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Nitric oxide and long-term outcomes after kidney transplantation: Results of the TransplantLines cohort study

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ABSTRACT

Impaired endogenous nitric oxide (NO) production may contribute to graft failure and premature mortality in kidney transplant recipients (KTR). We investigated potential associations of 24-h urinary NOx (NO_3^- + NO_2^-) excretion (uNOx) with long-term outcomes. uNOx was determined by HPLC and GC-MS in 698 KTR and in 132 kidney donors before and after donation. Additionally, we measured urinary nitroso species (RXNO) by gas-phase chemiluminescence. Median uNOx was lower in KTR compared to kidney donors (688 [393–1076] *vs.* 1301 [868–1863] before donation and 1312 [982–1853] μmol/24 h after donation, P < 0.001). During median follow-up of 5.4 [4.8–6.1] years, 150 KTR died (61 due to cardiovascular disease) and 83 experienced graft failure. uNOx was inversely associated with all-cause mortality (HR per doubling of uNOx: 0.84 [95% CI 0.75–0.93], $P < 0.001$) and cardiovascular mortality (HR 0.78 [95% CI 0.67–0.92], $P = 0.002$). The association of uNOx with graft failure was lost when adjusted for renal function (HR per doubling of uNOx: 0.89 [95% CI 0.76–1.05], $P = 0.17$). There were no significant associations of urinary RXNO with outcomes. Our study suggests that KTR have lower NO production than healthy subjects and that lower uNOx is associated with a higher risk of all-cause and cardiovascular mortality.

1. Introduction

Kidney transplantation is the preferred treatment option for patients with end stage renal disease [1]. Although kidney transplantation reduces mortality risk and improves quality of life for patients that were on dialysis before transplantation [2–4], there is still much to be gained in improving patient and graft survival. Particularly cardiovascular complications, such as hypertension and atherosclerosis, lead to premature death or decline in graft function, eventually resulting in a need for re-transplantation or return to dialysis [5–9]. With approximately 90,000 renal transplantations performed every year globally [10] and an aging population with a higher risk for chronic kidney disease (CKD), there is an increasing need to find new biological pathways/targets to improve patient and graft survival. Nitric oxide (NO) is a pluripotent cell signaling and effector molecule involved in the control and regulation of an enormous variety of physiological processes. NO not only plays a critical role in cardiovascular homeostasis [11], but also modulates fundamental processes spanning from cell proliferation over substrate utilization to energy production across all major organ systems [12,13]. In the kidney, NO plays an important role in tubular and glomerular hemodynamics, promoting natriuresis and diuresis [14].

Three isoforms of NO synthase (NOS) have been identified: inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS) [15].

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Abbreviation list

ADMA	Asymmetric dimethylarginine
BMI	Body mass index
BSA	Body surface area
cGMP/PKG	Cyclic guanosine monophosphate-dependent protein kinase G
CKD-EPI	Chronic kidney disease-epidemiology collaboration
CNI	Calcineurin Inhibitor
CRP	C-Reactive protein
CVA	Cerebrovascular accident
DBP	Diastolic blood pressure
eGFR	Estimated glomerular filtration rate
eNOS	Endothelial nitric oxide synthase
GC-MS	Gas chromatography mass spectrometry
HbA1c	Hemoglobin A1c
HPLC	High pressure liquid chromatography
iNOS	Inducible nitric oxide synthase
nNOS	Neural nitric oxide synthase
NO	Nitric oxide
NOx	Sum of nitrite and nitrate
NO2	Nitrite
NO 3	Nitrate
NT-proBNP	N-terminal pro b-type natriuretic peptide
KTR	Kidney transplant recipients
RXNO	Nitroso species
SBP	Systolic blood pressure
TIA	Transient ischemic attack
uNOx	Urinary nitrite and nitrate excretion

eNOS is the major enzyme in the vasculature that produces NO, regulating both systemic and glomerular hypertension [16,17]. A role for NO in both acute and chronic transplant rejection was suggested, with lower levels of urinary nitrite and nitrate excretion during acute rejection and increased iNOS versus decreased glomerular eNOS expression levels in chronic renal transplant failure [18,19]. This is in accordance with other pathophysiological conditions such as atherosclerosis and hypertension in which impaired eNOS activity and endothelial dysfunction are observed [20,21].

While many of NO's actions are mediated via the classical cyclic guanosine monophosphate-dependent protein kinase G (cGMP/PKG) pathway, a significant part appears to be mediated in a cGMP-independent manner through redox-dependent processes secondary to the nitrosation of protein moieties. The latter leads to the formation of nitroso species, a process by which a NO moiety is covalently incorporated into another biomolecule [22]. This may occur by either post-translational modification of sulfhydryl or amino moieties in proteins or via formation of low-molecular-weight nitrosothiols and nitrosamines (RXNO). Potential roles of such nitrosative modifications have been described in the regulation of insulin signaling, mitochondrial energy metabolism, mRNA transcription, stress signaling, and endoplasmic reticulum function [23]. While a circulating pool of nitrosated products has been demonstrated to exist in healthy humans [24,25], we know little about the dynamics of their formation and excretion and their relationship, if any, with clinical outcome. The above mentioned characteristics of NO radicals and RXNO help us to better understand the biological pathways associated with graft failure and transplant related death. Furthermore, they could also serve as a potential therapeutic target in transplant patients. To the best of our knowledge, the occurrence and possible role of urinary NO products and nitroso species in KTR has not been investigated.

