Urinary copper excretion is associated with long-term graft failure in kidney transplant recipients

Yepes-Calderon, M; Kremer, D; Post, A; Sotomayor, CG; Seidel, U; Huebbe, P; Knobbe, TJ; Lüersen, K; Eisenga, MF; Corpeleijn, E

Published in:
American Journal of Nephrology

DOI:
10.1159/000531147

AJN
American Journal
of Nephrology

Transplantation: Research Article

Am J Nephrol
DOI: 10.1159/000531147

Received: March 18, 2023
Accepted: April 27, 2023
Published online: May 19, 2023

Urinary Copper Excretion Is Associated with Long-Term Graft Failure in Kidney Transplant Recipients

Manuela Yepes-Calderón[a] Daan Kremer[a] Adrian Post[a]
Camilo G. Sotomayor[a, b] Ulrike Seidel[c] Patricia Huebbe[c] Tim J. Knobbe[a]
Kai Lüersen[c] Michele F. Eisenga[a] Eva Corpeleijn[d] Martin H. de Borst[a]
Gerjan J. Navis[a] Gerald Rimbach[c] Stephan J.L. Bakker[a]

[a]Division of Nephrology, Department of Internal Medicine, University Medical Center Groningen, Groningen, The Netherlands; [b]Clinical Hospital University of Chile, Independencia, Chile; [c]Institute of Human Nutrition and Food Science, University of Kiel, Kiel, Germany; [d]Department of Epidemiology, University Medical Center Groningen, Groningen, The Netherlands

Keywords
Copper · Oxidative stress · Kidney transplant · Graft failure

Abstract

Introduction: In chronic kidney disease, proteinuria increases urinary copper excretion, inducing oxidative tubular damage and worsening kidney function. We investigated whether this phenomenon occurred in kidney transplant recipients (KTRs). In addition, we studied the associations of urinary copper excretion with the biomarker of oxidative tubular damage urinary liver-type fatty-acid binding protein (u-LFABP) and death-censored graft failure. ***Methods:*** This prospective cohort study was performed in the Netherlands between 2008 and 2017, including outpatient KTR with a functioning graft for longer than 1 year, who were extensively phenotyped at baseline. Twenty-four-hour urinary copper excretion was measured by inductively coupled plasma mass spectrometry. Multivariable linear and Cox regression analyses were performed. ***Results:*** In 693 KTR (57% men, 53 ± 13 years, estimated glomerular filtration rate [eGFR] 52 ± 20 mL/min/1.73 m^2), baseline median urinary copper excretion was 23.6 (interquartile range 11.3–15.9) µg/24 h. Urinary protein excretion was positively associated with urinary copper excretion (standardized $\beta = 0.39$, $p < 0.001$), and urinary copper excretion was positively associated with u-LFABP (standardized $\beta = 0.29$, $p < 0.001$). During a median follow-up of 8 years, 109 (16%) KTR developed graft failure. KTR with relatively high copper excretion were at higher risk of long-term graft failure (hazard ratio [HR]: 1.57, 95% confidence interval [CI]: 1.32–1.86 per $\log_2$, $p < 0.001$), independent of multiple potential confounders like eGFR, urinary protein excretion, and time after transplantation. A dose-response relationship was observed over increasing tertiles of copper excretion (HR: 5.03, 95% CI: 2.75–9.19, tertile 3 vs. 1, $p < 0.001$). u-LFABP was a significant mediator of this association (74% of indirect effect, $p < 0.001$). ***Conclusion:*** In KTR, urinary protein excretion is positively correlated with urinary copper excretion. In turn, higher urinary copper excretion is associated with an independent increased risk of kidney graft failure, with a substantial mediating effect through oxidative tubular

karger@karger.com
www.karger.com/ajn

Correspondence to:
Manuela Yepes-Calderón, m.yepes.calderon@umcg.nl

damage. Further studies are warranted to investigate whether copper excretion-targeted interventions could improve kidney graft survival.

Introduction

Advances in the care of kidney transplant recipients (KTR) have led to short-term posttransplantation graft survival rates higher than 90% [1]. Similar success in long-term graft survival has not been achieved, and a vigorous search for potentially modifiable risk factors is ongoing to improve this outcome. Urinary excretion of copper has gained attention as one of these factors because it is increased with enhanced proteinuria, decreased estimated glomerular filtration rate (eGFR), and in patients with acute kidney injury [2–5]. Furthermore, free copper has the capacity to induce oxidative kidney tissue damage, potentially further compromising kidney function [6].

Human copper exposure occurs mainly through diet and environmental sources [7]. Copper homeostasis is tightly regulated and hardly affected by mild-to-moderate increases in copper intake [8]. About 30–50% of ingested copper is absorbed and transferred to the liver. There, the majority of copper is excreted through bile. A small amount of copper can be stored in the liver; or bound to carrier proteins, namely ceruloplasmin or albumin, and later secreted into plasma [8]. Under normal conditions, urinary copper excretion is 10–30 μg/24 h, lower than 3% of daily copper intake [3, 9]. However, an impairment of renal function is associated with increased urinary copper excretion and kidney copper load, potentially secondary to increased single nephron filtration and decreased reabsorption of its carrier proteins [2, 4].

