinary proteomic patterns, and possibly VTDB excretion (Kalay et al., 2023; Podestà et al., 2023; Zhang et al., 2017). Moreover, since VTDB is the primary carrier of vitamin D and is influenced by parathyroid hormone (PTH), its urinary levels may also reflect changes in mineral metabolism activity (Khundmiri, Murray, & Lederer, 2016). The unavailability of serum vitamin D and PTH levels in this cohort prevents exploration of whether VTDB fluctuations are related to disturbances in calcium-phosphate homeostasis, which is common in CKD. Future studies should include these variables to clarify VTDB's multifaceted role and enhance its clinical utility as a prognostic and mechanistic biomarker.

In addition to VTDB, other proteins also exhibited notable changes in abundance over time. Similarly, there was an increase of up to 68.97% in FETUA values at follow-up, although no significant association with eGFR was found. Conversely, FBN1 and CD44 levels decreased by 13.4% and 34.15%, respectively. Both proteins have been reported to be involved in renal tubular and ECM disorders (Chebotareva et al., 2023; Li et al., 2021); however, the clinical significance of their reduced urinary excretion in the context of CKD progression requires additional investigation.

FBN1, a microfibrillar protein, serves as a structural component in fibrotic kidney tissue in patients with CKD (Li et al., 2021). The relationship between FBN1 and kidney function, along with its urinary excretion, has not been extensively studied. CD44 is a transmembrane glycoprotein, and decreased levels of it in urine have been observed in IgA nephropathy and acute kidney transplant rejection (Prikryl et al., 2017; Sigdel et al., 2010). However, its correlation with eGFR has not been reported in these studies. These findings emphasize the evolving roles of VTDB, FBN1, and CD44 in CKD and their potential as biomarkers for clinical assessment.

4.3. Limitations

Both studies have limitations that must be acknowledged. First, the small sample size in both investigations restricts the strength of the conclusions, thereby limiting the generalizability of the findings. Additionally, the follow-up duration may not have been sufficient to capture long-term changes in CKD progression. Future research with larger multicenter cohorts and extended follow-up periods is essential to validate and expand on these observations. Second, a gender imbalance existed between the patient and control groups in both studies, introducing potential confounding factors that may have influenced the results. To enhance the reliability of future findings, studies should ensure a more balanced gender distribution. Third, both investigations focused exclusively on patients with stages 1–3 CKD, limiting the applicability of the results to later disease stages. The identified protein associations may differ in individuals with advanced CKD, highlighting the need for studies that encompass a broader range of disease severity. Fourth, the use of emPAI as a semiquantitative approach to estimate protein quantification has inherent limitations. The method does not provide absolute abundance, requiring validation through more sensitive and specific techniques, such as targeted mass spectrometry or immunoassays, to confirm the diagnostic potential of these findings. Furthermore, during the urine sample preparation for mass spectrometry analysis, high-abundance proteins were not depleted. This may have hindered the detection of low-abundance proteins, especially in patients with higher proteinuria at baseline. Fifth, the diagnosis of glomerular disease was not confirmed via kidney biopsy in either study, which could have influenced patient classification and subsequent data interpretation. Future studies should incorporate histopathological confirmation to strengthen diagnostic accuracy. Lastly, clinical data such as patient medication use, serum PTH and vitamin D levels were unavailable in our study. This limitation is particularly relevant for interpreting VTDB dynamics, as these factors influence VTDB levels and could confound its association with kidney function. Incorporating such data in future research would help clarify VTDB’s mechanistic role and enhance its clinical interpretability as a CKD biomarker.

4.4. Conclusion

This thesis demonstrated substantial differences in the type and quantity of urinary proteins between individuals with early-stage CKD and healthy controls. Several urinary proteins showed strong associations with kidney function, with some showing positive correlations and others demonstrating negative correlations. Proteins negatively associated with kidney function were mainly enriched in pathways related to tissue integrity, structural

maintenance, and immune regulation, while those with positive associations were linked to coagulation, ECM, cellular adhesion, and immune modulation.