In the present study, we measured 24 h urinary nitrite (NO_2^-)and nitrate (NO_3^-) as well as total RXNO (the sum of S- and N-nitroso species) excretion by high-performance liquid chromatography (HPLC) and gas-phase chemiluminescence, respectively, in stable KTR and healthy kidney donors before and after kidney donation. NO_2^- and NO_3^- are expressed as their sum: NOx. We studied associations of uNOx as well as 24 h-urinary NO_2^- and NO_3^- excretions, and RXNO excretion prospectively with all-cause mortality, cardiovascular mortality, and graft failure. Finally, we studied which factors (e.g. inflammatory, diabetic, and body composition) may be in the causal pathway of the prospective associations of uNOx with outcomes.

2. Methods and materials

2.1. Study population

The study protocol was approved by the Institutional Review Board (METc 2008/186), in adherence to the Declaration of Helsinki and Istanbul. This study is part of a larger cohort study of KTR and kidney donors in the northern region of the Netherlands; the TransplantLines Food and Nutrition cohort, Clinicaltrials.gov Nº NCT02811835. All stable KTR with a functional graft for >1 year without current or past malignancies (with exception of cured skin cancer) or active infections who visited our outpatient clinic of the University Medical Center Groningen (UMCG) between November 2008 and March 2011 were invited to participate. KTR with history of drug addiction or cancer other than cured skin cancer were excluded from participation in the study. In total, 706 KTR signed written informed consent (87% of invited). Of these, a total of 698 24-h urinary samples were available for NOx measurement. As a healthy reference group, we used 300 subjects who were evaluated prior to living kidney donation in our center during the same period, of which 297 signed written informed consent and 132 had available 24-h urinary samples for NOx measurements both before and after kidney donation. All 132 healthy subjects included in the current study were approved for kidney donation.

2.2. Data collection

All measurements were performed during a morning visit to the outpatient clinic after an 8–12 h overnight fasting period. All participants were instructed to collect urine over a 24 h period. In advance of the urine collection, chlorhexidine was added to the urine container as an antiseptic agent in order to prevent bacterial growth [26]. Upon completion of the 24-h urine collection, fasting venous blood samples anti-coagulated with lithium-heparin, sodium-fluoride and potassium-EDTA were obtained the following morning. Samples were aliquoted and kept frozen at −80 °C until analysis. The sum of urinary nitrite and nitrate excretion is widely used as a marker of systemic NO production [27,28]. Clinical and laboratory measurements have been described in detail in previous papers [29–31].

NOx clearance (mL/min) was calculated as:

$$\text{NOx clearance (mL/min)} = \frac{[\text{NOx}]_{\text{urine } (\mu\text{mol/L})}}{[\text{NOx}]_{\text{plasma } (\mu\text{mol/L})}} \times \frac{24\text{ h} - \text{urine volume (mL)}}{1440}$$

NOx fractional excretion (%) was calculated in the following way:

$$\text{FE}_{\text{NOx}}(\%) = 100 \times \frac{[\text{NOx}]_{\text{urine } (\mu\text{mol/L})} \times [\text{Creatinine}]_{\text{plasma } (\mu\text{mol/L})}}{[\text{NOx}]_{\text{plasma } (\mu\text{mol/L})} \times [\text{Creatinine}]_{\text{urine } (\mu\text{mol/L})}}$$

Kidney function was assessed by calculating the estimated glomerular filtration rate (eGFR) applying the latest Chronic Kidney Disease Epidemiology Collaboration equation with creatinine and cystatin C (CKD-EPI-creatinine-cystatin C) [32].

Information on participants' health status, medical history, and medication use was obtained from patient records. Information on smoking behavior was obtained by using a questionnaire. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2) and body surface area (BSA) according to the formula of Dubois and Dubois [33].

Data on nutrient and food intake was derived from a previously validated Food Frequency Questionnaire (FFQ) that has been described in detail before [34,35]. The FFQs were analyzed using the 2006 Dutch Food Composition Table (NEVO, Dutch Ministry of Health, Welfare, and Sport) [36]. FFQs that reported an energy intake of less than 500 or more than 5000 kcal/day were regarded unreliable and excluded from the analyses.

2.3. HPLC method for the determination of nitrate and nitrite

The HPLC method used employs ion chromatography with on-line reduction of NO_3^- to NO_2^- and subsequent post-column derivatization with the Griess reagent using a dedicated analysis system (ENO-20, Eicom, Kyoto, Japan) [37]. Samples were subjected to deproteinization using ice-cold methanol (1:1, v/v) followed by centrifugation. A total volume of 10 μL of sample was loaded onto the column, resulting in a detection limit of 10 nM at a signal:noise ratio of >3 for either anion.

2.4. GC-MS method for the determination of nitrate and nitrite

Nitrite and nitrate were also measured in 100-μL aliquots of urine samples by a fully validated GC-MS method as described earlier [38], except for the solvent extraction which was performed by ethyl acetate as described elsewhere [39]. The GC-MS method involves use of ^{15}N-labeled NO_3^- and NO_2^- as internal standards, derivatization of endogenous NO_3^- and NO_2^- (i.e., $^{14}NO_3^-$ and $^{14}NO_2^-$, respectively) and the ^{15}N-labeled NO_3^- and NO_2^- with pentafluorobenzyl bromide, solvent extraction of their pentafluorobenzyl derivatives with ethyl acetate and 1-μL aliquots injection in the splitless mode. The limits of detection of the GC-MS method are 0.2 fmol injected $NO_2^-$and 4.8 fmol injected NO_3^- corresponding to 2 nM NO_2^- and 48 nM NO_3^- using a 100-μL urine volume [39].

2.5. Assessment of urinary nitroso species via gas-phase chemiluminescence detection

Urinary RXNO concentrations were quantified in 24-h urine samples using reductive denitrosation by triiodide (I_3^-) with subsequent detection of the liberated NO by gas-phase chemiluminescence, as previously described [40]. Detailed description can be found in the supplementary data.