Copper overload has been proposed to contribute further to tubular damage and the progression of nephropathy [10–12]. Copper possesses unpaired electrons that promote and propagate-free radical reactions. It is also present in important redox enzymes such as cytochrome oxidase and copper-zinc super oxidase dismutase [13, 14]. This redox activity and the propensity of free copper ions to participate in the formation of reactive oxygen species are linked to cellular and tissue injury [6]. Furthermore, copper-related tissue damage has shown to be to some extent reversible in laboratory fibrosis models, which described that copper chelation therapy improved glomerular hypertrophy and cortex fibrosis [11, 15]. This makes copper an interesting unexplored opportunity for improving long-term kidney graft survival.

To date, no study has investigated a plausible association between posttransplant urinary copper excretion and the risk of graft failure. In the current study, we evaluated whether in KTR, increased urinary protein excretion or decreased eGFR were associated with higher urinary copper excretion. Next, we hypothesized that increased copper excretion was associated with kidney oxidative tubular damage, as measured by the highly specific biomarker urinary liver-type fatty-acid binding protein (u-LFABP) excretion [16]. Finally, we tested whether urinary copper excretion was associated with the risk of long-term graft failure in KTR.

Materials and Methods

Study Design and Population

In the TransplantLines Food and Nutrition Biobank and Cohort Study, all adult KTR with a functioning graft for ≥1 year, without a history of addiction or malignancy, who visited the University Medical Center of Groningen (The Netherlands) outpatient clinic between November 2008 and May 2011, were invited to participate. A total of 707 eligible KTR were enrolled after providing written informed consent. KTR missing baseline 24 h urinary copper measurements (n = 14) were excluded, resulting in 693 KTR, whose information is presented here. There were no significant differences in baseline characteristics between the included KTR and those missing urinary copper excretion measurements (online suppl. Table 1; for all online suppl. material, see https://doi.org/10.1159/000531147). The current study was approved by the Institutional Review Board (METc 2008/186) and adhered to the Declarations of Helsinki and Istanbul.

Data Collection and Calculations

Baseline clinical data were collected during a visit to the outpatient clinic. The detailed study protocol has been previously described [17]. Relevant information regarding medication, donor, and transplantation were extracted from the electronic patient records and the Groningen Renal Transplant Database [18].

According to a strict protocol, all KTR were asked to collect a 24 h urine sample the day before they visited the outpatient clinic. From these samples, urinary copper excretion was measured by inductively coupled plasma mass spectrometry (ICAP Q instrument, Thermo Fisher Scientific; Waltham, USA) at Synalb (Jena, Germany). Measurements were conducted following DIN EN ISO 17294-2: 2017-01 [19]. The test has a detection limit of 0.1 μg/L, an intra-assay variability of 0.3%, and inter-assay variability of 1%. u-LFABP was measured with an enzyme-linked immunosorbent assay (human u-LFABP assay kit 96 test; CMIC Holdings Co., Japan) [16].

Serum creatinine was determined by using the Jaffe reaction (MEGA AU510; Merck Diagnostica, Germany), and the eGFR was calculated by the serum creatinine-based chronic kidney disease epidemiology collaboration equation. Cumulative prednisolone

dose was calculated as the sum of the maintenance dose of prednisolone from transplantation to baseline.

Clinical End Points

The end point of this study was graft failure, defined as a restart of dialysis or the need for re-transplantation. Patients who died with a functioning graft were censored at the time of death. The continuous surveillance system of the outpatient clinic of our university hospital in close collaboration with affiliated hospitals ensures updated information on patient status and events of graft failure as assessed by a nephrologist. Follow-up was performed until December 2017, resulting in a median follow-up of 8.2 (interquartile range [IQR]: 4.7–8.9) years. General practitioners or referring nephrologists were contacted when the status of a KTR was unknown. No patients were lost to follow up.

Statistical Analysis

Data analyses, computations, and graphs were performed with GraphPad Prism version 7 software (GraphPad Software; San Diego, CA, USA) and R version 4.0.5 (R Foundation for Statistical Computing; Indianapolis, IN, USA). For descriptive statistics, data are presented as mean ± standard deviation (SD), for normally distributed data and as median (IQR) for variables with a non-normal distribution. Quantile-quantile plots were used to assess normality. Categorical data are expressed as number (percentage). A statistical significance level of $p < 0.05$ (two-tailed) was used for all analyses.

Differences at baseline among subgroups of KTR according to tertiles of urinary copper excretion were tested by one-way ANOVA for continuous variables with normal distribution, Mann-Whitney U test for continuous variables with non-normal distribution, and χ^2 test for categorical variables. Linear regression analyses were performed to assess the association of urinary protein excretion with urinary copper excretion. Adjustments were performed for age, sex, body surface area, and eGFR. The association between urinary copper excretion and u-LFABP was also tested by linear regression analyses. Adjustment was also performed for the variables above with the addition of urinary protein excretion. For regression analyses, urinary copper excretion, urinary protein excretion, and u-LFABP were $\log_2$ transformed; therefore, estimated regression coefficients are expressed per doubling of the variable of interest. Models were checked for the fulfillment of linear regression assumptions.

