# Urine Proteomics as a Source of Biological Information and Outcome Predictor in Living Kidney Transplantation

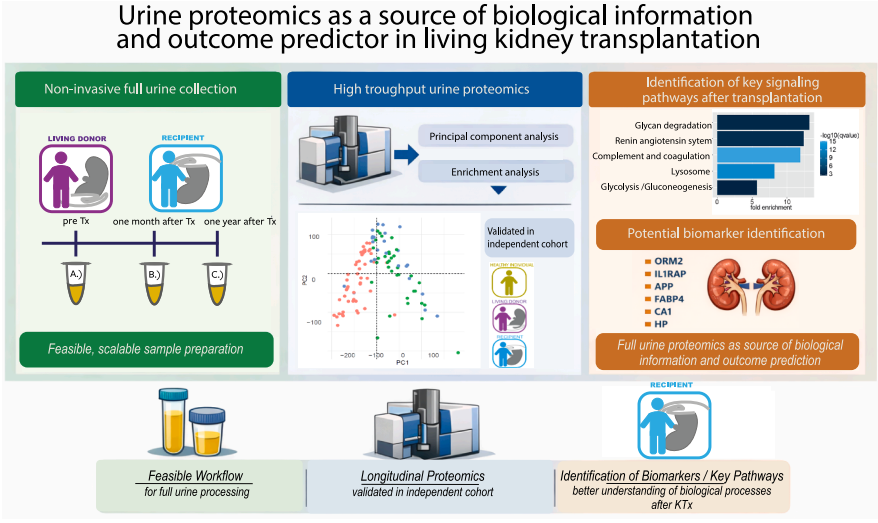

## Authors

K. Johanna R. Hoyer-Allo, Patrick Affeldt, Jan-Wilm Lackmann, Stefan Müller, Denise Buchner, Fabian Braun, Martin Richard Späth, Moritz Trappe, Sita Arjune, Joachim David Steiner, Marta Müller, Maximilian Buschmann, Inga Tometten, Jörg Timm, Johannes Stegbauer, Veronica Di Cristanziano, Christine Kurschat, Dirk Stippel, Katrin Bohl, and Roman-Ulrich Müller

## Correspondence

roman-ulrich.mueller@uk-koeln.de


## Graphical Abstract




## In Brief

This study shows that full-urine proteomics captures dynamic biological processes during living kidney transplantation and identifies non-invasive biomarkers predictive of graft outcomes. Distinct proteomic signatures differentiate donors from recipients and reveal inflammatory, immune, and metabolic pathways activated after transplantation. Key findings were confirmed in an independent validation cohort. Importantly, selected donor- and early post-transplant urinary proteins independently predict later kidney function and infection risk, highlighting urine proteomics as a tool for early risk stratification and personalized post-transplant monitoring.

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## Highlights

- Urine proteomics reveal dynamic molecular changes after living donor kidney transplant.
- Distinct donor and recipient proteome profiles confirmed across independent cohorts.
- Inflammation, immune response and coagulation pathways enriched post-transplant.
- Specific urinary proteins predict eGFR and infection risk up to 1 year.
- Non-invasive urine biomarkers may enhance post-transplant outcome monitoring.

# Urine Proteomics as a Source of Biological Information and Outcome Predictor in Living Kidney Transplantation

K. Johanna R. Hoyer-Allo[1,2,‡], Patrick Affeldt[1,3,‡], Jan-Wilm Lackmann[4], Stefan Müller[4], Denise Buchner[5], Fabian Braun[6], Martin Richard Späth[1], Moritz Trappe[1], Sita Arjune[1,7,8], Joachim David Steiner[1,9], Marta Müller[10], Maximilian Buschmann[10], Inga Tometten[11], Jörg Timm[11], Johannes Stegbauer[10,12], Veronica Di Cristanziano[3], Christine Kurschat[1,8], Dirk Stippel[5], Katrin Bohl[1,2,‡], and Roman-Ulrich Müller[1,2,10,‡*]

**Kidney transplantation (KTx) is the preferred treatment for kidney failure. However, post-transplant management is challenging due to the limited lifespan of transplanted organs. Current methods for monitoring post-transplant complications are invasive and have limitations. Therefore, there is an urgent need for novel non-invasive biomarkers. This study investigates the proteomic composition of urine to understand renal biology during the process of transplantation and to identify potential markers for outcome prediction. Urine samples were collected from donors before transplantation and from recipients 4 weeks and 1 year after transplantation. Proteomic analysis was performed using mass spectrometry and label-free quantification. Statistical analyses included principal component analysis (PCA) and enrichment analysis. The resulting key findings were confirmed in an independent validation cohort. In addition, correlative regression models to evaluate the relationship between protein abundance and clinical outcomes in the further course after transplantation were performed. 106 urine samples in the setting of 70 kidney transplantations were analyzed. PCA revealed distinct clustering of donor and recipient samples, indicating significant proteomic changes after transplantation. Hierarchical clustering and gene ontology analysis identified molecular changes as a response to transplantation and showed an over-representation of relevant pathways related to inflammation, cell immune response and coagulation in both the original and validation cohorts. Multivariate regression analysis, including linear and logistic regression, identified 11 potential protein biomarkers, including ORM2, IL1RAP, APP, and FABP4 as predictors of eGFR 12 months after transplantation and 1 HP as a predictor of infections within the first year after transplantation, respectively. This study underscores the potential of non-invasive urine proteomics for identifying biological processes involved in kidney transplantation and for enhancing post-transplant monitoring and outcome prediction. We identified 12 potential biomarkers with added value to standard clinical parameters linked to transplant outcomes, which will be promising candidates for future outcome monitoring after KTx.**

Chronic kidney disease (CKD) has emerged as a major public health challenge and a significant financial burden on healthcare systems worldwide (1). CKD exacerbates the global burden of morbidity and mortality, primarily through its impact on cardiovascular risk and end-stage renal disease (2). Currently, kidney transplantation (KTx) is the mainstay of treatment for patients with kidney failure, offering superior survival, improved quality of life, and cost-effectiveness compared to any form of dialysis (3, 4)

From the [1]Department II of Internal Medicine and Center for Molecular Medicine Cologne, and [2]CECAD, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany; [3]Institute of Virology, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany; [4]CECAD, University of Cologne, Faculty of Mathematics and Natural Sciences, Cologne, Germany; [5]Department of General, Visceral, and Transplant Surgery Cologne, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany; [6]III. Department of Medicine, University Medical Center Hamburg- Eppendorf, Hamburg, Germany; [7]Center for Molecular Medicine Cologne (CMMC), Cologne, Germany; [8]Center for Rare Diseases Cologne, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany; [9]Max Planck Institute for Biology of Ageing, Cologne, Germany; [10]Department of Nephrology, Medical Faculty, University Hospital Düsseldorf, and [11]Institute of Virology, Medical Faculty, University Hospital Düsseldorf, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany; [12]Cardiovascular Research Institute Düsseldorf (CARID), Medical Faculty, University Hospital Düsseldorf, Heinrich-Heine-University, Düsseldorf, Germany

‡These authors contributed equally to this work.

*For correspondence: Roman-Ulrich Müller, roman-ulrich.mueller@uk-koeln.de.