2.6. Measurements of amino acids

Urinary and plasma asymmetric dimethylarginine (ADMA) have been measured by GC-MS/MS which has been described in detail before [41–43].

2.7. Statistical analysis

All results presented were acquired from HPLC-derived data, unless stated that GC-MS-derived data was used. We cross-sectionally studied associations of uNOx with various baseline characteristics by applying univariable linear regression analyses. Since the distribution of uNOx was skewed, we log_2-transformed it. We compared baseline characteristics KTR and healthy donors by student's T-test, Wilcoxon's Rank test or chi-square test. We prospectively analyzed associations of sex-adjusted tertiles of uNOx with all-cause mortality using Kaplan-Meier analyses. Differences in survival between tertiles were tested by log-rank test. The associations of sex-adjusted tertiles of uNOx with cardiovascular mortality and graft failure were analyzed using cumulative incidence functions and Gray's test to take competing risks into account [44]. We analyzed associations of log_2-transformed uNOx as well as log_2-transformed 24-h NO_3^- and NO_2^- excretions with long-term outcomes by Cox proportional hazard analyses. Cox regression models were built in a stepwise fashion to avoid overfitting and to keep the number of predictors in proportion to the number of events [45,46]. A constant of 1 was added to the NO_2^- data before log-transformation, since there were many subjects with undetectable or very low NO_2^- excretion. The proportional hazards assumption was tested using the Schoenfeld residual test and was not violated ($P > 0.05$ for all outcomes). We first adjusted for potential confounders by applying non-cumulative models to reduce over-fitting of the Cox proportional hazard models. In secondary analyses, we adjusted the association uNOx for factors that are potentially in the causal pathway of the association with outcome. Since there are competing risks for the outcomes of cardiovascular mortality and graft failure, we performed competing risk analyses according to Fine and Gray [47]. In these analyses, a subhazard ratio is calculated which takes the competing risks for cardiovascular mortality (i.e. non-cardiovascular mortality) or graft failure (i.e. all-cause mortality) into account.

We presented normally distributed variables as mean ± standard deviation and skewed data as median [interquartile range] and nominal data as number (percentage). Results of the linear regression analyses were presented as a standardized beta-coefficient, those of the Cox regression analyses as a hazard ratio [95% confidence interval]. A *P*-value of ≤0.05 was regarded as statistically significant. To assess the agreement between the HPLC method and the GC-MS method for measuring urinary NO_3^- and NO_2^-, we performed Passing–Bablok regression analyses and Bland-Altman analyses. Urinary NO_3^- and NO_2^- data were log_2 transformed before analyses. A total of 693 and 683 samples were available for urinary nitrate method comparison and urinary nitrite method comparison, respectively.

We used IBM SPSS statistics version 23 (2015 IBM corporation, Armonk, New York, USA), R statistics version 3.2.3 (2015, R Foundation for Statistical Computing, Vienna, Austria), and Stata version 14 (2015, StataCorp, College Station, Texas, USA) for the statistical analyses. Method comparison analyses were performed using Analyse-IT (Analyse-it Software ltd, Leeds, United Kingdom), a statistical analysis add-in for Microsoft Excel. We produced figures with GraphPad Prism version 5.04 (2010, GraphPad Software, La Jolla, California, USA).

3. Results

3.1. Healthy control vs. KTR: baseline characteristics

Baseline characteristics of the study cohort are shown in Table 1. Of 698 KTR, 57% were male, compared to 46% of the healthy donors. Healthy donors and KTR were similar with regard to age and BMI. For donors, median time from kidney donation to the follow-up visit was 1.64 [1.61–1.87] months. After kidney donation, eGFR in healthy donors decreased from 96.6 ± 13.5 eGFR, ml/min/1.73 m^2 to 49.6 ± 11.1 ml/min/1.73 m^2 and mGFR decreased significantly from 104 ± 17.3 ml/min/1.73 m2 for donors pre-nephrectomy vs. 65.8 ± 9.8 ml/min/1.73 m2 for donors post-nephrectomy ($P < 0.001$). KTR had a significantly lower eGFR (Table 1: 45.1 ± 18.8 ml/min/1.73 m^2) compared to donors both before and after nephrectomy ($P < 0.001$). In KTR, median NO_3 concentration in 24-h urine was 285 [159–486] μmol/L ranging 3.64–18344 μmol/L, and median NO_2^- concentration in 24-h urine was 0.10 [0.02–0.21] μmol/L ranging 0.0–1397 μmol/L. In healthy donors, median NO_3 concentration in 24-h urine was 478 [330–747] μmol/L, ranging 31.2–4898 μmol/L, and median NO_2^- concentration was 0.03 [0.005–0.27] μmol/L, ranging 0.0–799 μmol/L. Post-nephrectomy,

Table 1
Difference between KTR and healthy subjects: association with log_2-transformed 24 h-urinary NOx excretion.