Prospective Analyses

The distribution of graft failure among subgroups of KTR according to tertiles of urinary copper excretion was visualized by Kaplan-Meier curves, with the difference among curves tested by the Log-rank test. Furthermore, to visualize the continuous associations of urinary copper excretion and u-LFABP with the risk of graft failure, these variables were plotted against the risk of graft failure, fitted by penalized splines (p-splines), to allow non-linearity. Next, Cox proportional-hazards regression analyses were performed by introducing urinary copper excretion as a continuous variable and, to assess dose-response, by contrasting the risk of being in the middle and higher tertile of urinary copper excretion against being in the lowest tertile. For these analyses, the competing risk of death with a functioning graft was accounted by censoring at the time of death, as previously described [20]. Initial adjustments were made for age, sex, and urinary volume (model 1). Successive Cox regression models were built in a forward stepwise fashion to avoid overfitting and keep the number of predictors proportional to the number of events. Adjustments were made for body surface area and eGFR (model 2); preemptive transplantation, time since transplantation, human leukocyte antigen mismatch, living donor, donor age, and donor sex (model 3); acute rejection treatment, cumulative prednisolone dose, calcineurin inhibitor usage, proliferation inhibitor usage (model 4); u-LFABP, urinary malondialdehyde excretion and high-sensitivity C-reactive protein (model 5); and urinary protein excretion (model 6). Models were checked for the fulfillment of Cox regression assumptions. Results are presented as {hazard ratio (HR) (95% confidence interval [CI]); p value}.

As secondary analyses, we performed classic mediation analyses according to Preacher and Hayes [21] to establish whether urinary copper excretion was a mediator in the known association between urinary protein excretion and graft failure [22] and whether u-LFABP mediated the association between urinary copper excretion and graft failure. As sensitivity analyses, we performed Cox regression analyses using the reported upper normal value of urinary copper excretion (30 μg/24 h) as a cut-off point [9] and contrasting the risk of graft failure of KTR above this threshold against the risk of KTR below it. Adjustment was performed by the models described above. We also tested the prospective association of urinary copper excretion with graft failure after the exclusion of patients (i) with eGFR <30 mL/min/1.73 m^2, (ii) who developed the outcome in the first year of follow-up, and (iii) who were outside the −2 and +2 SD of urinary copper excretion. These analyses were adjusted according to model 2 to avoid overfitting.

Results

Cross-Sectional Analyses at Baseline

The baseline characteristics of the study population are presented in Table 1. In total, 693 KTR (57% males, age 53 ± 13 years old) were included in the analyses. Median (IQR) urinary copper excretion was 23.57 (15.86–37.13) μg/24 h. The mean eGFR was 52 ± 20 mL/min/1.73 m^2, and the median 24 h urinary protein excretion was 0.20 (0.02–0.38) g. Compared to the lowest tertile of urinary copper excretion, KTR in the highest tertile were less often male ($p = 0.03$), had higher body surface area ($p = 0.04$), lower eGFR ($p < 0.001$), higher urinary protein excretion ($p < 0.001$), and urinary volume ($p < 0.001$). KTR on the highest tertile of urinary copper excretion also had higher urinary excretion of u-LFABP ($p < 0.001$) and malondialdehyde ($p < 0.001$). In addition, they less frequently received a kidney from a living donor ($p = 0.02$) and had higher plasma concentrations of pro-inflammatory markers, such as leukocyte count ($p = 0.03$) and high-sensitivity C-reactive protein ($p = 0.01$).

In linear regression analyses, urinary protein excretion was associated with urinary copper excretion (Std. $\beta = 0.39$, $p < 0.001$), independent of age, sex, body

Table 1. Baseline characteristics of study population by tertiles of urinary copper excretion

Baseline characteristics	Overall KTR	Tertiles of urinary copper excretion[π]			*p* value[¥]
		tertile 1	tertile 2	tertile 3	
N	693	231	231	231	—
Urinary copper excretion, µg/24 h	23.57 (15.86–37.13)	13.71 (11.32–15.87)	23.57 (20.34–26.46)	45.20 (37.05–66.04)	—
Demographic and anthropometric					
Age, years	53±13	53±13	53±12	53±13	0.83
Sex (male), *n* (%)	394 (57)	138 (60)	141 (61)	115 (50)	0.03
Body surface area, m^2	1.94±0.22	1.92±0.22	1.97±0.20	1.94±0.22	0.04
Kidney allograft function					
eGFR, mL/min/1.73 m^{2a}	52±20	58±18	52±22	47±20	<0.001
Urinary protein excretion, g/24 h	0.20 (0.00–0.38)	0.00 (0.00–0.22)	0.22 (0.00–0.35)	0.31 (0.00–0.94)	<0.001
Urinary volume, mL/24 h	2,439±799	2,230±617	2,419±743	2,667±943	<0.001
Urinary biomarkers of oxidative tubular damage and oxidative stress					
u-LFABP excretion, µg/24 h[b]	2.12 (0.92–7.52)	1.21 (0.62–2.08)	3.13 (1.11–8.54)	5.75 (1.53 –20.73)	<0.001
Urinary malondialdehyde excretion, µmol/24 h[c]	9.70 (5.87–15.7)	9.03 (5.35–13.6)	9.39 (5.70–15.4)	11.5 (7.34–17.9)	<0.001
Kidney transplant characteristics					
Preemptive transplantation, *n* (%)	106 (15)	44 (19)	28 (12)	34 (15)	0.11
Time since transplantation, years	5.39 (1.97–12.04)	5.64 (1.95–11.28)	5.39 (2.15–12.02)	5.13 (1.64–12.16)	0.62
Acute rejection, *n* (%)	185 (27)	56 (24)	67 (29)	62 (27)	0.51
HLA class I antibodies positive, *n* (%)	106 (15)	28 (12)	35 (15)	43 (19)	0.15
HLA class II antibodies positive, *n* (%)	120 (17)	35 (15)	38 (17)	47 (20)	0.31
Kidney donor characteristics					
Living donor, *n* (%)	234 (34)	94 (41)	68 (29)	72 (31)	0.02
Age, years[d]	43±15	43±15	42±16	44±15	0.61
Sex (male), *n* (%)[e]	350 (51)	122 (53)	113 (49)	115 (50)	0.84
Immunosuppressive therapy					
Cumulative prednisolone dose, g	18.6 (7.6–37.2)	18.6 (7.6–34.1)	19.0 (7.8–39.1)	18.5 (7.1–38.5)	0.92
Use of calcineurin inhibitor, *n* (%)	397 (57)	138 (60)	125 (54)	134 (58)	0.46
Use of proliferation inhibitor, *n* (%)	573 (83)	196 (85)	189 (82)	188 (81)	0.56
Cardiovascular history and lifestyle					
Systolic blood pressure, mm Hg[a]	136±18	133±16	136±18	138±19	0.06
Diastolic blood pressure, mm Hg[a]	82±11	81±11	83±11	83±11	0.22
Glycated hemoglobin, %[f]	5.8 (5.5–6.2)	5.8 (5.5–6.2)	5.9 (5.5–6.3)	5.8 (5.4–6.1)	0.82
Alcohol intake >30 g/day, *n* (%)[g]	30 (4)	14 (6)	4 (2)	12 (5)	0.06
Current smoker, *n* (%)[h]	81 (12)	22 (10)	34 (15)	25 (11)	0.20
Inflammation					
Leukocyte count, $10^9/L$[i]	8.13±2.62	7.7±2.32	8.40±2.62	8.21±2.86	0.03
High-sensitivity C-reactive protein, mg/L[j]	1.6 (0.7–4.6)	1.3 (0.6–3.5)	1.7 (0.8–4.7)	1.9 (0.8–5.5)	0.01