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Despite its advantages, post-transplant management requires rigorous monitoring due to the limited survival of transplanted organs, often leading to the need for repeat transplantation (5). Five-year graft survival after living donor kidney transplantation (LDKT) is higher than after deceased donor kidney transplantation (DDKT), with rates around 90% versus 81% in younger recipients and 81% versus 68% in older recipients in the United States. (6). Similar trends are reported internationally (7).

Rejection remains a major cause of graft loss. Clinical acute rejection affects 10 to 15% of patients in the first year and is responsible for about 17% of early graft failures, while chronic rejection causes most late graft losses (7). The gold standard for assessing post-transplant complications is invasive biopsy, which has several drawbacks, including bleeding risk with potential organ damage, sampling errors, and subjective interpretation (8). As a result, biopsies are usually performed only once graft dysfunction becomes clinically apparent, limiting the ability to detect subclinical graft injury at an early stage.

Non-invasive biomarkers such as urinary sediment, serum creatinine, estimated glomerular filtration rate (eGFR), and urinary albumin/protein excretion are currently used in clinical practice, although they tend to detect damage at a fairly late stage and provide little specificity (9). In particular, there is considerable heterogeneity in graft function after living donor kidney transplantation, especially regarding eGFR 1 year post-transplantation. This variability is not yet fully understood and limits the ability to stratify patients according to their risk for poorer outcomes. Consequently, clinicians currently lack reliable tools to identify patients at increased risk for adverse outcomes, such as rejection or progressive graft dysfunction, before irreversible injury occurs. The evaluation of a donor kidney is even more critical in the case of deceased donors, especially for kidneys with extended donor criteria (10). Therefore, there is a pressing need for biomarkers that can predict individual trajectories of kidney function early on. Such biomarkers could help guide the intensity of post-transplant monitoring, support clinical decision-making regarding the indication for diagnostic biopsy, and identify patients who may benefit from closer surveillance or earlier intervention. Such predictive tools could enable nephrologists to personalize post-transplant care—intensifying or reducing the need for invasive diagnostics and treatment based on biomarker-defined risk profiles.

Consequently, more specific and predictive diagnostic approaches are urgently needed—ideally based on a non-invasive and easily accessible source such as urine. Urine, which passes through all cells of the nephron, represents a promising avenue to complement histologic assessment. Since proteomics allows the comprehensive characterization of all proteins in a biological sample, differences in proteome composition may indicate pathological conditions, making proteins highly valuable for biomarker discovery.

Our study aims to characterize differences in the proteome composition of urine samples collected during the renal transplantation process. We focus on living donor kidney transplantation, where the need for individualized risk prediction is particularly high due to variability in long-term graft function, and results may be transferred to deceased donation in future investigations. Using mass spectrometry and label-free quantification, we analyzed the protein content of these samples, providing new insights into the biological processes involved in kidney transplantation. In addition, we correlated these findings with future kidney function, rejection, and post-transplant infections, providing preliminary insights into potential prognostic biomarkers.

## EXPERIMENTAL PROCEDURES

### *Experimental Design and Statistical Rationale*

Urine samples were collected from donors before transplantation and from recipients 4 weeks and 1 year after transplantation. Proteomic analysis was performed using mass spectrometry and label-free quantification.

### *Patient Recruitment*

Patients were recruited between 2014 and 2019 through the Departments of General, Visceral and Cancer Surgery, Division of Transplantation Surgery, and the Department II of Internal Medicine, Transplant Center Cologne, according to the approved ethics statements 11 to 310 and 15 to 032, Ethics committee, University of Cologne, Cologne, Germany. The study was conducted in accordance with the Declaration of Helsinki and the good clinical practice guidelines by the International Conference on Harmonization. The performed study is registered at the German Clinical Trials Registry under the ID: DRKS00010534.

In addition, validation was performed using full urine obtained from KTx donors, healthy individuals, and KTx recipients recruited between 2014 and 2025 at the University Hospitals Cologne and Duesseldorf (approved ethics statements 24-1181 and 2018-76-KFmgU).

### *Urine Collection*

Urine samples (≥50 ml) were collected as second morning midstream samples (timepoint A (donor preTx), B (4 weeks) and C (1 year)) into standard urine sample cups. Urine from validation cohorts was collected from healthy individuals and KTx donor preTx and from KTx recipients 4 to 8 weeks after KTx.

Within 90 min after sample preservation, preservatives and protease inhibitors were added to the final concentrations 6.6 mM $NaN_3$, 2.0 mM EGTA, 1.0 mM PMSF. Samples were stored at –80 °C without any additional freeze–thaw cycles.

*Outcome after KTx*—Besides measurement of eGFR infectious events, and events were defined as outcome parameters after KTx. Infectious events were defined as any reactivation or infection with BK polyomavirus (BKVPyV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV) in virus-specific PCR from blood and any other infection (viral, bacterial or fungal) requiring hospitalization.

KTx biopsies were performed upon indication, there were no protocol biopsies. Biopsy-proven rejection was classified according to the BANFF classification in the version of 2017 and considered all types of rejection including antibody-mediated (ABMR), T-cell mediated rejection (TCMR), borderline TCMR as well as C4d positivity.

Measurable DSA within the follow-up period that had not been present pre-KTX were considered de novo DSA (dnDSA). Data on the clinical outcome after KTx in the validation cohort was not available due to ongoing follow up in these studies.

### *Unbiased Proteomic Analysis*

*Data Acquisition*—Samples were analyzed by the Proteomics Facility on an Evosep One (Evosep) coupled to an Orbitrap Exploris 480 system equipped with FAIMS (Thermo Fisher Scientific). The Evosep One was run with the 60 SPD method using a recommended 15 cm column (150 μm inner diameter, 1.5 μm beads) and a 30 μm inner diameter stainless steel emitter (both Pepsep). The mass spectrometer was operated in data-independent acquisition FAIMS temperatures set to 99.5 °C (inner) and 95 °C (outer electrode, initial cohort)/85 °C (outer electrode, validation cohort), respectively and a constant compensation voltage of −50 V. MS1 scans were performed at 15k resolution and a range of 390 to 890 m/z. Staggered MS2 windows were set between 400 and 880 m/z with a width of 16 m/z, resulting in nominally 8 m/z windows for analysis after deconvolution using ProteoWizard 10 (initial cohort)/ProteoWizard 9 (validation cohort). MS2 acquisition was also performed at 15k resolution and 22 ms maximum IT.

Samples from HpH fractionation were analyzed on the same system with identical FAIMS settings and also with the 60 SPD gradient, but the mass spectrometer was run in data-dependent acquisition mode. MS1 scans were acquired between 390 and 890 m/z at a resolution of 60k. MS2 scans were acquired in a Top20 method with a resolution of 15k, an AGC target of 200% and 20 s dynamic exclusion. All MS2 scans were stored as centroid, MS1 scans as profile.

