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Urinary Post-Translationally Modified Fetuin-A Protein Is Associated with Increased Risk of Graft Failure in Kidney Transplant Recipients

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Keywords
Biomarkers · Fetuin-A · Graft failure · Kidney transplantation

Abstract

Introduction: Urinary fetuin-A has been identified as a biomarker for acute kidney injury and is proposed as a biomarker for early detection of kidney function decline. We investigated whether fetuin-A could serve as a marker of graft failure in kidney transplant recipients (KTRs). ***Methods:*** Data of KTR with a functioning graft ≥1 year that were enrolled in the TransplantLines Food and Nutrition Biobank and cohort study were used. Graft failure was defined as the need for re-transplantation or (re-)initiation of dialysis. Urinary fetuin-A was measured using an enzyme-linked immunosorbent assay kit that detected post-translationally modified fetuin-A in the urine (uPTM-FetA). In the main analyses, 24h uPTM-FetA excretion was used. In the sensitivity analyses, we excluded the outliers in 24h uPTM-FetA excretion, and we used uPTM-FetA concentration and uPTM-FetA concentration indexed for creatinine instead of 24h uPTM-FetA excretion. ***Results:*** A total of 627 KTRs (age 53 ± 13 years, 42% females) were included at 5.3 (1.9–12.2) years after transplantation. The estimated glomerular filtration rate (eGFR) was 52 ± 20 mL/min/1.73 m^2 and uPTM-FetA excretion was 34 (17–74) µg/24 h. During a median follow-up of 5.3 (4.5–6.0) years after baseline measurements, 73 (12%) KTRs developed graft failure. The association of 24h uPTM-FetA excretion with increased risk of graft failure was not constant over time, with increased risk only observed after 3 years from baseline measurements, independent of potential confounders including kidney function and 24 h urinary protein excretion (hazard ratio per doubling of 24h uPTM-FetA excretion = 1.31; 95% confidence interval = 1.06–1.61). This finding was robust in the sensitivity analyses. ***Conclusions:*** Our findings suggest that uPTM-FetA can be used as a marker for early detection of graft failure in KTR. Further studies are needed to confirm our findings.

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Introduction

End-stage kidney disease is a leading cause of morbidity and mortality and causes a major global health burden [1]. To date, kidney transplantation is a preferred treatment for end-stage kidney disease as it provides better quality of life and is most cost-effective compared to other kidney replacement therapy modalities [2, 3]. However, even after successful transplantation, kidney transplant recipients (KTRs) remain at risk of graft functional loss [4–6]. Looking at the patient's perspective, survival of the kidney graft is acknowledged to be more important than life itself, and the patients would rather die than return to dialysis [7, 8]. Thus, more efforts should be made to detect graft functional loss earlier so that appropriate treatment can be given and graft failure can be prevented.

In the current clinical setting, routine evaluation of graft function is based on serum creatinine and urinary protein excretion. In the cases with marked serum creatinine elevation or the presence of proteinuria, a kidney biopsy is considered. Unfortunately, these evaluations are imperfect, especially in regard to the graft prognosis prediction [9–11]. Therefore, there is a need for novel biomarkers that can be used to identify KTR at risk of graft function deterioration and subsequent graft failure.

Fetuin-A (also known as alpha-2-Heremans-Schmid glycoprotein) is a heterodimeric glycoprotein with 367 amino acid sequences containing 18 amino acids of a signal peptide, 282 amino acids of A-chain, 40 amino acid chains of connecting peptide, and 27 amino acids of B-chain that undergo various post-translational modifications before being secreted outside of the producing cells [12]. A previous study reported that urinary fetuin-A is elevated in patients with acute kidney injury (AKI), and recent evidence suggests that it is also linked to interstitial fibrosis/tubular atrophy [13, 14]. Both AKI and the presence of interstitial fibrosis/tubular atrophy after kidney transplantation are associated with unfavorable long-term graft outcomes [15, 16]. Urinary fetuin-A is also elevated in patients with chronic kidney diseases such as autosomal dominant polycystic kidney disease and focal segmental glomerular sclerosis [17, 18]. Next to that, urinary fetuin-A has previously been shown to be associated with kidney function decline in patients with type 2 diabetes and chronic kidney disease, and it can be used to predict the disease progression [19–22]. However, no study has been done to measure urinary fetuin-A in the KTR population and to investigate the plausible association with the graft outcome.

Because of the potential pathophysiological roles of fetuin-A in the occurrence of kidney function decline, we aimed to assess the association of urinary fetuin-A with clinical and biochemical parameters and to study its prospective association with graft failure among KTR. Additionally, we also aimed to investigate the association with graft function deterioration and all-cause mortality. To this end, we measured the fetuin-A using an enzyme-linked immunosorbent assay (ELISA) kit that detects post-translationally modified fetuin-A in the urine (uPTM-FetA).

Methods

This study was reported following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [23]. The completed checklist is provided in online supplementary Table 1 (for all online suppl. material, see https://doi.org/10.1159/000534829).