[π]Tertile 1: <18.32 µg/24 h. Tertile 2: 18.32–30.84 µg/24 h, Tertile 3: >30.84 µg/24 h. [¥]Differences were tested by ANOVA for continuous variables with normal distribution, Kruskal-Wallis test for continuous variables with non-normal distribution, and by χ^2 test for categorical variables. Data available in [a]690, [b]629, [c]692, [d]674, [e]677, [f]664, [g]631, [h]647, [i]691, and [j]650 patients. KTR, kidney transplant recipients; eGFR, estimated glomerular filtration rate; u-LFABP, urinary liver-type fatty-acid binding protein; HLA, human leukocyte antigen.

surface area, and eGFR. Among KTR with proteinuria, urinary copper excretion was significantly higher than among KTR without proteinuria (20.14 [14.65–30.20] vs. 39.91 [27.07–56.84] µg/24 h, $p < 0.001$). Urinary copper excretion was also associated with u-LFABP excretion (Std. $\beta = 0.29$, $p < 0.001$; shown in online suppl. Fig. 1), independent of the variables mentioned above and urinary protein excretion. Graphical analyses of the association of u-LFABP against the risk of graft failure as fitted by p-splines, revealed that patients with relatively higher u-LFABP had also a higher risk of graft failure (shown in online suppl. Fig. 2).

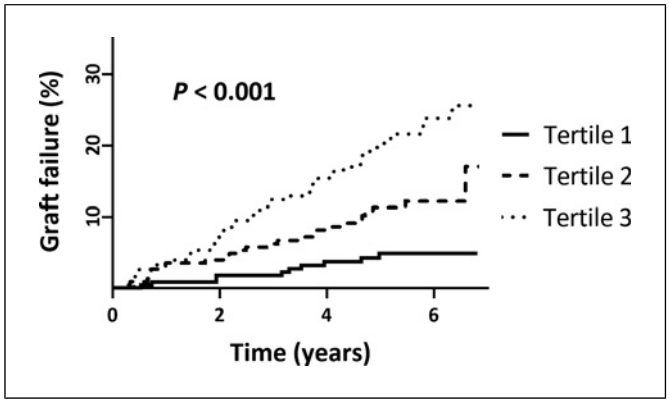

Fig. 1. Kaplan-Meier curves for graft failure according to tertiles of urinary copper excretion in KTR. Tertile 1: <18.32 μg/24 h; tertile 2: 18.32–30.84 μg/24 h; tertile 3: >30.84 μg/24 h. In total, 109 KTR developed graft failure (Tertile 1: 13, Tertile 2: 39, Tertile 3: 57). *p* value was calculated by Log-rank (Mantel-Cox) test. KTR, kidney transplant recipients.