*HpH Fractionation and Library Building*—For library building, a pool from all peptide digests was fractionated on an Infinity 1260 HPLC system (Agilent) using a 1 mm × 100 mm reversed phase column (Gemini, 3 μm, Phenomenex) at alkaline pH and 30 °C. The flow rate was 0.1 ml/min for the whole method. Prior to injection the column was equilibrated to 100% buffer A (5 mM ammonium bicarbonate in water) for 15 min. Peptides were loaded in a maximum volume of 100 μl. After two sample volumes were passed through the sample loop it was switched back to the bypass position and peptides were separated with a linear gradient of 0 to 30% buffer B (acetonitrile) within 38 min followed by an increase to 45% buffer B within 4 min. The column was regenerated using 85% buffer B for 5 min and re-equilibrated to start conditions for 15 min 48 Fractions were collected from 6 min to 46 min and concatenated to 12 final pools by combining every fourth fraction. Fractions were dried in a vacuum centrifuge, and peptides were dissolved in 10 μl 5% acetic acid in 2% acetonitrile prior to analysis.

After data acquisition, the project-specific spectral library was built in Fragpipe 16.0 (9) using easyPQP option following the "library building" workflow with the only allowed modification being fixed carbamidomethylation at cysteine and MS1 and MS2 boundaries were matched to the acquisition parameters.

The library search was performed against the Uniprot canonical Human proteome database (UP5640, downloaded 04.01.2022), comprising 20,335 entries, using trypsin as the digestion enzyme with two missed cleavages allowed. Precursor and fragment ion mass tolerances were set to 20 ppm. The FDR threshold was set to 1% at both the peptide and protein levels. A full list of peptides included in the DDA-based spectral library, including peptide sequence, precursor charge and m/z, observed modifications and scores, is provided in Supplemental Table S1 (initial cohort)/Supplemental Table S3 (validation cohort).

*Data Processing in DIA-NN*—Sample data was processed in DIA-NN 1.8.111 using the HpH library generated in Fragpipe and annotating it with the Uniprot canonical Human reference proteome (UP5640, downloaded 04.01.2022) using standard settings. MS1 and MS2 boundaries were matched to the acquisition and library generation parameters, and the additional command line parameter "–report-lib-info" was used. Afterward, results were filtered for at least six fragments per precursor, and MaxLFQ quantified using the DIA-NN R-package. Only proteins with at least one unique peptide were used for data analysis. For proteins identified based on a single unique peptide, annotated MS/MS spectra are provided in Supplementary File 1 (initial cohort)/Supplementary File 2 (validation cohort).

DIA searches were conducted with one missed cleavage allowed. Precursor and fragment ion mass tolerances were set to 20 ppm. Carbamidomethylation at cysteine was used as a fixed modification, and no variable modifications were included. The FDR threshold was set to 1% at both the peptide and protein levels. A list of all proteins quantified in the study, including protein accession numbers, number of unique peptides, and MaxLFQ-based quantitation values, is provided in Supplemental Table S2 (initial cohort)/Supplemental Table S4 (validation cohort).

*Data Analysis*—Statistical analysis of DIA-NN output was performed using R (version 4.3.1, R Project for Statistical Computing, Vienna, Austria). A standard PCA was done for all proteins using the R function prcomp. For validation purposes, PCA with healthy individuals, KTx donors and recipients, as well as random remeasurements of samples from timepoints A, B, and C, were performed.

The enrichment analysis was done using the R package topGO [[_aajr_2021]. topGO: Enrichment Analysis for Gene Ontology. R package version 2.46.0.; https://doi.org/10.1093/bioinformatics/btl140]. For the heatmap and clustering the R package pheatmap was used [https://CRAN.R-project.org/package=pheatmap].

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was performed using the function enrichKEGG from the R package clusterProfiler (version 4.11.1). The resulting tables were sorted for q-value. The fold enrichment of the top five pathways was plotted (11). Visualization of KEGG pathway maps was performed with the R package pathview [https://doi.org/10.1093/bioinformatics/btt285].

*Experimental Design and Statistical Rationale for Initial Cohort*—In total, data from 70 kidney transplant donors and recipients were included in the study for the initial cohort. Due to sample availability, 48, 34, and 24 samples were analyzed at timepoints A, B, and C, respectively, representing biological replicates. No technical replicates were included.

To assess associations between protein levels and clinical outcomes (eGFR, rejection, infection), linear and logistic regression models with leave-one-out cross-validation (LOOCV) were employed. This approach is particularly suited for limited sample sizes as it reduces overfitting and provides robust error estimation. A mean model was generated across all LOOCV iterations for each protein and outcome.

While acknowledging the moderate sample size, especially at timepoint C, LOOCV provides a statistically valid framework to identify proteins with consistent correlations across samples. To ensure robustness, only the top 10 proteins per endpoint were selected for further adjustment by clinical confounders, and all findings were interpreted with caution regarding statistical power.

### *Outcome Prediction through Regression Models*

Detected proteins were correlated by linear and logistic regression models to the outcome after transplantation (eGFR, rejection, infectious events) after 1 year (timepoint A, B) and after 2 years (timepoint C). The eGFR was estimated using the CKD-EPI Creatinine Equation (12). Infectious events in the further course after transplantation were defined as any CMV, BKV and/or symptomatic infections with need of

inpatient treatment. Rejections were defined as any biopsy-proven cellular and or humoral rejection.

Models were generated using a leave-one-out cross-validation procedure. Here, all values but one are used as the training set (n-1) to calculate a linear model using the R function lm or glm for linear or logistic regression, respectively. The left-out measurement (the "test set") is calculated using the method predict. lm and the difference between the predicted and true value is used to calculate the prediction error of the model. The process is repeated according to the number of measured proteins (n), resulting in n linear models. Using these models, a mean linear/logistic model is generated for each detected protein. The top 10 strongest correlated proteins for each endpoint were then adjusted for confounders/baseline parameters such as sex, age, donor eGFR and underlying kidney disease.

*Manuscript Preparation*—The original draft of the manuscript was written by the authors without the support of artificial intelligence. ChatGPT was used to optimize wording in the discussion and to obtain a shortened, concise final version based on the manuscript draft.

## RESULTS

### *Clinical Characteristics of Donors and Recipients in Living Donor Kidney Transplantation*

We collected urine samples over the course of 69 living and 1 deceased donor transplantations (only timepoint C).

Figure 1*A* shows the study design. In the case of living donation, urine was harvested from donors the day before transplantation (sample A). Recipient urine was obtained 4 weeks (sample B) and 1 year after transplantation (sample C). 48 Samples were available at timepoint A, 34 at timepoint B and 24 at timepoint C (Supplemental Fig. S1; Supplemental Table S1).

Table 1 summarizes the clinical characteristics of the donors and corresponding recipients. The mean age of donors was 56 years, with a higher proportion of women than men (38 vs 32). The median donor creatinine level was 0.82 mg/dl. Recipients were generally younger and predominantly male. Most transplantations were preemptive living donor transplants (AB0 compatible), followed by ABO-incompatible transplants. Underlying kidney disease groups were primarily genetic, followed by autoimmune diseases.

Table 2 shows the number of adverse events and median eGFR values at one and 2 years after transplantation. Notably, there were 17 rejections at 1 year and only one rejection in the second year after transplantation. 21 infections (as defined in the methods) had occurred at 1 year and five in the second year, including CMV and BKV infections. The median eGFR (CKD-EPI) value remained relatively stable over the 2 years (1 year: 51.00 ml/min, 2 years: 55.56 ml/min.

### *Global Proteomic Profile Analysis and Impact of Baseline Characteristics*

A principal component analysis (PCA) revealed that donor samples clustered separately from recipient samples, which globally appeared similar at 4 weeks and 1 year post-transplantation (Fig. 1*B*). This finding remains unchanged when only including patients for whom samples of all timepoints were available (Fig. S1).