Design and Study Population

In this prospective cohort study, we used data from the TransplantLines Food and Nutrition Biobank and cohort study (NCT02811835). All adult KTR visiting the University Medical Center Groningen (UMCG) outpatient clinic between November 2008 and March 2011 with a functioning graft for at least 1 year after transplantation, without any drug or alcohol addiction or systemic illnesses, were invited to participate in this cohort [24]. During the recruitment period, 817 KTRs were invited, of whom 707 (87%) agreed to participate and gave written informed consent. For the current study, participants with missing 24h uPTM-FetA excretion measurements were excluded from the analyses. This study was conducted in accordance with the Declaration of Helsinki and Istanbul, and the study protocol was approved by the Institutional Review Board of UMCG (METc 2008/186).

The primary end-point of this study was graft failure, defined as the need for re-transplantation or (re-)initiation of dialysis. The secondary end-points were graft function deterioration (graft failure or doubling of serum creatinine) and all-cause mortality [25]. For graft failure and graft function deterioration end-points, KTRs who died with a functioning graft were censored at the time of death. End-points were recorded until September 2015. With the continuous surveillance system of the UMCG outpatient clinic, no KTRs were lost to follow-up.

Clinical Parameters

All baseline measurements were performed during a morning visit to the outpatient clinic. Blood pressure was measured thrice with 15 min intervals, and then the results were averaged. Measurements were performed using a semi-automatic device (Dinamap 1846, Critikon, Tampa, USA). Bodyweight and height were measured with the participants wearing indoor clothing without shoes. Body mass index was calculated as weight in kilograms divided by height in meters squared (kg/m^2), and body surface area was estimated in meters squared (m^2) using DuBois and DuBois formula [26]. Diabetes was defined according to the American Diabetes Association criteria [27]. The estimated glomerular filtration rate (eGFR) was calculated using the creatinine-based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [28]. Primary CMV infection was defined as CMV infection occurring in a previously seronegative KTR prior to transplantation with a kidney from a seropositive donor.

Secondary CMV infection was defined as CMV infection occurring in a KTR which was seropositive prior to transplantation. Relevant donor, recipient, and transplant information were extracted from the medical records, as described previously [24].

Laboratory Methods and uPTM-FetA Measurement

Blood samples were drawn at the outpatient clinic in the morning after an overnight fasting period (approximately 8–12 h), which included no medication intake. For the urine collection, all participants were instructed to collect their 24 h urine during the day before their visit.

uPTM-FetA was measured using a novel Human uPTM3-DKD ELISA kit (CE IVD Marked, Bio Preventive Medicine Corp., Hsinchu, Taiwan; Trade name: DNlite-IVD103) [29]. This assay detects post-translationally modified fetuin-A protein that contains the connecting peptide. Total urinary protein excretion was determined using the Biuret reaction (MEGA AU 150, Merck Diagnostica, Darmstadt, Germany). Other biochemical parameters, including serum creatinine and high-sensitivity C-reactive protein (hs-CRP), were measured using routine laboratory methods.

Statistical Analyses

All data were analyzed using SPSS version 28.0 (IBM Corp., Armonk, NY, USA) and R version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). For all analyses, p value <0.05 was considered statistically significant. The distribution of continuous variables was assessed by visually inspecting the histograms and quantile-quantile plots. Normally distributed variables were presented as mean ± standard deviation, skewed variables as median (interquartile range), and categorical variables as frequency (valid percentage). Univariable linear regression analyses were performed to assess the associations between 24h uPTM-FetA excretion and clinical and biochemical parameters. After univariable analyses, we adjusted 24h uPTM-FetA excretion for sex, serum creatinine, and 24 h urinary protein excretion ≥0.5 g/24 h. During the linear regression analyses, skewed variables were $\log_2$-transformed to fulfill the assumption for linear regression. If $\log_2$ transformation could not fulfill the assumption, the variables were re-categorized into categorical variables according to the median value or acceptable cut-off levels.

Kaplan-Meier curves were used to visualize the difference in graft and patient survival among subgroups of KTR according to the median level 24h uPTM-FetA excretion (<34 μg/24 h vs. ≥ 34 μg/24 h), and the significances of the differences between subgroups were calculated using the log-rank test. Cox proportional hazard regression analyses were used to assess the associations of 24h uPTM-FetA excretion with graft failure, graft function deterioration, and all-cause mortality, where the associations were adjusted for potential confounders. Variables that were associated with 24h uPTM-FetA excretion in the unadjusted linear regression analyses were considered as potential confounders. In model 1, 24h uPTM-FetA excretion was adjusted for age, sex, and time after transplantation at inclusion ($\log_2$). In model 2, we additionally adjusted for eGFR. In model 3, we additionally adjusted for 24 h urinary protein excretion ($\log_2$). In model 4, we additionally adjusted KTR clinical characteristics (systolic blood pressure, body surface area, smoking status, and CMV infection status) and the use of proliferation inhibitors. In model 5, we additionally adjusted for the donor characteristics (type of donor, donor age, and donor sex). In the full model (model 6), we additionally adjusted for hs-CRP ($\log_2$).