Prospective Analyses

During follow-up, 109 (16%) KTR developed graft failure. The most frequent cause was chronic rejection (75%) followed by recurrence of the primary disease (8%; online suppl. Table 2). Kaplan-Meier curves by tertiles of KTR according to urinary copper excretion are shown in Figure 1. In total, graft failure occurred in 13 (6%), 39 (17%), and 57 (25%) of KTR in the first, second, and third tertile of copper excretion, respectively ($p_{\text{log-rank}}$ <0.001). In agreement, graphical analyses of the association of urinary copper excretion against the risk of graft failure as fitted by p-splines, revealed that patients with relatively higher urinary copper excretion had also a higher risk of graft failure (shown in online suppl. Fig. 2). Cox regression analyses revealed that higher urinary copper excretion was associated with a higher risk of graft failure (HR: 1.57 [95% CI: 1.32–1.86] per $\log_2$; $p < 0.001$). This association remained significant after adjustment for age, sex, urinary volume, eGFR, and body surface area. Further adjustment for transplant-related characteristics, immunosuppressive therapy, inflammation and oxidative stress biomarkers, and urinary protein excretion did not materially affect the association. Consistently, being in the highest tertile of urinary copper excretion when compared to the lowest tertile was associated with a five-fold increased risk of death-censored graft failure (HR: 5.03 [95% CI = 2.75–9.19]; $p < 0.001$), also independent of adjustments by potential confounders (Table 2).

Mediation Analyses

We tested the mediation of urinary copper excretion in the association between urinary protein excretion and graft failure and of u-LFABP in the association between urinary copper excretion and graft failure (shown in Fig. 2). We found that urinary copper excretion mediated 8% of the association between urinary protein excretion and graft failure (total effect β = 0.34 [95% CI = 0.26–0.41], indirect effect β = 0.03 [95% CI = 0.002–0.05]; $p_{\text{indirect effect}}$ = 0.03). u-LFABP mediated 74% of the association between urinary copper excretion and graft failure (total effect β = 0.23 [95% CI = 0.14–0.31], indirect effect β = 0.17 [95% CI = 0.12–0.11]; $p_{\text{indirect effect}}$ <0.001).

Sensitivity Analyses

In sensitivity analyses, consistent with our primary analyses, KTR with a urinary copper excretion higher than 30 μg/24 h had a two-fold risk of long-term graft failure when compared to KTR with a urinary copper excretion below this threshold (HR: 2.44 [95% CI = 1.68–3.57]; $p < 0.001$), independent of adjustments by potential confounders (online suppl. Table 3). Furthermore, urinary copper excretion remained associated with the risk of graft failure after the exclusion of patients (i) with eGFR <30 mL/min/1.73 m^2 (HR: 1.56 [95% CI = 1.25–1.95] per $\log_2$; $p < 0.001$), (ii) who presented the outcome within the first year of follow-up (HR: 1.46 [95% CI: 1.19–1.79] per $\log_2$; $p < 0.001$) and (iii) patients outside the −2 and +2 SD of urinary copper excretion (HR: 1.66 [95% CI = 1.27–2.18] per $\log_2$; $p < 0.001$; online suppl. Table 4).

Discussion

In a large cohort of outpatient KTR, urinary protein excretion was associated with increased urinary copper excretion. In turn, urinary copper excretion was associated with increased oxidative tubular damage as assessed by u-LFABP. Moreover, relatively higher urinary copper excretion was independently associated with a higher risk of long-term graft failure. Urinary copper excretion mediated the association of urinary protein excretion with graft failure, and oxidative tubular damage mediated the association of urinary copper excretion with graft failure. Our findings align with previous studies that showed an increased urinary copper excretion during impaired renal function, especially if proteinuria is present [2–5]. Moreover, these findings highlight the suggested potential role of urinary copper overload as an enhancer of oxidative tubular damage [10–12]. To our knowledge, this is the first study to investigate the consequences of this in the kidney posttransplantation setting.

Table 2. Association between urinary copper excretion and graft failure in KTR

	Urinary copper excretion distribution							Urinary copper excretion, per log_2		
	tertile 1	tertile 2			tertile 3					
	Ref	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value
Crude	Ref	3.13	1.67–5.86	<0.001	5.03	2.75–9.19	<0.001	1.57	1.32–1.86	<0.001
Model 1	Ref	3.28	1.75–6.14	<0.001	5.92	3.20–10.93	<0.001	1.62	1.37–1.92	<0.001
Model 2	Ref	2.15	1.13–4.07	0.02	3.35	1.78–6.29	<0.001	1.44	1.18–1.75	<0.001
Model 3	Ref	2.87	1.52–5.44	<0.001	5.20	2.79–9.73	<0.001	1.64	1.36–1.98	<0.001
Model 4	Ref	3.45	1.83–4.48	<0.001	6.36	3.43–11.82	<0.001	1.62	1.37–1.92	<0.001
Model 5	Ref	3.60	1.82–7.13	<0.001	5.72	2.89–11.33	<0.001	1.49	1.23–1.82	<0.001
Model 6	Ref	3.09	1.65–5.80	<0.001	3.98	2.11–7.53	<0.001	1.27	1.03–1.57	0.03

In total, 109 KTR developed graft failure (tertile 1: 13, tertile 2: 39, tertile 3: 57). Multivariable Cox regression analyses were performed. Model 1: adjustment for age, sex, and urinary volume. Model 2: model 1 + body surface area and estimated glomerular filtration rate. Model 3: model 1 + preemptive transplantation, time since transplantation, human leukocyte antigen mismatch, living donor, donor age, and donor sex. Model 4: model 1 + acute rejection treatment, cumulative prednisolone dose, calcineurin inhibitor usage, proliferation inhibitor usage. Model 5: model 1 + urinary liver-type fatty-acid binding protein, urinary malondialdehyde excretion, and high–sensitivity C–reactive protein. Model 6: model 1 + urinary protein excretion. KTR, kidney transplant recipients; HR, hazard ratio; CI, confidence interval.