	KTR (n = 698)	St. β	P	Donors pre-nephrectomy (n = 132)	St. β	P	Donors after nephrectomy (n = 132)
Demographics							
Age, years	54.6 [44.8–62.9]	−0.085	**0.03**	52.6 [45.6–59.5]	−0.165	0.06	53.5 [46.2–60.3]
Sex, *n* (%) male	396 (56.7)	0.127	**0.001**	61 (46.2)*	0.124	0.16	61 (46.2)
Body composition							
Weight, kg	80.4 ± 16.6	0.090	**0.02**	80.1 ± 12.8	0.028	0.76	79.3 ± 13.0
BMI, kg/m^2	26.0 [23.2–29.4]	0.002	0.97	25.4 [23.7–28.4]	0.008	0.93	25.0 [23.6–28.4]
Cardiovascular parameters							
Systolic pressure, mmHg	136 ± 18	−0.042	0.27	127 ± 13*	−0.002	0.98	121 ± 11*
Diastolic pressure, mmHg	83 ± 11	0.032	0.40	78 ± 8*	−0.048	0.59	75 ± 9*
Number of antihypertensive drugs, *n* (%)[a]							
0	82 (11.7)	Ref.	Ref.	106 (80.3)*	Ref.	Ref.	106 (80.3)*
1	192 (27.5)	−0.029	0.62	12 (9.1)*	0.053	0.55	10 (7.6)*
≥2	424 (60.7)	−0.118	**0.04**	10 (7.6)*	−0.028	0.76	13 (9.8)*
NT-proBNP, ng/L	254 [108–614]	−0.162	**<0.001**	39 [21–72] *	−0.085	0.36	55 [32–104]*
Kidney function parameters							
Urinary urea excretion, mmol/24 h	383 [308–456]	0.267	**<0.001**	400 [339–479]*	0.281	<0.001	381 [305–475]
Urinary creatinine excretion, μmol/24 h	11.3 [9.2–14.0]	0.281	**<0.001**	12.5–10.2 – 16.3]*	0.217	0.01	12.3 [9.5–15.3]*
eGFR, ml/min/1.73m^2[b]	45.1 ± 18.8	0.184	**<0.001**	96.6 ± 13.47*	0.235	0.01	49.6 ± 11.1*
mGFR, ml/min1.73m^2	49.1 ± 18.2†	0.153	**0.03**	104.0 ± 17.3*	0.183	0.06	65.8 ± 9.8*
Protein excretion, g/24 h	0.19 [0.02–0.37]	−0.060	0.11	0.02 [0.02–0.09]*	−0.052	0.56	0.02 [0.02–0.10]*
Proteinuria (>0.5 g/24 h), *n* (%)	155 (22.2)	−0.023	0.54	0*	N/A		0*
Nitric oxide							
Urinary NO_2^- excretion, μmol/24 h	0.21 [0.04–0.49]	0.100	**0.01**	0.07 [0.01–0.73]	0.094	0.28	0.07 [0.02–0.31]*
Urinary NO_3^- excretion, μmol/24 h	683 [385–1071]	0.600	**<0.001**	1296 [865–1836]*	0.874	**<0.001**	1300 [982–1844]*
Urinary NOx excretion, μmol/24 h	688 [393–1076]	N/A	N/A	1301 [868–1863]*	N/A	N/A	1312 [982–1853]*
Urinary nitroso excretion, μmol/24 h	0.28 [0.16–1.15]	0.011	0.77	0.27 [0.18–0.42]	−0.190	**0.03**	0.32 [0.19–0.53]
Plasma NO_2^-, μmol/L	0.29 [0.17–0.48]	0.025	0.52	0.08 [0.06–0.10]*	−0.096	0.34	0.11 [0.07–0.18]*
Plasma NO_3^-, μmol/L	39.7 [27.1–58.8]	0.218	**<0.001**	21.5 [19.9–29.5]*	0.514	**<0.001**	35.1 [29.8–46.8]
Plasma NOx, μmol/L	40.1 [27.8–59.6]	0.219	**<0.001**	22.3 [16.2–30.1]*	0.480	**<0.001**	35.2 [30.1–47.0]
NOx fractional excretion, %	19.2 [12.2–27.2]	0.479	**<0.001**	31.2 [22.0–48.8]*	0.358	**<0.001**	30.6 [24.4–39.5]*
NOx clearance, mL/min	11.5 [6.5–19.8]	0.472	**<0.001**	43.1 [30.8–59.1]*	0.498	**<0.001**	33.8 [23.7–42.8]*
Plasma ADMA, μmol/L	0.61 ± 0.12	−0.024	0.53	0.53 ± 0.08*	0.062	0.49	0.58 ± 0.09*
Urinary ADMA excretion, μmol/24h	31.4 ± 13.2	0.32	**<0.001**	60.5 ± 16.0*	0.38	**<0.001**	40.2 ± 11.5*
Systemic drugs that interact with the NO system							
RAAS inhibitor usage, n (%)	327 (46.8)	0.025	0.51	10 (7.6)*	−0.015	0.88	14 (10.6)*
B-adrenoreceptor blocker usage, n (%)	441 (63.2)	−0.092	**0.02**	7 (5.3)*	−0.069	0.48	11 (8.3)*
PDE5 inhibitor usage, n (%)	4 (0.6)	−0.002	0.96	0	N/A	N/A	Data N/A
Statin usage	371 (53.2)	−0.044	0.35	4 (3.0)*	0.026	0.80	5 (3.8)*
Diabetes							
Diabetes, n (%)[c]	166 (23.8)	−0.090	**0.02**	1 (0.8)*	−0.068	0.44	3 (2.3)*
Use of antidiabetic drugs, *n* (%)	107 (15.3)	−0.059	0.12	0 (0)*	N/A	N/A	0 (0)*
Glucose, mmol/L	5.3 [4.8–6.0]	−0.053	0.16	5.3 [5.1–5.6]	0.036	0.68	5.2 [4.9–5.4]
HbA_{1c}, %	5.8 [5.5–6.2]	−0.075	**0.05**	5.6 [5.4–5.8]*	−0.067	0.45	5.5 [5.4–5.7]*
Inflammation							
CRP, mg/L	1.6 [0.7–4.5]	−0.120	**0.002**	1.2 [0.5–2.3]*	−0.023	0.81	Data N/A
Blood leukocyte, x10^9/l	8.1 ± 2.6	−0.061	0.11	6.6 ± 1.9*	−0.232	**0.01**	Data N/A
Serum albumin, g/L	43 ± 3	0.138	**<0.001**	45 ± 2*	0.001	0.99	Data N/A
Lipids							
Total cholesterol, mmol/L	5.12 ± 1.12	−0.110	**0.004**	5.37 ± 1.05*	−0.149	0.09	5.5 ± 1.0
HDL cholesterol, mmol/L	1.30 [1.10–1.60]	−0.019	0.62	1.70 [1.38–2.05]*	−0.75	**0.001**	1.40 [1.10–1.65]
LDL cholesterol, mmol/l	2.90 [2.30–3.50]	−0.056	0.14	3.80 [3.23–4.28]*	0.30	0.27	3.50 [3.10–4.00]*
Triglycerides, mmol/L	1.68 [1.24–2.28]	−0.124	**0.001**	1.13 [0.83–1.59]*	−0.003	0.97	1.44 [1.10–1.99]
Cardiovascular disease history							
Myocardial infarction, *n* (%)	35 (5.0)	0.039	0.31	Data N/A			Data N/A
CABG and/or PCI, *n* (%)	54 (7.7)	−0.044	0.25	Data N/A			Data N/A
CVA and/or TIA, *n* (%)	40 (5.7)	−0.074	**0.05**	Data N/A			Data N/A
Smoking behavior, *n* (%)[d]							
Never	274 (39.3)	Ref.	Ref.	63 (47.7)*	Ref.	Ref.	Data N/A