Schoenfeld residuals on functions of time were performed to evaluate the proportional hazards assumption in the final Cox regression model of each end-point. Graft failure and graft function deterioration violated the proportional hazards assumption ($p = 0.004$ and $p = 0.005$, respectively), whereas all-cause mortality was not ($p = 0.073$). Therefore, Cox models with time-dependent covariates were used to calculate hazard ratios (HRs) over time for graft failure and graft function deterioration [30, 31]. HR was presented as per doubling of 24h uPTM-FetA excretion with 95% confidence intervals (95% CIs).

For the primary end-point, we assessed the potential interactions of age, sex, eGFR, and 24 h urinary protein excretion with 24h uPTM-FetA excretion by adding interaction terms to the full model. For the sensitivity analyses, we re-evaluated the association of 24h uPTM-FetA excretion with graft failure after excluding the outliers. Outliers in this study were defined as values deviating more than two standard deviations from the mean of the $\log_2$ 24h uPTM-FetA excretion [32]. Next to that, we used uPTM-FetA concentration and uPTM-FetA concentration indexed for creatinine (uPTM-FetA/creatinine ratio) instead of 24h uPTM-FetA excretion.

For all cross-sectional analyses, the original non-imputed dataset was used, and variables with >20 missing values (3.16%) were reported in the table footnotes. For all prospective analyses, multiple imputations using fully conditional specification were performed using the R package "mice" (number of multiple imputations = 10) to account for missing data in variables other than 24h uPTM-FetA excretion.

Results

Baseline Characteristics

In total, 627 KTRs were included in this study. The flowchart of the study population selection is presented in the online supplementary Figure 1. The mean age was 53 ± 13 years, 42% were female, the median time after transplantation was 5.3 (1.9–12.2) years, and the mean eGFR was 52 ± 20 mL/min/1.73 m^2. Median 24h uPTM-FetA excretion was 34 (17–74) μg/24 h. Median 24 h urinary protein excretion was 0.21 (0.01–0.34) g/24 h, of which 504 (81%) had a urinary protein excretion <0.5 g/24 h and only 8 (1%) had a urinary protein excretion >3 g/24 h. More detailed baseline characteristics of the study population are presented in Table 1. 24h uPTM-FetA excretion was negatively correlated with eGFR and positively correlated with plasma creatinine concentration, urinary albumin, and 24 h urinary protein excretion (Fig. 1)

Cross-Sectional Associations of 24h uPTM-FetA Excretion with Clinical and Biochemical Parameters

The strongest association of 24h uPTM-FetA excretion in the univariable linear regression analyses was with 24h urinary protein excretion ≥0.5 g/24 h (standardized β coefficient [Std. β] 0.94, $p < 0.001$). Additionally, 24h uPTM-FetA excretion was also significantly associated

Table 1. Baseline characteristics and linear regression analysis for 24h uPTM-FetA excretion