Copper is present at low concentrations in fluids and tissues [23]. It is obtained mainly through diet or environmental exposure [7]. Copper-rich food includes shellfish, whole grains, beans, and nuts [24]. In healthy individuals, urinary copper excretion is very low, accounting for less than 3% of the daily copper intake since it is mainly bound to proteins that do not cross the glomerular membrane or are reabsorbed in the tubules [4]. However, several studies have found that urinary copper excretion increases when renal function is impaired. Yang et al. [2] described this phenomenon in a large population cohort, in which urinary copper was shown to be inversely associated with eGFR. Also, multiple studies have shown an increase in urinary copper in patients with chronic kidney disease and microalbuminuria compared to patients without microalbuminuria [4]. In our cohort, urinary protein excretion was significantly associated with urinary copper excretion. Furthermore, KTR with proteinuria had significantly higher values of urinary copper excretion when compared to KTR without proteinuria and higher values than those previously reported for healthy individuals [3, 9]. This augmented copper excretion is proposed to be caused by increased protein filtration, including copper carriers, when the glomerular membrane is affected [2]. Noteworthy, this association was independent of adjustment by eGFR, implying that in KTR, decreased graft function is not necessary for an increase in urinary copper excretion. Furthermore, urinary copper excretion mediated the known association between urinary protein excretion and the risk of kidney graft failure [22, 25]. Suggesting that an increase in copper filtration when proteinuria is present could be one of the pathophysiological mechanisms by which proteinuria contributes to the progression of nephropathy.

We found that urinary copper excretion is independently associated with the risk of long-term graft failure in KTR. Copper possesses unpaired electrons that make it prone to participate in redox reactions. Therefore, under conditions of copper overload, there is more propagation of reactive oxygen species and lipid peroxidation [10–14]. KTR in the highest tertile of urinary copper excretion also had higher urinary excretion of oxidative stress biomarkers, e.g., malondialdehyde, and we found a positive independent association between posttransplantation urinary copper excretion and the biomarker of tubular oxidative injury u-LFABP. Importantly, u-LFABP mediated the association between urinary copper excretion and graft failure. u-LFABP is an intracellular lipid chaperon that, in the kidney, is only present in the proximal tubule. u-LFABP function is to eliminate lipid peroxides by transferring them to the lumen, and its synthesis is upregulated in the presence of oxidative challenge. Therefore, in this scenario, u-LFABP urinary excretion also increases [26, 27]. These findings support the theory that copper can induce cellular toxicity and oxidative tissue injury [10–12]. Additionally, fibrotic kidneys possess a

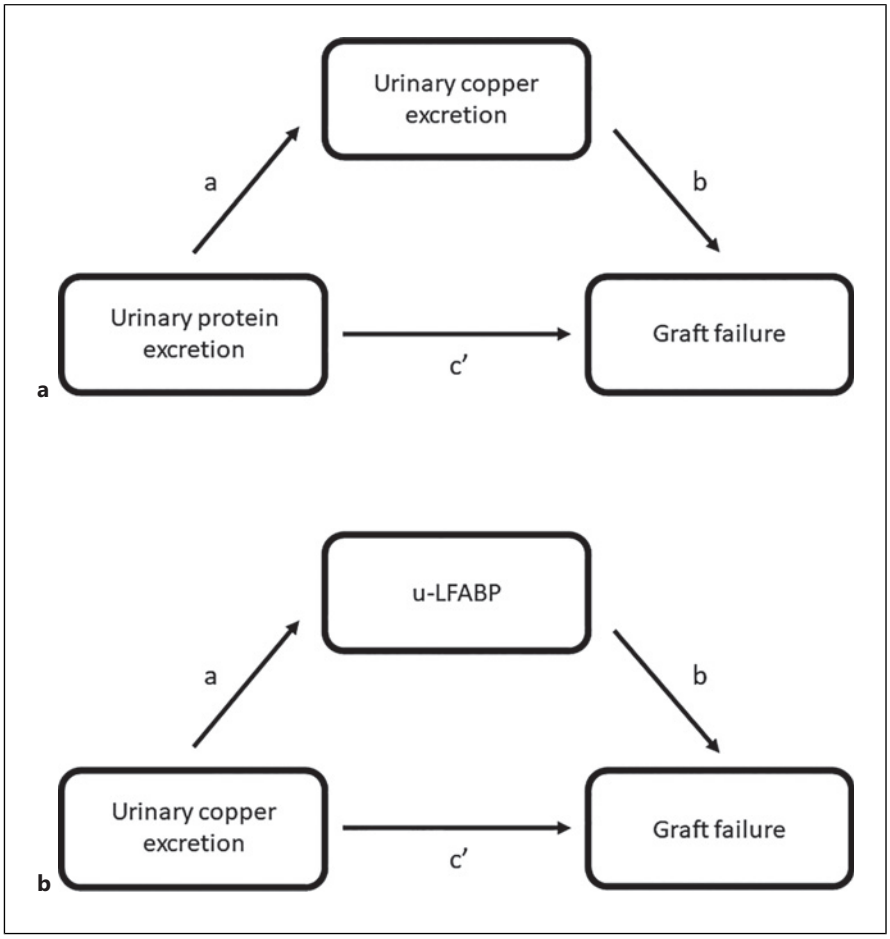

Fig. 2. Mediation analyses. We tested the potential mediation of (a) urinary copper excretion in the association between urinary protein excretion and graft failure and (b) u-LFABP in the association between urinary copper excretion and graft failure. The indirect effect is calculated as a*b, and the total effect is calculated as a*b + c'. Confidence intervals for the indirect and total effects are corrected after running 2,000 bootstrap samples. The magnitude of mediation is calculated as the indirect effect divided by the total effect. u-LFABP, urinary liver-type fatty-acid binding protein.

reduced concentration of copper-related defenses, namely metallothionein [28], an intracellular metal-binding protein that protects tissues from oxidative stress [8]. Importantly, the damage caused by copper overload appears reversible to some extent. Animal and laboratory model studies that tested the effect of copper-specific chelation therapy on diabetic nephropathy showed an improvement after treatment in albumin/creatinine ratio, glomerular hypertrophy, and the presence of fibrosis markers in the kidney cortex [11, 15, 29].