In addition, separate clustering of donor and recipient samples in PCA could be confirmed in the validation cohort (Fig. S2, *A* and *B*), consisting of n = 51 donors/healthy individuals, n = 14 KTx recipients, 4 to 8 weeks after KTx. Besides, n = 29 samples from the initial cohort were re-measured to confirm comparability of measurements between runs (n = 14 timepoint A, n = 11 timepoint B, n = 4 timepoint C; Supplemental Table S5).

To further explore the impact of baseline characteristics, we visualized potential confounding factors such as sex, preemptive status, donation type, age, baseline eGFR (Fig. 1, *C–H*) and disease type by mapping them onto the PCA (Fig. S3). The results revealed no clustering based on these baseline characteristics.

### *In-depth Analysis of Differential Protein Abundance Across Timepoints Reveals Changes in Transport Machinery, Inflammation, Cell Death, and NF-Kappa B Signaling*

Comparing the 20 most abundant proteins measured at each time point, we found that 55% of these proteins were consistently present across all time points (Fig. 2*A*). These included common urinary proteins such as uromodulin (UMOD), albumin (ALB), alpha-1-microglobulin/bikunin precursor (AMBP), ribonuclease A family member 1 (RNASe1), and Kininogen-1 (KNG1). Hierarchical clustering revealed that donor samples differed significantly from other time points with respect to the top 20 proteins, suggesting a transplant-related shift (Fig. 2*B*). Proteins such as epidermal growth factor (EGF), keratin 1 (KRT1), keratin 9 (KRT9), and serpin family G member 1 (SERPING1) were only present in the donor samples within the top 20 proteins. Conversely, retinol binding protein 4 (RBP4), orosomucoid 1 and 2 (ORM1/2), Alpha-2-Glycoprotein 1, Zinc-Binding (AZGP1), Immunoglobulin Kappa Variable 3 to 20 (IGKV3-20), and lymphatic vessel endothelial receptor 1 (LYVE1) were enriched at 4 weeks and 1 year after transplantation.

Interestingly, increased abundance of RBP4, ORM1/2, AZGP1, IGKV3 and LYVE1 could be confirmed in the hierarchical clustering of top 20 proteins in the validation cohort post KTx (Fig. S4*A*).

Gene Ontology (GO) analysis revealed significant changes in the urine proteome at different time points compared to the initial donor FU proteome. Here, we found endosomal sorting complexes required for transport (ESCRT) as an overlapping pattern between 1 month and 1 year after transplantation (Fig. S4, *C* and *D*). To overcome limitations of inter-individual differences and obtain a more general idea of regulated GO-Terms in the course of kidney transplantation, initial and validation cohorts were screened for overlapping terms in both cohorts. In total, 15 terms with enriched proteins of acute inflammation

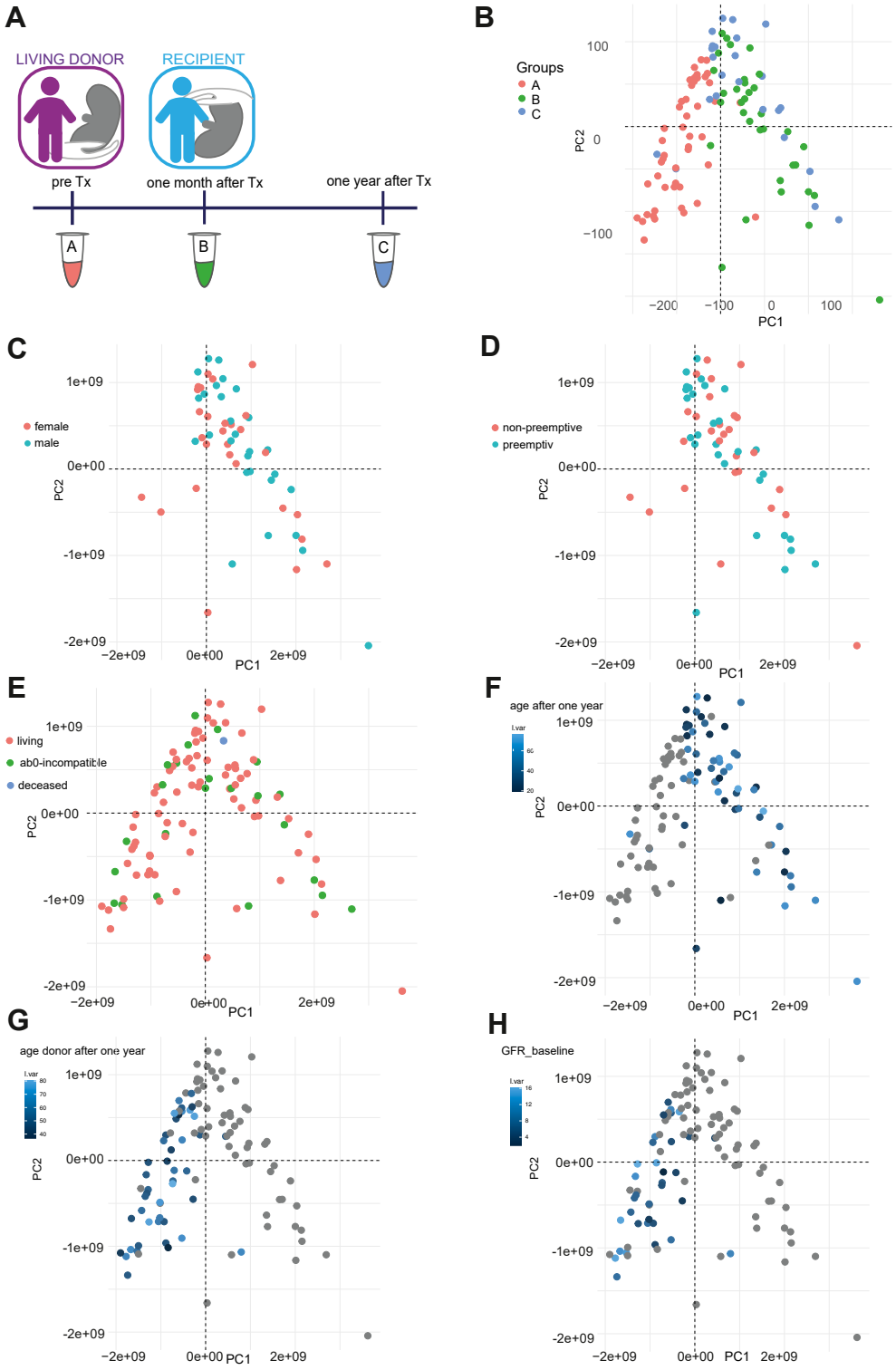


FIG. 1. **Quantitative urinary proteomics in living donor transplantation: Timepoint in respect towards the time of transplantation is the key factor defining changes in the urinary proteome profile.** *A*, schematic overview of the employed sampling protocol during living donor transplantation, *A*: donor sample; *B*: recipient sample 1 month after transplantation; *C*: recipient sample 1 year after transplantation. *B*, principal component analysis of the proteomic data sets of all three groups (*A*: donor samples; *B*: recipient samples 1 month post-transplantation; *C*: recipient samples 1 year post-transplantation). *C*, principal component analysis based on the sex of the recipient 1:female 2:male. *D*, principal component analysis differentiated for non-preemptive kidney transplantation (0) and preemptive kidney transplantation (1). *E*, Principal component analysis based on the type of kidney donation 1:living donation 2:AB0-incompatible donation 3:deceased donation. *F*, principal component analysis based on the age of the recipient 1 year after transplantation. *G*, principal component analysis based on the age of the donor 1 year after transplantation. *H*, principal component analysis based on the eGFR baseline.