Baseline variables	Total subjects (N = 627)	Linear regression with 24h uPTM-FetA excretion as dependent variable		Adjusted for sex, serum creatinine, and urinary protein excretion ≥0.5 g/24 h	
		std. β (95% CI)	p value	std. β (95% CI)	p value
24h uPTM-FetA excretion (μg/24 h)[#]	34 (17–74)	N.A.	N.A.	N.A.	N.A.
Clinical characteristics					
Female sex	266 (42)	−0.22 (−0.38 to −0.06)	**0.006**	–	–
Age, years	53±13	−0.07 (−0.15 to 0.01)	0.068	−0.03 (−0.10 to 0.04)	0.4
Primary renal disease					
Unknown	100 (16)	*Ref.*		*Ref.*	
Glomerulonephritis	165 (26)	−0.07 (−0.32 to 0.18)	0.6	−0.18 (−0.41 to 0.04)	0.1
Interstitial nephritis	76 (12)	−0.02 (−0.32 to 0.28)	0.9	−0.11 (−0.38 to 0.16)	0.4
Cystic kidney disease	128 (20)	−0.13 (−0.39 to 0.13)	0.3	−0.20 (−0.44 to 0.04)	0.097
Other congenital/hereditary disease	32 (5)	−0.22 (−0.62 to 0.18)	0.3	−0.39 (−0.75 to −0.03)	**0.035**
Renal vascular disease	35 (6)	−0.12 (−0.51 to 0.27)	0.6	−0.30 (−0.65 to 0.05)	0.091
Diabetic nephropathy	28 (5)	0.17 (−0.25 to 0.59)	0.4	0.00 (−0.38 to 0.38)	1.0
Other multisystem diseases	46 (7)	−0.07 (−0.42 to 0.28)	0.7	−0.14 (−0.45 to 0.18)	0.4
Other	17 (3)	−0.09 (−0.60 to 0.43)	0.7	−0.23 (−0.69 to 0.24)	0.3
Body surface area, m^2	1.95±0.22	0.10 (0.03–0.18)	**0.009**	0.04 (−0.04 to 0.12)	0.4
BMI,[#] kg/m^2	26 (23–29)	0.07 (−0.01 to 0.15)	0.093	0.05 (−0.02 to 0.12)	0.2
Systolic blood pressure, mm Hg	136±17	0.09 (0.02–0.17)	**0.019**	0.01 (−0.06 to 0.08)	0.8
Hypertension	261 (42)	0.11 (−0.05 to 0.27)	0.2	−0.04 (−0.18 to 0.11)	0.6
History of cardiovascular disease	153 (24)	0.02 (−0.16 to 0.21)	0.8	0.01 (−0.16 to 0.17)	0.9
Diabetes	144 (23)	0.03 (−0.16 to 0.22)	0.8	0.03 (−0.14 to 0.19)	0.8
Smoking status					
Never	248 (42)	*Ref.*		*Ref.*	
History of smoking	267 (45)	0.10 (−0.07 to 0.28)	0.2	0.10 (−0.05 to 0.26)	0.2
Current smoking	73 (12)	0.28 (0.02–0.54)	**0.033**	0.21 (−0.03 to 0.45)	0.079
Alcohol consumption status					
0 g/day	54 (10)	*Ref.*		*Ref.*	
0–10 g/day	354 (63)	0.02 (−0.13 to 0.14)	0.9	0.10 (−0.16 to 0.36)	0.4
>10 g/day	153 (27)	0.13 (−0.08 to 0.20)	0.4	0.20 (−0.09 to 0.49)	0.2
CMV infection status					
Negative	420 (73)	*Ref.*		*Ref.*	
Primary infection	67 (12)	−0.01 (−0.26 to 0.24)	0.9	−0.12 (−0.35 to 0.11)	0.3
Secondary infection	88 (15)	−0.20 (−0.43 to 0.02)	0.080	−0.20 (−0.41 to 0.00)	0.053
Transplant-related characteristics					
First kidney transplantation	564 (90)	0.14 (−0.12 to 0.40)	0.3	0.18 (−0.06 to 0.42)	0.1
Pre-emptive transplantation	93 (15)	−0.03 (−0.25 to 0.19)	0.8	0.03 (−0.17 to 0.23)	0.8
Time after transplantation, years*	5.3 (1.9–12.2)	N.A.	N.A.	N.A.	N.A.
Time after transplantation >5 years	338 (54)	−0.23 (−0.39 to −0.08)	**0.004**	−0.27 (−0.41 to −0.13)	<**0.001**
Living donor	222 (35)	0.19 (0.03–0.35)	**0.025**	0.25 (0.10–0.39)	**0.001**
Donor age, years	43±15	0.13 (0.05–0.21)	**0.001**	0.11 (0.04–0.19)	**0.003**
Donor female sex	295 (48)	0.21 (0.05–0.37)	**0.009**	0.12 (−0.02 to 0.27)	0.1
Presence of HLA antibodies	161 (26)	−0.01 (−0.19 to 0.17)	0.9	−0.09 (−0.26 to 0.07)	0.3
HLA class I antibodies positive	99 (16)	−0.02 (−0.23 to 0.20)	0.9	−0.06 (−0.26 to 0.14)	0.6
HLA class II antibodies positive	110 (17.5)	0.01 (−0.19 to 0.22)	0.9	−0.10 (−0.29 to 0.09)	0.3
History of delayed graft function	44 (7)	0.12 (−0.19 to 0.43)	0.4	0.00 (−0.27 to 0.28)	1.0
History of rejection	171 (27)	0.09 (−0.09 to 0.27)	0.3	−0.04 (−0.20 to 0.13)	0.7
Immunosuppressive medication use					
Prednisolone	620 (99)	0.00 (−0.75 to 0.74)	1.0	−0.09 (−0.76 to 0.58)	0.8
Calcineurin inhibitor	367 (59)	0.06 (−0.10 to 0.22)	0.5	−0.03 (−0.18 to 0.12)	0.7
Proliferation inhibitor	517 (83)	0.18 (−0.03 to 0.38)	0.096	0.23 (0.04–0.41)	**0.016**
mTOR inhibitor	22 (3.5)	0.12 (−0.30 to 0.55)	0.6	0.04 (−0.35 to 0.42)	0.8

Table 1 (continued)

Baseline variables	Total subjects (N = 627)	Linear regression with 24h uPTM-FetA excretion as dependent variable		Adjusted for sex, serum creatinine, and urinary protein excretion ≥0.5 g/24 h	
		std. β (95% CI)	p value	std. β (95% CI)	p value
Laboratory measurements					
Hemoglobin, mmol/L	8.2±1.1	−0.16 (−0.24 to −0.09)	**<0.001**	−0.06 (−0.14 to 0.03)	0.2
Hemoglobin A1C,* %	5.8 (5.5–6.2)	N.A.	N.A.	N.A.	N.A.
Hemoglobin A1C >6.5%, n (%)	82 (14)	0.01 (−0.22 to 0.25)	0.9	0.07 (−0.15 to 0.28)	0.5
Leukocyte, 10^9/L	8.2±2.6	0.00 (−0.08 to 0.08)	1.0	0.0 (−0.07 to 0.07)	1.0
hs-CRP,# mg/L	1.6 (0.7–4.5)	0.09 (0.01–0.17)	**0.033**	0.05 (−0.02 to 0.12)	0.2
Serum creatinine,# µmol/L	124 (99–160)	0.34 (0.26–0.41)	**<0.001**	–	–
eGFR, mL/min/1.73 m^2	52±20	−0.27 (−0.34 to −0.19)	**<0.001**	–	–
Urinary albumin excretion,# mg/24 h	38 (11–171)	0.45 (0.38–0.52)	**<0.001**	0.29 (0.18–0.39)	**<0.001**
Urinary protein excretion,* g/24 h	0.21 (0.01–0.34)	N.A.	N.A.	N.A.	N.A.
Urinary protein excretion ≥0.5 g/24 h	120 (19)	0.94 (0.76–1.13)	**<0.001**	–	–