The current study comprises a large cohort of outpatient extensively phenotyped KTR, which allowed adjustment for several potential confounders. Furthermore, all study subjects were closely monitored, without losses to follow-up. On the other hand, this is a single-center study and we did not possess repeated measurements of urinary copper excretion. However, previous studies suggest that accounting for intraindividual variability of biomarkers increases their predictive properties. Therefore, the higher the intraindividual variation of urinary copper excretion would be, the bigger the expected benefit of repeated measurements in strengthening the association we describe in this study [30]. Next, as with any observational study, a causal relationship between urinary copper excretion, tubular damage, and graft failure cannot be established. Urinary copper excretion could merely be a marker of disease progression. However, multiple studies support the idea of a direct harming effect of copper on kidney tissue [6, 11]. As with any observational study, unmeasured confounding may occur, despite the substantial number of potentially confounding variables for which we adjusted in our analyses.

In conclusion, in outpatient KTR, an increased urinary protein excretion is associated with enhanced copper excretion. In turn, urinary copper excretion associates with a higher risk of long-term graft failure, possibly through the induction of oxidative tubular damage. This is the first study to describe a relationship between posttransplantation urinary copper overload and kidney graft failure. Further studies are warranted to assess

whether copper excretion-targeted interventions represent a potential strategy for improving long-term graft survival in KTR, particularly among those with proteinuria.

Statement of Ethics

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

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was based on the TransplantLines Food and Nutrition Biobank and Cohort Study (TxL-FN), funded by the Top Institute Food and Nutrition of the Netherlands (grant A-1003). This collaboration project is co-financed by the Dutch Ministry of Economic Affairs and Climate Policy through the PPP allowance made available by the Top Sector Life Sciences & Health to stimulate public-private partnerships.

Author Contributions

Gerjan J. Navis, Gerald Rimbach, and Stephan J.L. Bakker designed the study; Daan Kremer, Ulrike Seidel, Patricia Huebbe, Tim J. Knobbe, Kai Lüersen, and Gerald Rimbach performed the measurements and provided the data; Manuela Yepes-Calderón, Martin H. de Borst, and Stephan J.L. Bakker analyzed the data; Adrian Post, Daan Kremer, Camilo G. Sotomayor, Tim J. Knobbe, Michele F. Eisenga, Eva Corpeleijn, Martin H. de Borst, and Stephan J.L. Bakker provided critical review, advice, and consultation throughout; Manuela Yepes-Calderón and Adrian Post made the figures; Manuela Yepes-Calderón, Adrian Post, Camilo G. Sotomayor, Martin H. de Borst, and Stephan J.L. Bakker drafted and revised the paper; all authors approved the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are not publicly available due to privacy of research participants but are available from principal investigator S.J.L.B (s.j.l.bakker@umcg.nl) upon reasonable request.