(*continued on next page*)

Table 1 (*continued*)

	KTR (n = 698)	St. β	P	Donors pre-nephrectomy (n = 132)	St. β	P	Donors after nephrectomy (n = 132)
Ex	299 (42.8)	−0.045	0.28	28 (21.2)*	0.04	0.68	Data N/A
Current	83 (11.9)	0.063	0.13	28 (21.2)*	0.05	0.62	Data N/A
Alcohol intake, n (%)							
0–10 g/24 h	468 (67.0)	Ref.	Ref.	58 (43.9)*	Ref.	Ref.	Data N/A
10–30 g/24 h	138 (19.8)	0.013	0.74	30 (22.7)*	0.034	0.75	Data N/A
>30 g/24 h	30 (4.3)	0.053	0.18	5 (3.8)*	0.129	0.23	Data N/A
Dietary intakes							
Vegetables (g/day)	106 [68–149]	0.102	**0.01**	Data N/A			Data N/A
Fruit (g/day)	123 [66–232]	0.066	0.10	Data N/A			Data N/A
Legumes and nuts (g/day)	11 [4–22]	0.057	0.16	Data N/A			Data N/A
Magnesium (mg/day)	331 ± 90	0.182	**<0.001**	352 ± 93*			Data N/A
Plasma magnesium	0.95 ± 0.12	−0.02	0.69	Data N/A			Data N/A
Urinary sodium excretion	146 [114–190]	0.19	**<0.001**	192 [148–247]*			165 [130–210]*
Urinary potassium excretion	73 ± 24	0.28	**<0.001**	87 ± 28*			Data N/A

Abbreviations: ADMA: asymmetric dimethylarginine. BMI: body mass index; CABG: coronary artery bypass grafting; CRP: C-reactive protein; CVA: cerebrovascular accident; HbA_{1c}: hemoglobin A1c; KTR: kidney transplant recipients; NT-proBNP: N-terminal pro b-type natriuretic peptide; PCI: percutaneous coronary intervention; PDE5: phosphodiesterase type 5; RAAS: renin-angiotensin aldosterone system.; st. β: standardized beta; TIA: transient ischemic attack.
Data N/A: data is not available.
Data are presented as mean ± standard deviation (SD) for normally distributed variables, median [interquartile range] for variables with a skewed distribution, and nominal data as number (percentages).
* Significant difference compared with KTR.
†data only available for 201 KTR
[a] Percentages do not add up to 100% due to missing cases.
[b] CKD-EPI creatinine-cystatin C.
[c] Defined as blood glucose ≥7 mmol/L, $HbA_{1c} \geq 6.5$, and/or use of antidiabetic drugs.
[d] Percentages do not add up to 100% because of rounding.

median 24-h urine NO_3^- concentration was 594 [454–890] μmol/L and median 24-h urine NO_2^- concentration was 0.03 [0.01–0.12] μmol/L. Mean 24-h urine volume was 2.44 ± 0.80 L in KTR vs. 2.69 ± 0.80 L in healthy donors before nephrectomy and 2.23 ± 0.76 L post-nephrectomy. Urinary NO_3^- excretion was significantly lower in KTR when compared to healthy donors (683 [385–1071] μmol/24 h vs. 1301 [868–1863]μmol/24 h, $P < 0.001$ before nephrectomy and 1312 [982–1853] μmol/24 h, $P < 0.001$ post nephrectomy). Since NO_3^- makes the greatest contribution to uNOx levels, the same was true for uNOx (688 [393–1076] μmol/24 h vs. 1296 [865–1836]μmol/24 h, $P < 0.001$ before nephrectomy and 1300 [982–1844] μmol/24 h, $P < 0.001$ post-nephrectomy). By contrast, median urinary NO_2^- was higher in KTR compared to donors (0.21 [0.04–0.49] μmol/24 h vs. 0.07 [0.01–0.73] μmol/24 h, $P = 0.08$ before nephrectomy and 0.07 [0.02–0.31], $P = 0.001$ post-nephrectomy). Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) were higher in KTR compared to healthy donors before and after nephrectomy (Table 1). uNOx was associated with younger age, less usage of antihypertensive drugs, less usage of β-adrenoreceptor blocking drugs, lower NT-proBNP, less diabetes and lower HbA_{1c}, lower CRP, cholesterol and triglycerides.