Normally distributed variables were presented as mean ± standard deviation, skewed variables as median (interquartile range), and categorical variables as number (valid percentage). Smoking status was missing in 39 patients, alcohol consumption status was missing in 66 patients, CMV infection status was missing in 52 patients, Hemoglobin A1C was missing in 21 patients, and hs-CRP level was missing in 32 patients. All other variables had missing value in <20 patients. eGFR, estimated glomerular filtration rate as calculated using the creatinine-based CKD-EPI formula; HLA, human leukocyte antigen; hs-CRP, high-sensitivity C-reactive protein; mTOR, mammalian target of rapamycin; Std. β, standardized β coefficient; uPTM-FetA, urinary post-translationally modified fetuin-A; BMI, body mass index. #Variables were $\log_2$ transformed to fulfill the assumptions in linear regression analyses. *Linear regression analysis could not be performed.

with 24h urinary albumin excretion (Std. β 0.45, $p < 0.001$) serum creatinine (Std. β 0.34, $p < 0.001$), and eGFR (Std β −0.27, $p < 0.001$). Furthermore, female sex was also significantly associated with higher 24h uPTM-FetA excretion (Std. β −0.22, $p = 0.006$). Older donors, living donors, the use of proliferation inhibitors, and 24 h urinary albumin excretion were significantly associated with higher 24h uPTM-FetA excretion independent of sex, serum creatinine, and 24 h urinary protein excretion ≥0.5 g/24 h. In contrast, longer duration after transplantation and congenital disease as the primary kidney disease were negatively associated with 24h uPTM-FetA excretion (Table 1).

Prospective Analyses of the Association between 24h uPTM-FetA Excretion and Graft Failure

During a median follow-up of 5.3 (4.5–6.0) years after baseline measurements, 73 (12%) KTRs developed graft failure. The most frequent cause of graft failure was chronic graft dysfunction (77%) and recurrence of primary kidney disease (10%). Other causes of graft failure include vascular problems and infection. Rates of graft failure were 4% and 19% in KTRs with baseline 24h uPTM-FetA excretion below and above the median, respectively ($p_{\log\ \text{rank}} < 0.001$) (Fig. 2). The association of 24h uPTM-FetA excretion with the risk for graft failure was not constant over time ($p_{\text{schoenfeld residue}} = 0.004$). 24h uPTM-FetA excretion was associated with graft failure within 1 year or between 1 and 3 years after baseline measurements in the unadjusted model; however, the association was lost after adjustment for confounders (HR 0.99, 95% CI: 0.77–1.27 and 0.96, 95% CI: 0.78–1.19, respectively). In contrast, 24h uPTM-FetA excretion was associated with an increased risk of graft failure after 3 years since baseline measurements (HR 1.85, 95% CI: 1.55–2.22), and the association remained significant even after adjustment for potential confounders (HR 1.31, 95% CI: 1.06–1.61) (Table 2). There was no interaction between 24h uPTM-FetA excretion and sex, age, eGFR, or 24 h urinary protein excretion for the associations of 24h uPTM-FetA excretion with graft failure (all $p_{\text{interaction}} > 0.05$).

Sensitivity Analyses

We identified 34 (5.4%) KTRs with outliers (11 KTRs with 24h uPTM-FetA excretion <3.33 µg/24 h and 23 KTRs with uPTM-FetA excretion >408 µg/24 h). After the exclusion of outliers, 24h uPTM-FetA excretion remained independently associated with the risk of graft failure only after 3 years since baseline measurements (HR 1.77, 95% CI: 1.25–2.49) (online suppl. Table 2). In