References

1 Poggio ED, Augustine JJ, Arrigain S, Brennan DC, Schold JD. Long-term kidney transplant graft survival: making progress when most needed. Am J Transplant. 2021 Aug;21(8):2824–32.
2 Yang F, Yi X, Guo J, Xu S, Xiao Y, Huang X, et al. Association of plasma and urine metals levels with kidney function: a population-based cross-sectional study in China. Chemosphere. 2019 Jul;226:321–8.
3 Gardner DS, Allen JC, Goodson D, Harvey D, Sharman A, Skinner H, et al. Urinary trace elements are biomarkers for early detection of acute kidney injury. Kidney Int Rep. 2022 Jul;7(7):1524–38.
4 Ito S, Fujita H, Narita T, Yaginuma T, Kawarada Y, Kawagoe M, et al. Urinary copper excretion in type 2 diabetic patients with nephropathy. Nephron. 2001;88(4):307–12.
5 Gembillo G, Labbozzetta V, Giuffrida AE, Peritore L, Calabrese V, Spinella C, et al. Potential role of copper in diabetes and diabetic kidney disease. Metabolites. 2022 Dec;13(1):17.
6 Hordyjewska A, Popiołek Ł, Kocot J. The many "faces" of copper in medicine and treatment. Biometals. 2014 Aug;27(4):611–21.
7 Taylor AA, Tsuji JS, Garry MR, McArdle ME, Goodfellow WL, Adams WJ, et al. Critical review of exposure and effects: implications for setting regulatory health criteria for ingested copper. Environ Manage. 2020 Jan;65(1):131–59.
8 Gaetke LM, Chow-Johnson HS, Chow CK. Copper: toxicological relevance and mechanisms. Arch Toxicol. 2014 Nov;88(11):1929–38.
9 Anstee Q, Jones D. Hepatology. 23rd ed. In: Hobson R, Strachan M, Penman I, Ralston S, editors. Davidson's principles and practice of medicine. Philadelphia: Elsevier; 2018.
10 Ahmad S, Ärnlöv J, Larsson SC. Genetically predicted circulating copper and risk of chronic kidney disease: a mendelian randomization study. Nutrients. 2022 Jan;14(3):509.
11 Niu Y, Zhang Y, Zhu Z, Zhang X, Liu X, Zhu S, et al. Elevated intracellular copper contributes a unique role to kidney fibrosis by lysyl oxidase mediated matrix crosslinking. Cell Death Dis. 2020 Mar;11(3):211.
12 Jiang C, Ye H, Cui L, Pai P, Wang G. Relationship of serum copper and zinc with kidney function and urinary albumin to creatinine ratio: cross-sectional data from the NHANES 2011–2016. Eur J Clin Nutr. 2022 Dec;76(12):1748–54.
13 Bacou E, Walk C, Rider S, Litta G, Perez-Calvo E. Dietary oxidative distress: a review of nutritional challenges as models for poultry, swine and fish. Antioxidants. 2021 Mar;10(4):525.
14 Guengerich FP. Introduction to Metals in Biology 2018: copper homeostasis and utilization in redox enzymes. J Biol Chem. 2018 Mar;293(13):4603–5.
15 Saifi MA, Godugu C. Copper chelation therapy inhibits renal fibrosis by modulating copper transport proteins. BioFactors. 2022 Mar;48(4):934–45.
16 Yepes-Calderón M, Sotomayor CG, Pena M, Eisenga MF, Gans ROB, Berger SP, et al. Urinary liver-type fatty acid-binding protein is independently associated with graft failure in outpatient kidney transplant recipients. Am J Transplant. 2021 Apr;21(4):1535–44.
17 van den Berg E, Engberink M, Brink E, van Baak M, Gans R, Navis G, et al. Dietary protein, blood pressure and renal function in renal transplant recipients. Br J Nutr. 2013;109(8):1463–70.
18 Eisenga MF, Gomes-Neto AW, van Londen M, Ziengs AL, Douwes RM, Stam SP, et al. Rationale and design of TransplantLines: a prospective cohort study and biobank of solid organ transplant recipients. BMJ Open. 2018 Dec;8(12):e024502.

19 Weber KS, Ratjen I, Enderle J, Seidel U, Rimbach G, Lieb W. Plasma boron concentrations in the general population: a cross-sectional analysis of cardio-metabolic and dietary correlates. Eur J Nutr. 2022 Apr;61(3):1363–75.
20 Noordzij M, Leffondre K, van Stralen KJ, Zoccali C, Dekker FW, Jager KJ. When do we need competing risks methods for survival analysis in nephrology? Nephrol Dial Transplant. 2013 Nov;28(11):2670–7.
21 Agler R, de Boeck P. On the interpretation and use of mediation: multiple perspectives on mediation analysis. Front Psychol. 2017 Nov;8:1984.
22 Borrego Hinojosa J, Gentil Govantes MA, Cabello Díaz M, Rodriguez Benot A, Mazuecos Blanca A, Osuna Ortega A, et al. Progression of urinary protein excretion after kidney transplantation: a marker for poor long-term prognosis. Nefrologia. 2015 Jul;35(4):374–84.
23 Focarelli F, Giachino A, Waldron KJ. Copper microenvironments in the human body define patterns of copper adaptation in pathogenic bacteria. PLoS Pathog. 2022 Jul;18(7):e1010617.
24 Bost M, Houdart S, Oberli M, Kalonji E, Huneau J-F, Margaritis I. Dietary copper and human health: current evidence and unresolved issues. J Trace Elem Med Biol. 2016 May;35:107–15.
25 Stevens KK, Patel RK, Methven S, Clancy MJ, Fox JG, Jardine AG, et al. Proteinuria and outcome after renal transplantation. Transplantation. 2013 Jul;96(1):65–9.
26 Susantitaphong P, Siribamrungwong M, Doi K, Noiri E, Terrin N, Jaber BL. Performance of urinary liver-type fatty acid: binding protein in acute kidney injury–a meta-analysis. Am J Kidney Dis. 2013 Mar;61(3):430–9.
27 Zou C, Wang C, Lu L. Advances in the study of subclinical AKI biomarkers. Front Physiol. 2022 Aug;13:960059.
28 Lu Y-J, Wu Y-J, Chen L-J, Ko B-S, Chang T-C, Wu Y-J, et al. Reduced expression of metallothionein-I/II in renal proximal tubules is associated with advanced chronic kidney disease. Toxins. 2021 Aug;13(8):568.
29 Gong D, Lu J, Chen X, Reddy S, Crossman DJ, Glyn-Jones S, et al. A copper(II)-selective chelator ameliorates diabetes-evoked renal fibrosis and albuminuria, and suppresses pathogenic TGF-β activation in the kidneys of rats used as a model of diabetes. Diabetologia. 2008 Sep;51(9):1741–51.
30 Koenig W, Sund M, Fröhlich M, Löwel H, Hutchinson WL, Pepys MB. Refinement of the association of serum C-reactive protein concentration and coronary heart disease risk by correction for within-subject variation over TimeThe MONICA augsburg studies, 1984 and 1987. Am J Epidemiol. 2003 Aug;158(4):357–64.