TABLE 1
*Baseline characteristics of the initial cohort (n = 70)*

| Baseline characteristics of the cohort (n = 70) | Donor | Recipient |
|---|---|---|
| Age (IQR) | 56 (14) | 48 (29) |
| Sex (%) | | |
| female | 38 (54.3) | 30 (42.9) |
| male | 32 (45.7) | 40 (57.1) |
| Transplantation | | |
| deceased | 1 (1.3) | |
| living | 54 (77.1) | |
| living ABOi | 15 (21.4) | |
| renal disease (%) | | |
| Immunological | | 15 (21.4) |
| genetic | | 25 (35.7) |
| diabetic/hypertension | | 7 (10.0) |
| other | | 23 (32.9) |
| Baseline eGFR ml/min (IQR) | 96.42 (23,52) | |

Representation of age and sex of donors and recipients, type of transplantation of the donor and type of renal disease of the recipients as well as baseline eGFR of the donor.
IQR, interquartile range.

response, transmembrane receptor activities, defense response and immunoglobulin binding were identified (Fig. 2; Fig. S4, *B–E*).

KEGG analysis of the early timepoint, 4 weeks after transplantation, revealed significant differences in pathways implicated in kidney disease, such as the complement and coagulation pathway, renin–angiotensin system and glycolysis/gluconeogenesis as well as lysosome and glycan degradation-associated proteins compared to baseline (Fig. 2*E*). These key pathways had also been identified as key pathways in our validation cohort (Fig. S4*F*). One year post-transplantation, the pathways associated with lysosomes and complement and coagulation signalling, as well as the renin–angiotensin system, were still among the most enriched pathways. Besides, the KEGG analysis identified cell adhesion molecules and ECM-receptor interaction as differentially regulated pathways comparing this later time point with baseline samples (Fig. 2*F*).

TABLE 2
*Outcome in the further course after KTx*

| Event | n |
|---|---|
| ***First year*** | |
| Rejection | 17 |
| Infection | 21 |
| CMV | 9 |
| BKV | 4 |
| EBV | 0 |
| ***Second year*** | |
| Rejection | 1 |
| Infection | 5 |
| CMV | 5 |
| BKV | 0 |
| EBV | 0 |
| ***Total*** | |
| Rejection | 17 |
| Infection | 23 |
| CMV | 11 |
| BKV | 4 |
| EBV | 0 |
| ***Kidney function (IQR)*** | |
| eGFR ml/min 1year | 1.495 (0.63) |
| eGFR ml/min 2years | 1.395 (0.70) |

Number of adverse events (rejection or infection with CMV (cytomegalovirus), BKV (BK polyomavirus), EBV (Epstein-Barr virus) and median eGFR at one and 2 years after transplantation.
IQR, interquartile range.

### *Identification of Protein Markers Predicting Transplant Outcome*

To explore the potential of proteomic data in predicting transplant outcomes, we performed a correlation for clinical endpoints such as eGFR, rejection, and infection (endpoints further defined in the methods section). The resulting candidate protein lists were filtered for the 10 candidates with the lowest error (lowest RSS squared) and highest stability (lowest stdev RSS squared) when correlated with estimated GFR, the occurrence of an infection, or rejection (Fig. 3*A*).

In a second step, these models were adjusted for clinical baseline parameters (Fig. 3, *B–C*) via logistic (infection and rejection endpoint) and linear regression (eGFR). After adjusting for baseline parameters, none of the protein candidates remained significant for the rejection endpoint. For the infection endpoint, only Haptoglobin (HP) at timepoint B was negatively and independently correlated ($p < 0.05$) to occurrence of infections within the first year after transplantation (Supplemental Fig. S5*A*).

Concerning prediction of eGFR after 1 year through candidate proteins measured at timepoint A, the following candidates remained significant after adjusting for baseline parameters: Interleukin 1 Receptor Accessory Protein (IL1RAP), Intracellular Cholesterol Transporter 2 (NPC2), Amyloid-beta precursor protein (APP), Collagen Type XVIII Alpha 1 Chain (COL18A1), Orosomucoid 2 (ORM2), Dermatopontin (DPT), and human prion protein gene (PRNP) (Fig. 3, *B* and *C*, Supplemental Fig. S5, *B–F*). In addition, proteins such as carbonic anhydrase (CA1), immunoglobulin lambda variable 2 to 11 (IGLV2-11), and fatty acid binding protein 4 (FABP-4) at 4 weeks post-transplant remained significant predictors for eGFR at 1 year (Fig. 3*D*, Supplemental Fig. S5, *G* and *H*). For the timepoint C, only C1Q and collagen domain containing adiponectin (ADIPOQ) were independent predictors of eGFR after 2 years (Supplemental Fig. S5*I*).

Multivariate regression of all significant protein candidates in a single analysis, as well as the clinical parameters, did not reveal any meaningful results, probably due to the low sample size compared to the number of tested variables (data not shown).

To minimize the number of tested variables, we prefiltered all significant variables for a $p$-value <0.02 after adjusting for baseline parameters and tested them together with donor and