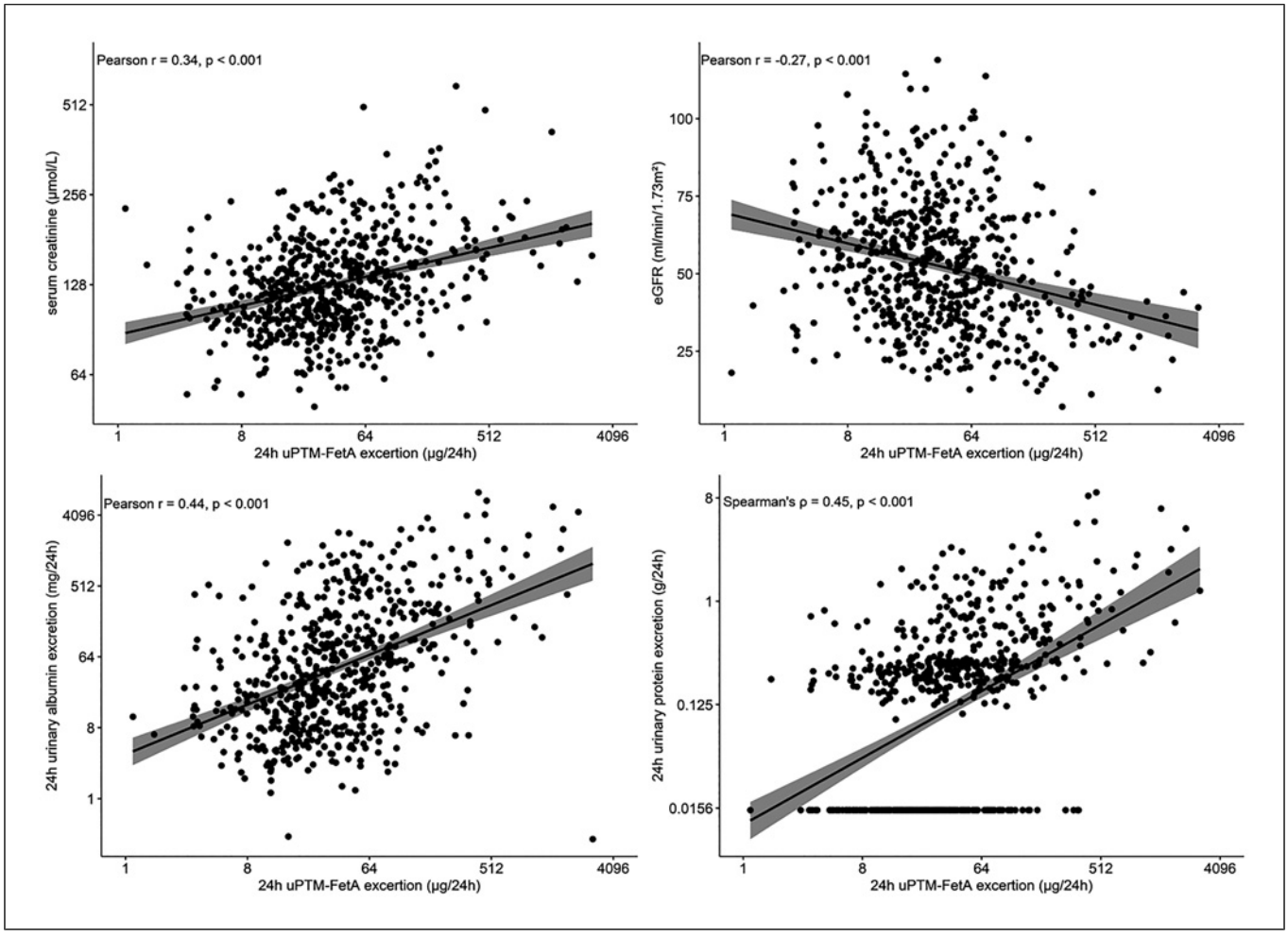

Fig. 1. Scatter plots and visual presentation of the correlation of 24h uPTM-FetA with the kidney function parameters, 24h urinary albumin excretion, and 24h urinary protein excretion. eGFR, estimated glomerular filtration rate; uPTM-FetA, urinary post-translationally modified fetuin-A.

the other sensitivity analyses, we used uPTM-FetA concentration and uPTM-FetA/creatinine ratio instead of 24h uPTM-FetA excretion. Similarly, uPTM-FetA concentration and uPTM-FetA/creatinine ratio were also significantly associated with graft failure only after 3 years since baseline measurements, even after adjustments for potential confounders (HR 1.35, 95% CI: 1.10–1.65 and HR 1.36, 95% CI: 1.10–1.67, respectively) (online suppl. Tables 3, 4).

Secondary Analyses of the Association between 24h uPTM-FetA and Graft Function Deterioration and All-Cause Mortality

During a median follow-up of 5.3 (4.0–6.0) years after baseline measurements, 121 (19%) KTRs developed graft function deterioration. Rates of graft function deterioration were 9% and 30% in KTRs with baseline 24h uPTM-FetA excretion below and above the median, respectively ($p_{\log\ rank} < 0.001$) (online suppl. Fig. 2). The association of 24h uPTM-FetA excretion with the risk for graft failure was not constant over time ($p_{schoenfeld\ residue} = 0.005$). 24h uPTM-FetA excretion was significantly associated with an increased risk of graft function deterioration only after 3 years since baseline measurements (HR 1.64, 95% CI: 1.41–1.92), and the association remained significant even after the adjustment for potential confounders (HR 1.39, 95% CI: 1.17–1.65) (online suppl. Table 5).