Plasma NO_2^-, NO_3^- and NOx were significantly lower in kidney donors before nephrectomy compared to KTR, while NOx clearance and fractional excretion were higher in healthy kidney donors. Of note, after nephrectomy, there was no significant difference in plasma NO_3^- and NOx between KTR and healthy donors.

3.2. Transplant-related baseline characteristics of KTR

The transplantation characteristics of the KTR are shown in Table 2. Participants had a median transplant vintage of 5.4 [1.8–12.1] years and past dialysis vintage of 27 [9–51] months. There were 399 KTR who used a calcineurine inhibitor (57.2% of all KTR). High uNOx was associated with a lower dialysis vintage and lower frequency of calcineurin inhibitor use (all $P < 0.05$).

3.3. Associations of urinary NOx, NO_3^-, and NO_2^- excretions with long-term outcomes

During 5.4 [4.8–6.1] years of follow-up, 150 (22%) KTR died, of which 61 (41%) died due to cardiovascular disease. In the highest sex-stratified tertile of uNOx, 31 out of 233 (13.3%) died, while this was 44 out of 233 (18.9%) in the middle tertile and 75 out of 232 (32.3%) in the tertile with the lowest uNOx (log-rank test $P < 0.001$, Fig. 1). KTR who died during follow-up had a significantly lower uNOx compared to those who survived during follow-up (489 μmol/24 h versus 738 μmol/24 h, $P < 0.001$). We present cumulative incidence curves for the association of sex-stratified tertiles of uNOx with cardiovascular mortality in Fig. 2A. There was a significant difference in cumulative incidence of cardiovascular mortality between the sex-adjusted tertiles ($P < 0.001$ for cardiovascular mortality, $P = 0.02$ for competing risk of non-cardiovascular mortality).

Graft failure occurred in 83 KTR of which 73.5% resulted from chronic rejection, 9.6% from recurrence of primary renal disease, 3.6% from rejection of the graft, 3.6% from infection of the graft, 3.6% from infections not related to the graft, 2.4% from vascular problems, and 2.4% from miscellaneous causes. We found that KTR in the highest stratified tertile of uNOx experienced less graft failure ($n = 17$, 7.3%) compared to KTR in the middle ($n = 29$, 12.4%) and lowest tertiles ($n = 37$, 15.9%) (log-rank test $P = 0.003$). Patients experiencing graft failure had significantly lower uNOx compared to those who did not experience graft failure (526 μmol/24 h versus 708 μmol/24 h, $P = 0.001$). We present cumulative incidence curves for the associations of sex-stratified tertiles of uNOx with death-censored graft failure in Fig. 2B. There was a significant difference in cumulative incidence of death-censored graft failure between the sex-adjusted tertiles ($P = 0.01$ for graft failure, <0.001 for the competing risk of death).

3.4. Causal path analyses of the associations with long-term outcomes

We performed Cox regression analyses of the associations of uNOx with long-term outcomes, adjusted for potential confounders of uNOx (Supplemental Table S1). High uNOx was significantly associated with lower risk of all-cause and cardiovascular mortality (HR [95%CI]: 0.84

Table 2
Transplantation characteristics.

	KTR ($n = 698$)	Association with urinary log_2 NOx excretion	
		St. β	*P*
Primary kidney disease, *n* (%)[a]			
Tubular interstitial disease	83 (11.9)	−0.032	0.49
Polycystic renal disease	145 (20.8)	0.005	0.92
Dysplasia and hypoplasia	28 (4.0)	0.026	0.53
Renovascular disease	40 (5.7)	−0.021	0.62
Diabetes mellitus	35 (5.0)	−0.030	0.48
Other/unknown cause	116 (16.6)	Ref.	Ref.
Time between transplantation and baseline, years	5.35 [1.84–12.1]	0.012	0.75
Pre transplantation dialysis time, months	26.5 [9.0–51.0]	−0.077	**0.04**
Living donor transplantation, *n* (%)	237 (34.0)	0.074	0.05
Ischemia times			
Cold ischemia times, h	15.4 [2.9–21.3]	−0.044	0.25
Warm ischemia times, min	40 [33–50]	−0.064	0.09
Rejection before baseline, *n* (%)	186 (26.6)	0.007	0.86
Number of transplantations, *n* (%)			
1	630 (90.3)	Ref.	Ref.
2 or more	68 (9.7)	−0.065	0.09
Immunosuppressive medication			
Prednisolone dosage, mg/24 h	10.0 [7.5–10.0]	0.045	0.23
CNI usage[b], *n* (%)	399 (57.2)	−0.087	**0.02**
Tacrolimus trough level[e]	7.4 ± 2.8	−0.078	**0.04**
Cyclosporine trough level[e]	118 ± 55	−0.026	0.50
Proliferation inhibitor usage[c], *n* (%)	579 (83.0)	0.033	0.39
mTOR inhibitor usage[d], *n* (%)	20 (2.9)	0.054	0.15

Abbreviations: CNI: calcineurin inhibitor; eGFR: estimated glomerular filtration rate; KTR: kidney transplant recipient; mTOR: mammalian target of rapamycin. Data are presented as mean ± standard deviation (SD) for normally distributed variables, median [interquartile range] for variables with a skewed distribution, and nominal data as number (percentages).

[a] Percentages do not add up to 100% because of rounding.

[b] e.g. tacrolimus.

[c] e.g. mycophenolate mofetil.

[d] Sirolimus or everolimus.

[e] For this analysis, only the subjects that used the medication were included. For tacrolimus, there were 106 trough levels available. For cyclosporine, there were 258 trough levels available.