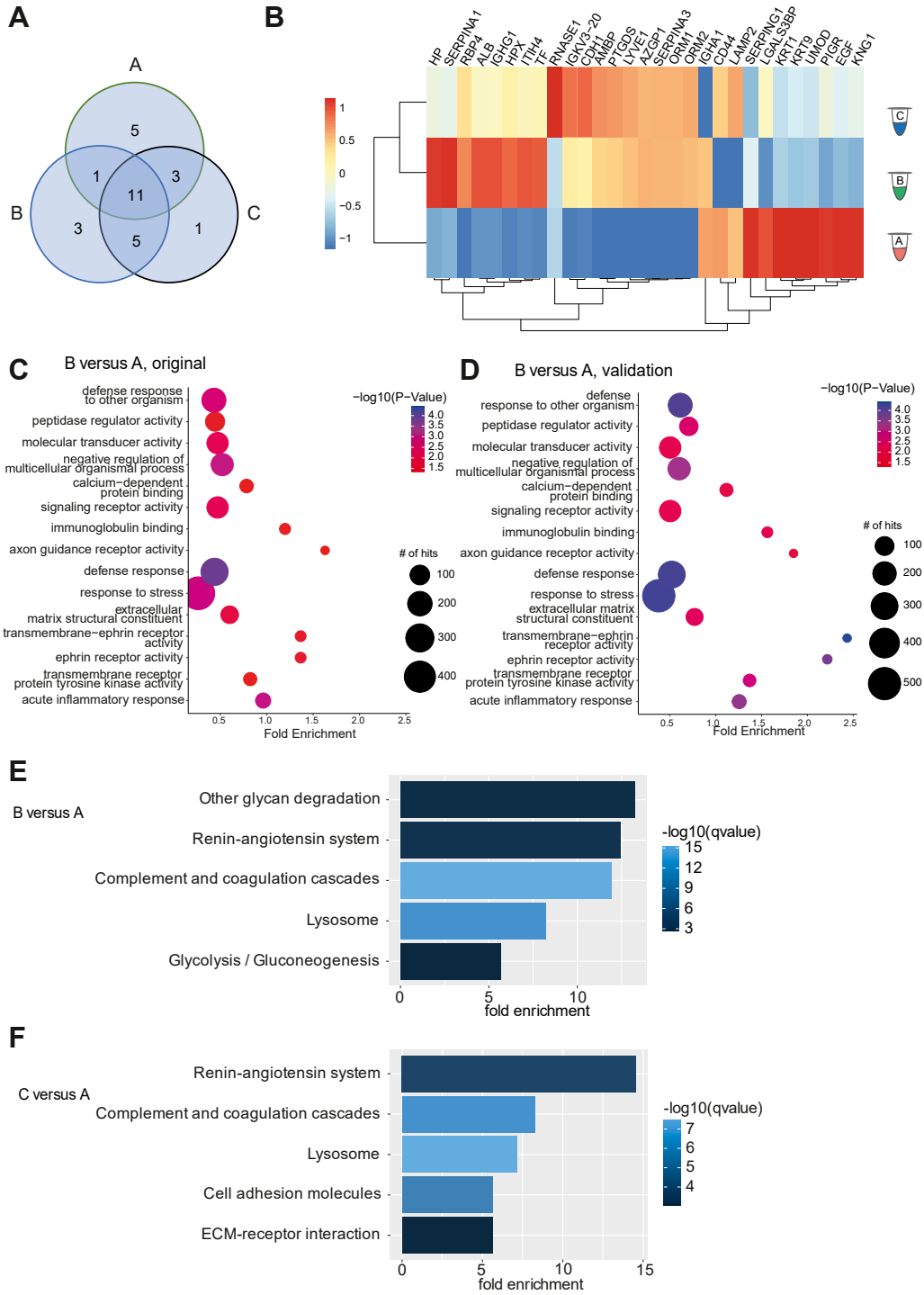


FIG. 2. **Identification of key signaling pathways in the allograft's response to transplantation.** *A*, Venn diagram showing the overlap of the 20 most abundant urinary proteins measured at each timepoint (*A*: donor samples; *B*: recipient samples 1 month; *C*: recipient samples 1 year). *B*, Heatmap of the pooled list of the 29 proteins resulting from the 20 most abundant urinary proteins for each timepoint of sample collection (*A*: donor samples; *B*: recipient samples 1-month post-transplantation; *C*: recipient samples 1 year post-transplantation). Expression is visualized in z-score. *C* and *D*, GO term analysis of protein fold changes compared to donor sample. Overlapping significant GO terms of initial and validation cohort for both timepoints after transplantation compared to donor samples. *p* value depicted as color code, number of annotated proteins corresponding to bubble size, black GO terms indicating biological processes (*C*: Timepoint *B* vs *A*, *D*: Timepoint *C* vs *A*). *E* and *F*) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for comparison of pathway regulation between different timepoints and baseline. *E*, Top five significant q values of pathways timepoint *B* vs. *A*. *F*, top five significant q values of pathways timepoint (*C*) vs. (*A*).

**A**

| Rejection A 1y | Rejection B 1y | Rejection C 2Y | Infection A 1y | Infection B 1y | Infection C 2y | eGFR A 1y | eGFR B 1y | eGFR C 2y |
|---|---|---|---|---|---|---|---|---|
| VPS37D | COL15A1 | PGK1 | FREM2 | SPINT1 | CFHR4 | IL1RAP* | CA1* | COMP |
| PTK7 | CTSD | ARSF | SLC12A1 | CD160 | A2ML1 | NPC2* | MRC1 | CRIM1 |
| CD300LG | EPHB6 | CLEC14A | TPI1 | PKM | SPRR3 | APP** | IGLV2-11* | LTBP4 |
| WDR1 | SOD3 | ATP6V1A | GPC4 | EPS8 | COL12A1 | CLEC14A | VPS4A | ARRDC1 |
| L1CAM | IST1 | COL3A1 | EFEMP1 | VCP | PROS1 | COL18A1* | GPX3 | FLOT2 |
| NPEPPS | CPM | H4C1 | PLS3 | CTSA | APCS | PAPPA2 | GSN | IGLV5-45 |
| PROS1 | DNASE1 | UPK1A | IGHV6-1 | MSN | IGKV3-20 | ORM2* | ANGPTL3 | MPST |
| CADM1 | ARSA | CA2 | OPCML | SERPINA1 | UMOD | DPT* | PTGDS | ADIPOQ* |
| KIF12 | CD2AP | BCAM | AKR1B1 | F11 | A2M | PRNP** | COTL1 | MELTF |
| F11 | PGLS | BDH2 | HP* | C4BPA | PRSS3 | FREM2 | FABP4* | CHMP3 |

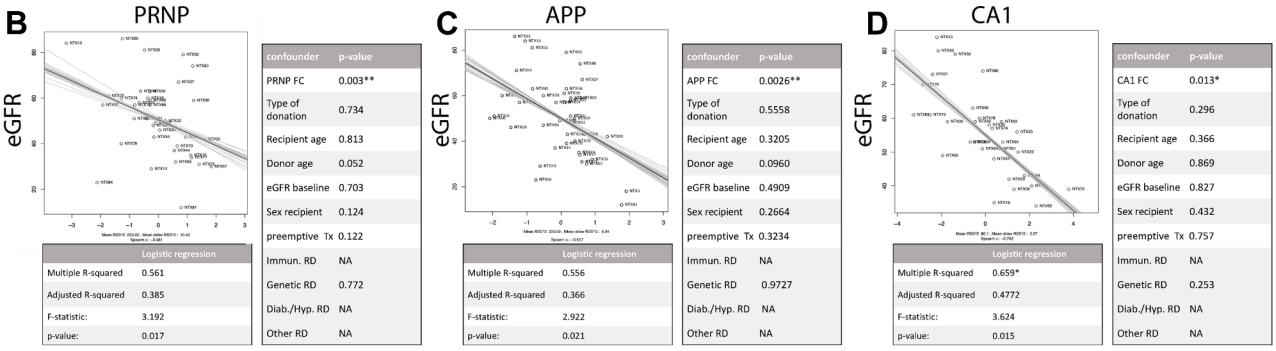


FIG. 3. **Linear regression analyses reveal urinary protein signatures in donor and early post-transplant samples predicting outcome.** *A*, table for top 10 proteins regarding error and stability in correlation with the individual endpoints (separately for each timepoint, pos. (*blue*)/ neg. (*red*) correlation with endpoints indicated by color). *B*, linear regression model for correlation of eGFR and protein FC of PRNP at timepoint A, adjusted for confounders. Strength of correlation is expressed in *p*-values. Given on the x-axis FC- of protein. Given on the y-axis eGFR 1 year after transplantation. *C*, linear regression model for correlation of eGFR and protein FC of APP at timepoint *A*, adjusted for confounders. Strength of correlation is expressed in *p*-values. Given on the x-axis FC- of protein. Given on the y-axis eGFR 1 year after transplantation. *D*, linear regression model for correlation of eGFR and protein FC of CA1 at timepoint A, adjusted for confounders. Strength of correlation is expressed in *p*-values. Given on the x-axis FC- of protein. Given on the y-axis eGFR 1 year after transplantation.

recipient age as cofactors in a new linear regression model. For timepoint B, the correlation between CA1 and eGFR after 1 year was still significant, whereas no such correlation was observed for timepoint A (Supplemental Table S2). For timepoint C, no analysis was performed as only *p*-value of ADIPOQ reached the cutoff of $p < 0.02$.