During a median follow-up of 5.4 (4.9–6.1) years after baseline measurements, 132 (21%) KTRs died. There were no significant differences in the mortality rates between KTRs with 24h uPTM-FetA excretion below

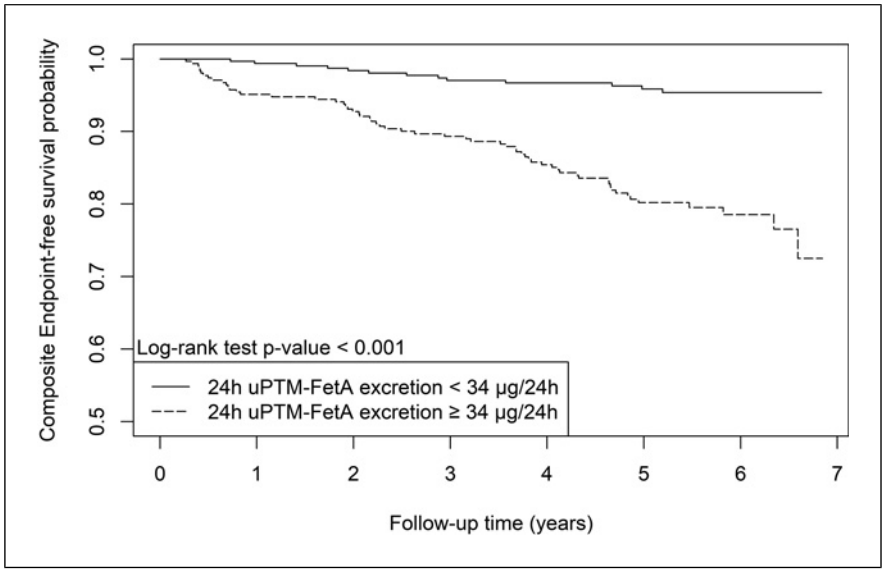

Fig. 2. Kaplan-Meier curve for death-censored graft failure below and above the median of 24 h urinary post-translationally modified fetuin-A excretion.

Table 2. Prospective analysis of the association of 24h uPTM-FetA excretion with death-censored graft failure in 627 KTRs

$n_{events}/n_{at\ risk}$	Continuous analyses of 24h uPTM-FetA excretion (per doubling)					
	<1 year		1–3 year		>3 year	
	17/627		24/600		32/534	
model	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Crude	1.46 (1.17–1.84)	**0.001**	1.36 (1.11–1.66)	**0.003**	1.85 (1.55–2.22)	<**0.001**
Model 1	1.46 (1.16–1.82)	<**0.001**	1.36 (1.12–1.65)	**0.002**	1.86 (1.56–2.22)	<**0.001**
Model 2	1.19 (0.94–1.50)	0.1	1.13 (0.93–1.39)	0.2	1.51 (1.26–1.82)	<**0.001**
Model 3	0.96 (0.76–1.23)	0.8	0.93 (0.76–1.15)	0.5	1.24 (1.02–1.50)	**0.028**
Model 4	1.00 (0.78–1.28)	1.0	0.96 (0.78–1.20)	0.7	1.31 (1.07–1.61)	**0.008**
Model 5	1.00 (0.78–1.28)	1.0	0.97 (0.78–1.20)	0.8	1.32 (1.07–1.62)	**0.008**
Model 6	0.99 (0.77–1.27)	1.0	0.96 (0.78–1.19)	0.7	1.31 (1.06–1.61)	**0.011**

Cox proportional-hazard regression analyses were performed to assess the association of 24 h uPTM-FetA excretion with the risk of death-censored graft failure (i.e., the need for re-transplantation or (re-)initiation of dialysis). Model 1 was adjusted for age, sex, and time after transplantation at inclusion (log_2). Model 2 was further adjusted for eGFR based on creatinine-based CKD-EPI formula. Model 3 was further adjusted for 24 h urinary protein excretion (log_2). Model 4 was further adjusted for KTR clinical characteristics (systolic blood pressure, body surface area, smoking status, and CMV infection status) and the use of proliferation inhibitors. Model 5 was further adjusted for donor characteristics (type of donor, donor age, and donor sex). Model 6 was further adjusted for serum high-sensitivity C-reactive protein (log_2). 95% CI, 95% confidence interval; HR, hazard ratio; uPTM-FetA, urinary post-translationally modified fetuin-A.

(19%) and above (23%) the median level ($p_{log\ rank} = 0.2$) (online suppl. Fig. 3). Unadjusted and adjusted Cox regression models showed no prospective association between 24h uPTM-FetA excretion and all-cause mortality (online suppl. Table 6).

Discussion

In this cohort of 627 KTRs, the strongest association of 24h uPTM-FetA excretion was with 24 h urinary protein excretion ≥0.5 g/24 h. In addition to that, shorter time

after transplantation, living donor, older donor age, congenital disease as the primary kidney disease, the use of proliferation inhibitors, and 24 h urinary albumin excretion were independently associated with higher 24h uPTM-FetA excretion. In the prospective analyses, 24h uPTM-FetA excretion was independently associated with an increased risk of graft failure. However, the risk was not constant over time, with increased risk only observed beyond 3 years after baseline measurements. The association was robust in several sensitivity analyses. Similarly, 24h uPTM-FetA excretion was associated with graft function deterioration only after 3 years since baseline measurements. In contrast, 24h uPTM-FetA excretion was not associated with an increased risk of mortality.