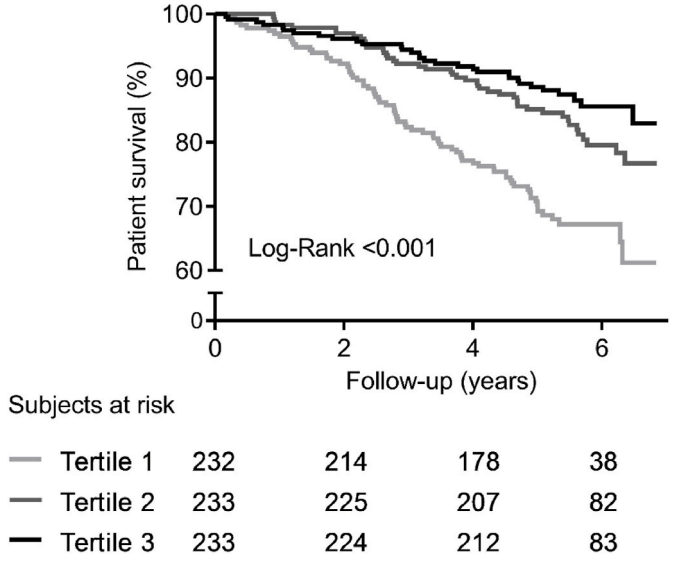

Fig. 1. Kaplan-Meier analysis of the associations of urinary NOx excretion with all-cause mortality in KTR.
Kaplan-Meier curve displayed for survival, with log-rank test P < 0.001 Abbreviations: KTR: kidney transplant recipient.

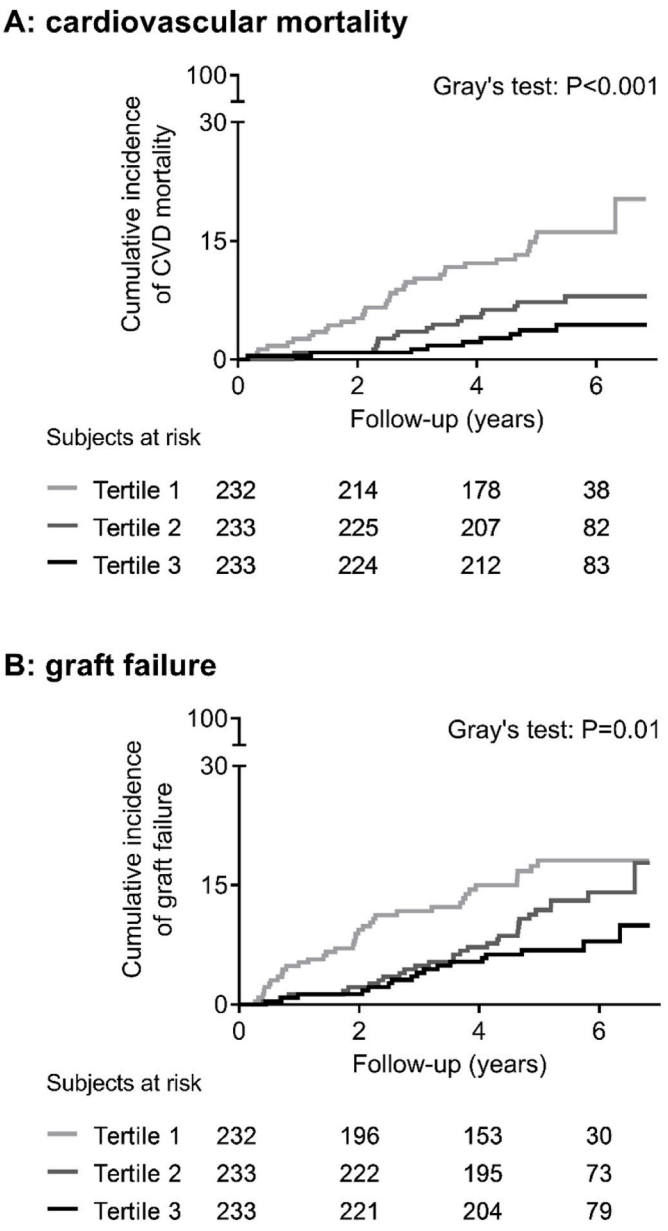

Fig. 2. Cumulative incidence functions of the associations of urinary NOx excretion with CVD mortality and death-censored graft failure in KTR.
Difference between tertiles (according to Gray's test): A. cardiovascular mortality: P < 0.001; B: death-censored graft failure $P = 0.01$. Abbreviations: KTR: kidney transplant recipient; CVD: cardiovascular disease.

[0.76–0.92], $P < 0.001$ and HR [95%CI]: 0.80 [0.69–0.92], $P = 0.002$, respectively). Per doubling of uNOx, the unadjusted risk of premature all-cause mortality decreases by 16% and the risk of premature cardiovascular mortality by 20%. The associations with mortality remained materially unchanged when adjusted for potential confounders (Table S1, models 1–5). We also analyzed associations of uNOx with graft failure and found a protective association of higher uNOx with lower risk of graft failure (HR [95%CI]: 0.81 [0.71–0.91], $P < 0.001$). This association, however, lost significance when corrected for eGFR (Table S1, model 5).

Next to the total uNOx, we performed Cox regression analyses on the NO_2^- and NO_3^- excretions (Table S2). High urinary NO_3^- excretion was significantly associated with lower risk of all-cause and cardiovascular mortality independent of adjustment for all potential confounders (Table S2, models 1–5) (HR [95%CI]: 0.83 [0.76–0.92], $P < 0.001$ for

all-cause mortality and HR [95%CI]: 0.79 [0.69–0.91], $P < 0.001$ for cardiovascular mortality). Similar to NOx, NO_3^- excretion showed a protective effect against graft failure, however, this association was also strongly dependent on baseline eGFR (Table S2, model 5). Urinary NO_2^- excretion was not associated with a lower risk of all-cause and cardiovascular mortality. However, high urinary NO_2^- excretion was associated with lower risk of graft failure, independent of potential confounders including eGFR (Table S2, model 5: HR [95%CI]: 0.60 [0.40–0.92], $P < 0.02$).