## DISCUSSION

In this study, we performed a comprehensive proteomic analysis of urine samples collected throughout the renal transplantation process, including the donor phase, organ procurement, preservation, and post-transplant surgery. Our findings underscore the potential relevance of donor-derived molecular markers for improving early stratification of transplant outcomes. Although outcomes after living donor kidney transplantation are generally favorable, considerable interindividual variability remains. The higher incidence of early graft dysfunction in deceased donor transplantation underscores the need for robust, noninvasive biomarkers for early risk stratification. Our study focuses on proteomic changes in full urine, providing novel insights into the molecular processes occurring in the early post-transplantation period and during long-term recovery. A major challenge of using proteomics in clinical samples is the wide dynamic range of protein abundance and the variability introduced by clinical and sample handling differences (13). Although the present study focuses on living donor kidney transplantation, the proteomic signatures and analytical approach described here may also be highly relevant for deceased donor kidney transplantation. In this context, urinary proteomics holds particular promise for predicting clinically important outcomes such as delayed graft function, acute rejection, and long-term graft function (eGFR), where early molecular markers are urgently needed to guide post-transplant management.

As described for SUV, sample clusters of donor and 4 weeks timepoint show the greatest difference. In the further course, 1-year samples appear to converge toward the baseline values (14). Notably, baseline characteristics such as sex, disease type, age and baseline GFR contributed much less—if at all—to differences in urinary proteome profiles than the timepoint in respect to the time of transplantation. Further investigation of individual protein abundances and overall processes identified by gene ontology terms provided a deeper understanding of the transplant response. Among the

top 20 proteins with the highest abundance, 55% were consistently detectable across all time points. Proteins, such as UMOD, ALB and AMBP, interpreted as a sign of coprecipitation of the classical free proteins in urine, were also present in previous proteomic analyses of urine or urinary extracellular vesicles (14–16). Sustained post-transplant increases in urinary RBP4, AZGP1, and LYVE1—validated in our validation cohort—likely reflect tubular injury and tissue remodeling[_bernard_et_al_1987] but were not associated with graft function, rejection, or other clinical outcomes in our analysis. Proteins uniquely detected 4 weeks after transplantation in the initial and validation cohorts, such as Serpina1, may indicate inflammation or immune activation (17, 18). Their presence might reflect early graft adaptation processes, though their clinical relevance remains to be clarified, as they were not associated with long-term function or rejection in this study.

Gene ontology (GO) term analysis revealed significant changes in ESCRT complexes at both post-transplantation time points (B, C) compared to the healthy donor state. ESCRT proteins, which are involved in various cellular processes such as endosomal trafficking and plasma membrane repair, seem to play a crucial role in transplant survival and rejection by mitigating necroptosis-induced damage (19, 20). However, upregulation of ESCRT complexes could not be confirmed in the validation cohort. Conversely, regulation of inflammatory and immune response could be confirmed as key mechanistic adaptations after transplantation (21).

One year after transplantation, we observed significant enrichment in pathways involving extracellular vesicles and lysosomes, which are associated with monocyte recruitment to damaged tissue and renal recovery after ischemia-reperfusion injury (22–24).

KEGG analysis revealed notable differences in the complement and coagulation cascades between time points, highlighting the initial immune response to ischemia-reperfusion and allograft exposure. This is in line with previous studies, suggesting that early blockade of the complement system may be beneficial (25–27). Changes in the renin–angiotensin–aldosterone system (RAAS) at both time points underscore its importance in blood pressure regulation and graft adaptation. This is consistent with previous studies linking it to clinical outcomes in transplantation (28–30). Lysosomes also showed significant changes. Since autophagy is triggered by starvation and inflammation - key stressors in transplantation - it likely plays a role in graft homeostasis (31). Glycan degradation, particularly of the endothelial glycocalyx during IRI, contributes to vascular permeability, leukocyte adhesion, and inflammation, thereby compromising graft function (32). Metabolic pathways, including glycolysis and gluconeogenesis, were also affected. Enhanced glycolysis protects against oxidative stress during IRI (33), while impaired gluconeogenesis, as seen in chronic kidney disease, may exacerbate metabolic disturbances and graft dysfunction (34). Finally, changes in cell adhesion molecules (CAMs) and ECM-receptor interactions reflect tissue repair and adaptation to the recipient environment. Dysregulation of these pathways may promote immune cell recruitment, fibrosis, and chronic allograft dysfunction (35, 36). Collectively, these pathways highlight interconnected metabolic, structural, and signaling processes involved in graft adaptation. These associations should be viewed as hypothesis-generating, as enrichment reflects relative protein abundance rather than direct pathway activation. For example, the increase in cell adhesion molecules and ECM-receptor interactions observed at 1 year may reflect ongoing tissue remodeling or chronic inflammation, potentially contributing to the development of fibrosis and chronic allograft dysfunction. Sustained pathway enrichment over time should therefore be interpreted cautiously, as it may indicate maladaptive processes rather than recovery.

Besides being a resource for the biological temporal changes after transplantation, we hypothesized that the full urine proteome may serve as a valuable resource for identifying biomarkers predictive of transplant outcomes. From a clinical perspective, such urinary proteomic biomarkers may serve as decision-support tools to guide the intensity of post-transplant monitoring and to inform the indication for diagnostic biopsy. In particular, they may help identify patients at increased risk for adverse outcomes before overt functional deterioration occurs, while potentially reducing unnecessary invasive procedures in low-risk individuals.

By using binary and linear regression analysis, we aimed to detect potential prognostic markers regarding infection, rejection or predictive outcome. Both the binary and the linear regression models were adjusted for confounders. Our study identified several donor-derived urinary proteins significantly associated with renal function 12 months post-transplant. These results highlight the potential of pre-transplant urinary proteomic profiling as a noninvasive tool for early risk stratification. The identified markers span diverse biological functions, including immune modulation, structural remodeling, metabolism, and stress response, all of which are highly relevant to graft resilience.

Inflammatory and immune-related proteins, such as interleukin-1 receptor accessory protein (IL1RAP) and orosomucoid 2 (ORM2), likely reflect pre-existing inflammatory activity in the donor organ (37). Of particular interest, ORM2 was consistently among the top 20 enriched proteins detected after KTx and conversely was diminished before transplantation in both the initial and validation cohorts. Notably, lower pre-KTx ORM2 levels were negatively associated with eGFR at 1 year, highlighting a potential link between baseline ORM2 expression and long-term graft function. These findings point toward a critical role for ORM2 in early immun-processes in the course of kidney transplantation. ORM2 is an acute-phase glycoprotein with broad immunomodulatory properties (38). It is typically upregulated during systemic

inflammation and has been shown to modulate neutrophil activity, limit excessive complement activation, and influence T-cell responses. In the transplant setting, altered ORM2 expression may therefore mirror the inflammatory status of the donor organ, which is increasingly recognized as a determinant of post-transplant immune activation and alloimmune risk.