Human fetuin-A in its single-chain precursor form comprises an A-chain, a connecting peptide, and a B-chain, in addition to a signaling peptide [12]. Subsequently, this precursor protein undergoes post-translational modifications by means of glycosylation and proteolysis [33, 34]. During proteolytic processing, the connecting peptide region is removed from the precursor protein, and the single-chain form is converted into a mature two-chain form linked by a disulfide bond [34]. Under certain conditions in which proteolysis does not occur, the single-chain fetuin-A undergoes phosphorylation instead, primarily at the serine residue in the connecting peptide region [35, 36]. The ELISA kit used in this study exclusively detects the latter post-translationally modified form of fetuin-A.

In healthy adults, the kidney does not express fetuin-A [37]. However, proximal tubule epithelial cells (PTECs) develop the ability to express fetuin-A upon injury and release it to the luminal side of the tubule. Zhou et al. [13] have shown that in cisplatin-induced and ischemia/reperfusion-induced AKI rat models, fetuin-A was predominantly present in the urinary exosome fraction instead of the non-exosomal fraction, indicating that fetuin-A is produced by the PTEC. Recently, Rudloff et al. [38] also showed that PTEC can locally produce fetuin-A under hypoxic conditions after stimulation by hypoxia-inducible transcription factors. It has been postulated that the presence of fetuin-A in the proximal tubules aids in protecting the kidney from hypoxia-induced kidney inflammation by preventing the shift of macrophages to pro-inflammatory macrophages M1, and from hypoxia-induced fibrosis by antagonizing transforming growth factor-β signaling [38].

Both acute and chronic kidney injury are tightly associated with the occurrence of hypoxia [39]. In the setting of kidney transplantation, the kidney is exposed to various conditions that will enhance hypoxia and hypoxic injury, such as ischemia and reperfusion during organ donation and post-operative vascular complications [40]. Among all structures within the kidney, PTEC are the most sensitive and vulnerable as these cells are highly active and have a high oxygen demand [41]. Because hypoxia plays a major role in the progression of kidney disease and may present in the relatively early stages of kidney injury even before the structural injury occurs [42, 43], early identification of this condition may be beneficial to prevent further deterioration. In both cisplatin-induced and ischemia/reperfusion-induced AKI, urinary fetuin-A excretion increased before the surge of serum creatinine and also before the injury was present morphologically [13]. In the current study, we found that uPTM-FetA was significantly associated with increased risk of graft failure and graft function deterioration after 3 years since baseline measurements. As one of the most important requirements for a biomarker is to reflect an underlying pathophysiology of the disease [44], measurement of uPTM-FetA in the urine may offer additional benefit as a biomarker for earlier detection of graft injury in KTR over currently used parameters such as serum creatinine or proteinuria [45, 46].

This study has three important limitations that need to be mentioned. First, this study was performed in a single center in the Netherlands, with an over-representation of the Caucasian population. Second, as this was an observational study, residual confounding may still exist despite the number of potentially confounding factors that we have adjusted for, and the nature of this study does not allow for hard conclusions on causality. Third, we did not adjust the *p* values for multiple testing because this was an exploratory study [47]. Nevertheless, this was the first study to evaluate the prospective association of urinary fetuin-A with long-term outcomes in kidney transplantation setting.

In conclusion, 24h uPTM-FetA excretion is significantly associated with lower kidney function and higher 24-h urinary albumin and protein excretion. Prospectively, 24h uPTM-FetA excretion is independently associated with an increased risk of graft failure and graft function deterioration beyond 3 years after measurements. Our findings suggest that uPTM-FetA can be used as a clinical marker to allow earlier detection of graft failure in KTR. Further studies with larger and more heterogenous KTR populations are needed to confirm our study findings.

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Statement of Ethics

This study protocol was reviewed and approved by the University Medical Center Groningen (UMCG) Review Board, approval number (METc 2008/186). Written informed consent was obtained from participants to participate in the study.

Conflict of Interest Statement

DK, L-MC, and SJLB received travel reimbursement from Bio Preventive Medicine Corp. for attending the World Congress of Nephrology 2023. T-LT is a full-time employee and holds stocks at Bio Preventive Medicine Corp, the company that developed the assay to measure uPTM-FetA in the current study. All other authors have no conflict of interest to declare.

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Author Contributions

J.v.d.B., S.P.B., T.-L.T., and S.J.L.B. designed the work. F.F.A., D.K., and S.J.L.B. retrieved and analyzed the data. C.A.t.V.-K., G.D.L., and L.-M.C. interpreted the data for the work. F.F.A. drafted the work, and all other authors revised it critically for important intellectual content. All authors approved the final version of the manuscript.

Data Availability Statement

Public sharing of individual participant data was not included in the informed consent of the TransplantLines Biobank and cohort study, but data can be made available to interested researchers upon reasonable request by sending an e-mail to the data manager of the TransplantLines Biobank and cohort study (datarequest.transplantlines@umcg.nl).

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