Moreover, longitudinal analysis revealed dynamic changes in protein associations over time, suggesting their potential role in CKD progression. Notably, VTDB shifted from a negative to a positive correlation with kidney function, while CD44 and FBN1 displayed the opposite trend, suggesting the evolving biological significance of these proteins in the disease's pathophysiology.

These findings highlight the potential of urinary proteomics in monitoring CKD progression and identifying biomarkers essential for early detection, prognosis, and disease stratification while reflecting injury and recovery processes. Specifically, the VTDB, FETUA, B2MG, and AMBP proteins may serve as non-invasive diagnostic tools for detecting early kidney dysfunction and tubular damage. Meanwhile, CERU, A1BG, and LV39 could be utilized to monitor preserved kidney function. The dynamic changes observed in FBN1, CD44, and VTDB levels also suggest their prognostic utility in tracking disease progression or therapeutic response over time.

Clinically, these biomarkers could assist in personalized treatment planning, helping to identify patients at a higher risk of progression or those who respond well to therapy. Additionally, early biomarker-based detection may reduce the need for invasive procedures such as kidney biopsies in diagnostics and disease stratification.

To translate these findings into clinical practice, further validation through multicenter studies with larger cohorts and extended follow-up periods is needed. Additionally, the integration of targeted proteomics and immunoassays will be critical in establishing the clinical relevance and specificity of these biomarkers. Expanding research to include patients across all CKD stages, as well as patients with diabetes and diabetic nephropathy, will also enhance the understanding of how urinary proteins influence disease progression, ultimately contributing to more effective and personalized therapeutic approaches.

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Appendix A

Written Informed Consent Form Template

Introduction. You are invited to participate in a research study entitled "Development and validation of hybrid Brillouin-Raman spectroscopy for non-invasive assessment of mechano-chemical properties of urine proteins as biomarkers of kidney diseases."

Procedures.

The goal of the project is to develop and validate the non-contact assessment of visco-elastic and chemical properties of human urine proteins as biomarkers of kidney disease. The project ultimately aims to development of an optical spectroscopic sensor for rapid, non-contact monitoring of urine samples from patients in clinical settings. Participants will be asked to collect 5 ml venous blood samples (during the routine blood tests in hospital sittings) and spot urine samples once at the hospitalization. No other invasive or interventional procedures or treatment recommendations related to this study are allowed. All other diagnostic and treatment protocols will be provided according to the hospital regulations and locally approved guidelines by responsible physicians, independent of this proposed project. This blood and urine sample collection will take approximately 15-20 minutes to complete.

Risks. The potential risks of participating in this study are: there are no risks related to blood/urine collections.

Benefits. Anticipated benefits from this study add the potential benefits to patients with proteinuria and nephrotic syndrome. Identifying the most important types of urine proteins based on their visco-elasticity, amino-acid profile, and molecular weight responsible for the most severe cases of proteinuria and progressive renal function decline will further prognosis and predict the adverse outcomes. Based on the results of the study, diagnostic and predictive models would be suggested that offers benefit to any patients with proteinuria and nephrotic syndrome.

Compensation. No tangible compensation will be given. A copy of the research results will be available at the conclusion of the study disseminated as research articles and reports.

Confidentiality & Privacy. Any information that is obtained during this study will be kept confidential to the full extent possible. All efforts, within reason, will be made to keep your personal information in your research record confidential, but total confidentiality cannot be guaranteed. Case report forms (CRF) will be created for each participant, including 6-digital numbers, which will be used for further coding to all patient's data. Only primary care physicians will know each patient's identity and the

corresponding 6-digit number assigned to the patient. However, primary care physicians will be blinded from the results of the study, and the research team will be blinded from the patients ID.

Voluntary Nature of the Study. Participation in this study is strictly voluntary, and if an agreement to participate is given, it can be withdrawn at any time without prejudice.

Points of Contact. It is understood that should any questions or comments arise regarding this project or a research-related injury be received, the Principal Investigator, ***Dr. Abduzhappar Gaipov, +7(701)379-0637, abduzhappar.gaipov@nu.edu.kz*** should be contacted. Any other questions or concerns may be addressed to the Nazarbayev University Institutional Research Ethics Committee, resethics@nu.edu.kz.

Statement of Consent.

I,

_____

_____,

Give my voluntary consent to participate in this study.