In this context, ORM2 may serve not only as a biomarker of donor-derived immune activation, but also as a functional mediator influencing how the graft interfaces with the recipient's immune system during the early post-transplant period. Its dynamic regulation before and after KTx further suggests that ORM2 could integrate signals from donor inflammation, peri-transplant injury, and recipient immune responses. IL1RAP, a key component of proinflammatory IL-1 signaling, has been linked to congenital nephrotic syndrome, while inhibition of the IL-1 pathway shows renoprotective effects (37, 39, 40). Proteins related to extracellular matrix remodeling and structural integrity (DPT, COL18A1), lipid metabolism (NPC2), and cellular stress or injury (PRNP, APP) indicate early fibrotic, metabolic, and subclinical damage in donor kidneys (41–46). Their detection in pre-procurement urine highlights the potential of urinary proteomics for donor risk assessment and transplant outcome prediction. The fact that the most significant predictors of long-term transplant function originated from donor urine (timepoint A) highlights the possibility that certain grafts may already display molecular signs of vulnerability or senescence even before transplantation. This raises the hypothesis that donor-derived biomarkers may identify organs with lower intrinsic resilience, which could inform allocation decisions or perioperative management strategies. Understanding pre-transplant proteomic profiles may be essential in predicting how a graft will respond to ischemia-reperfusion injury and adapt to the host environment. Especially in the context of deceased donation and extended donor criteria, noninvasive biomarkers may be a future tool for evaluating donor kidneys.

Compared to proteins detected in donors, post-transplant protein profiles provide insights into dynamic biological processes such as immune adaptation and the graft's response to ischemia-reperfusion injury. Urinary proteins detected 4 weeks after kidney transplantation, including IGLV2-11, carbonic anhydrase 1 (CA1), and FABP4, were associated with graft function after 1 year. While IGLV2-11's function remains largely unknown, it may indicate immune activity or structural changes within the graft (https://www.ncbi.nlm.nih.gov/gene?Cmd=DetailsSearch&Term=28816). CA1 showed an independent correlation to eGFR after 1 year and regulates renal acid–base balance, ensuring normal urine acidification; experimental data suggest preserved or enhanced CA1 activity may improve kidney performance (47). ADIPOQ was associated with longer-term eGFR and reflects inflammatory and cardiometabolic pathways (48–50). FABP4, a key regulator of metabolic and inflammatory signaling, is strongly linked to kidney injury and influences transplant outcomes through effects on inflammation, NF-kappaB activation, and ER stress-induced apoptosis (51–58). Collectively, these proteins are promising prognostic biomarkers and potential therapeutic targets.

Urinary biomarkers have been increasingly studied for predicting kidney allograft outcomes, particularly early detection of rejection, tubular injury, and delayed graft function. Chemokines such as CXCL9 and CXCL10 complement conventional markers like serum creatinine or proteinuria (59–61). While KIM-1 and NGAL indicate tubular injury (62). mRNA, microRNA, and exosomal profiling provide additional insights into graft status.

Our findings contribute by providing a comprehensive full-urine proteomic profile across clinically relevant time points, highlighting RBP4, FABP4, ORM2, and CDH1 as potential prognostic markers. RBP4 reflects proximal tubular dysfunction alongside KIM-1 and NGAL. Using full urine samples without vesicle enrichment offers a simplified, scalable approach for biomarker detection.

PCK2 was identified as a predictor of long-term transplant function via targeted mass spectrometry in urinary extracellular vesicles (suEVs) (14). While our study focuses on full urine, these findings emphasize the complementary value of different urine proteomic approaches for predicting outcomes.

This study has several limitations. First, the sample size was limited and only a subset of patients provided samples at all timepoints, although subset PCA analyses showed clustering comparable to the full dataset and protein abundance, KEGG, and GO-term results were reproduced in an independent validation cohort. A major strength is the robust identification of proteins associated with future outcomes independently of classical clinical markers, analyzed separately for each time point. Very early post-transplant urine samples were not collected due to logistical constraints and potential contamination, which limits assessment of early graft immune responses. Proteomic findings were not correlated with histology, which remains a critical next step for functional validation.

Although our study focuses on living donor kidney transplantation, the identified approaches and biomarkers could also be valuable for deceased donor transplants. Only a single deceased donor was included, precluding meaningful clustering or stratification by donation type, so conclusions mainly apply to living donation. Given the greater variability and poorer outcomes in deceased donation, applying proteomic tools to predict delayed graft function, rejection, and long-term outcomes is a crucial next step. Taken together, our data highlight the value of temporal full-urine proteomic profiling for understanding graft adaptation and generating prognostic biomarker hypotheses, and future validation in larger, prospectively collected cohorts with targeted quantification will be essential for clinical translation.

## DATA AVAILABILITY

Raw MS data of the initial cohort were deposited on the PRIDE platform with accession number PXD057361. reviewer_pxd057361@ebi.ac.uk

The MS data of the validation cohort have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD071468

reviewer_pxd071468@ebi.ac.uk

This article contains supplemental data.

*Supplemental data*—This article contains supplemental data.

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*Conflict of Interest*—The authors declare that they do not have any conflicts of interest with the content of this article.

*Abbreviations*—The abbreviations used are: ALB, albumin; ABMR, antibody-mediated rejection; ADIPOQ, collagen domain containing adiponectin; AMBP, alpha-1-microglobulin/bikunin precursor; APP, Amyloid-beta precursor protein; AZGP1, Alpha-2-Glycoprotein 1, Zinc-Binding; BKVPyV, BK polyomavirus; CA1, carbonic anhydrase; CKD, Chronic kidney disease; CMV, Cytomegalovirus; COL18A1, Collagen Type XVIII Alpha 1 Chain; dnDSA, de novo DSA; DPT, Dermatopontin; EBV, Epstein-Barr virus; EGF, epidermal growth factor; eGFR, estimated glomerular filtration rate; ESCRT, endosomal sorting complexes required for transport; ESRD, end-stage renal disease; FABP-4, fatty acid binding protein 4; FoxO, Forkhead box O; GO, gene ontology; Hif-1, hypoxia-inducible factor-1; HP, Haptoglobin; IGKV3-20, Immunoglobulin Kappa Variable 3 to 20; IGLV2-11, immunoglobulin lambda variable 2 to 11; IL1RAP, Interleukin 1 Receptor Accessory Protein; KNG1, Kininogen-1; KRT1, keratin 1; KRT9, keratin 9; KTx, Kidney transplantation; LYVE1, lymphatic vessel endothelial receptor 1; NF-kappa B, nuclear factor kappa B; NPC2, Intracellular Cholesterol Transporter 2; ORM2, Orosomucoid 2; PCA, principal component analysis; PI3K-AKT, signaling phosphoinositide-3-kinase–protein kinase B/Akt; PRNP, human prion protein gene; RBP4, retinol binding protein 4; RNASe1, ribonuclease A family member 1; SERPING1, serpin family G member 1; TCMR, T-cell mediated rejection; UMOD, uromodulin.

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