The researchers clearly explained to me the background information and objectives of the study and what my participation in this study involves.

I understand that my participation in this study is voluntary. I can, at any time and without giving any reason, withdraw my consent, and this will not have any negative consequences for me.

I understand that the information collected during this study will be treated confidentially.

Signature:_____Date: _____

Researcher:

Signed_____Date_____

Appendix B

24-hour Urine Sample Collection

A 24-hour urine sample is a collection of urine samples multiple times within 24 hours. A storage container is used for 24-hour urine collection. Urine specimens should be collected between the first day of 6 am and the second day of 6 am.

Urine Collection Steps

- Label a container with a name, date of birth, and sample collection date and time.
- Wash hands each time thoroughly collecting the urine.
- On Sunday, at 6 am, flush the first urine specimen upon rising from sleep and note the time. Collect the urine starting from the second urine specimen.
- Finish the urine collection the next day at 6 am.

After the first flushed specimen, all urine must be stored in the cold on ice or in a fridge at +4 °C for 24 hours and immediately transported to medical personnel.

Early Morning Urine Sample Collection

A sterile container is used for urine collection. Mid-stream first-morning specimen should be collected immediately after a night's sleep. The container with urine should be stored in a sealed bag in a fridge at +4 °C in case someone cannot hand it over to medical personnel within one hour after urine collection. It should not be stored longer than 24 hours.

Urine Collection Steps

- Label a container with a name, date of birth, and sample collection date and time.
- Wash hands thoroughly.
- Avoid contacting the container with legs and clothing.
- Avoid touching the inside of the container and the lid.
- Urinate the first portion of urine into the urinal, bedpan, or toilet, momentarily pause the urine flow and collect the second portion "mid-stream" of 100 mL urine into the container.
- Urinate the remaining third portion of urine into the urinal, bedpan, or toilet.
- Close the container immediately.
- Wash hands thoroughly.

Appendix C

Table C1

Characteristics of Urinary Proteins Identified as Potential Biomarkers in CKD Patients

UniProt entry name	Molecular weight (kDa)	Association with eGFR	Clinical relevance	CKD-related role (from literature)	References
A1BG	54	Positive	Potential marker of preserved kidney function.	Diagnostic relevance in pediatric steroid-resistant nephrotic syndrome; acute phase increase.	Brasileiro-Martins et al., 2024; Piyaphanee et al., 2011
AMBP	39	Negative	Potential marker of kidney dysfunction.	Elevated in tubular injury (diabetic nephropathy/glomerulopathies); antioxidant role in PTECs.	Amatruda et al., 2022; Kalantari et al., 2013; Kristiansson et al., 2020
B2MG	14	Negative	Potential marker of kidney dysfunction.	Marker of tubular injury; may promote oxidative stress in PTECs.	Argyropoulos et al., 2017; Fels et al., 2019; Jaswanth et al., 2019;
CD44	81	F: Positive → negative	Potential marker for disease progression and declining kidney function.	Decreased levels were observed in IgA nephropathy and acute kidney transplant rejection.	Prikryl et al., 2017; Sigdel et al., 2010
CERU	122	Positive	Potential marker of preserved kidney function.	Potential tubular toxicity in acidic proteinuric microenvironments; high-molecular-weight protein positively associated with eGFR (preserved function).	Ito et al., 2001; Kalantari et al., 2013
FBN1	312	F: Positive → negative	Potential marker for disease progression and declining kidney function.	Structural component of fibrotic kidney tissue.	Li et al., 2021
FETUA	39	Negative	Potential marker of kidney dysfunction.	Renoprotective properties (anti-inflammatory/anti-fibrotic); a marker of CKD progression.	Bassey et al., 2022; Chekol et al., 2022; Piazzon et al., 2015
LV39	12	Positive	Potential marker of preserved kidney function.	Role unclear in CKD.	
VTDB	53	Negative	Potential marker of kidney dysfunction.	Marker of tubular damage and predictive marker for IgA nephropathy severity.	Kalantari et al., 2013; Mirković et al., 2013
		F: Negative → positive	Potential marker for monitoring improvement in kidney function.		

Note. F = shift of association with eGFR over time in the follow-up study.