We adjusted the associations of uNOx with all-cause mortality, cardiovascular mortality, and graft failure for factors that may lie in the causal pathway of these associations, in order to assess their significance in the associations (Table 3). The association of uNOx with all-cause mortality remained independent of adjustment for biomarkers of inflammation (model 2), cardiovascular disease (model 3), diabetes (model 4), muscle mass (model 5), and urinary ADMA excretion (model 6). The association of uNOx with cardiovascular mortality also remained materially unaffected by adjustment for potential causal pathway factors. The association of uNOx with graft failure was not significant. Urinary NO_3^- excretion showed very similar associations as uNOx and the association of high urinary NO_3^- excretion with lower risk of all-cause and cardiovascular mortality did not materially change when adjusted for factors that potentially lie in the causal pathway of these associations (Table 4). The association of high urinary NO_2^- excretion with lower risk of graft failure remained unaffected after adjustment for the potential causal pathway factors (Table 4). In addition, adjustment for vegetable intake, magnesium intake, urinary sodium excretion, and potassium excretion did not materially change the association between uNOx and mortality (Supplemental Table S7). In Table 5, we present competing risk analyses according to Fine and Gray.[47] The subhazard ratios of cardiovascular mortality and graft failure do not materially differ from the hazard ratios of the regular Cox regression analyses presented in Table 3.

An overview of the most important findings can be found in Fig. 3, were we display the influence of uNOx on different outcomes.

3.5. Cox regression analyses of GC-MS-measured data

In addition to the analyses with HPLC-measured data, we sought to confirm the associations of urinary NOx, NO_2^- and NO_3^- excretions with long-term outcomes using an independent method of analysis, GC-MS. Indeed, outcomes showed very similar results as the HPLC-measured urinary excretions, for both the associations with long-term outcomes and the causal pathways analyses (Tables S3 and S4).

Table 4
Causal path analyses of associations of log_2-transformed urinary NO_2^- with NO_3^- excretions and long-term outcomes.

All-cause mortality	Log_2 NO_3^- excretion		Log_2 NO_2^- excretion[a]	
	HR [95% CI]	*P*	*HR [95% CI]*	*P*
Crude	0.83 [0.76–0.92]	<0.001	0.97 [0.84–1.11]	0.65
Model 1	0.81 [0.72–0.91]	<0.001	0.85 [0.68–1.06]	0.15
Model 2	0.83 [0.72–0.94]	0.004	0.73 [0.54–0.97]	0.03
Model 3	0.82 [0.72–0.94]	0.004	0.80 [0.61–1.04]	0.10
Model 4	0.84 [0.74–0.95]	0.01	0.88 [0.71–1.08]	0.22
Model 5	0.88 [0.77–1.00]	0.05	0.86 [0.70–1.07]	0.18
Model 6	0.85 [0.74–0.96]	0.01	0.86 [0.70–1.07]	0.18
CVD mortality	*HR [95% CI]*	*P*	*HR [95% CI]*	*P*
Crude	0.79 [0.69–0.91]	0.001	1.02 [0.85–1.23]	0.80
Model 1	0.73 [0.61–0.88]	<0.001	0.88 [0.62–1.24]	0.45
Model 2	0.74 [0.60–0.90]	0.003	0.86 [0.60–1.23]	0.41
Model 3	0.74 [0.60–0.92]	0.01	0.92 [0.66–1.29]	0.64
Model 4	0.77 [0.63–0.94]	0.01	0.91 [0.67–1.25]	0.57
Model 5	0.78 [0.64–0.94]	0.01	0.89 [0.63–1.24]	0.48
Model 6	0.78 [0.64–0.95]	0.02	0.89 [0.64–1.24]	0.49
Graft failure	*HR [95% CI]*	*P*	*HR [95% CI]*	*P*
Crude	0.81 [0.71–0.92]	0.001	0.80 [0.59–1.07]	0.13
Model 1	0.91 [0.77–1.06]	0.22	0.60 [0.40–0.92]	0.02
Model 2	0.93 [0.78–1.11]	0.43	0.55 [0.34–0.88]	0.01
Model 3	0.91 [0.77–1.09]	0.31	0.58 [0.38–0.91]	0.02
Model 4	0.93 [0.79–1.10]	0.41	0.59 [0.38–0.91]	0.02
Model 5	0.92 [0.78–1.09]	0.33	0.61 [0.40–0.92]	0.02
Model 6	0.92 [0.78–1.08]	0.29	0.58 [0.37–0.91]	0.02

Model 1	Crude + adjustments for age, sex, weight, proteinuria, total dialysis time, smoking behavior, alcohol intake, and eGFR.
Model 2	Model 1 + CRP and serum albumin
Model 3	Model 1 + NT-proBNP, SBP, total cholesterol, HDL cholesterol, antihypertensive use, and cerebrovascular events before baseline.
Model 4	Model 1 + medical history of diabetes and HbA1c
Model 5	Model 1 + urinary creatinine excretion
Model 6	Model 1 + urinary ADMA excretion

Abbreviations: ADMA: asymmetric dimethylarginine; CI: confidence interval; CNI: calcineurin inhibitor; CRP: c-reactive protein; CVD: cardiovascular disease; eGFR: estimated glomerular filtration rate; HR: hazard ratio; NT-proBNP: N-terminal pro B-type natriuretic peptide; SBP: systolic blood pressure.
Data are presented as hazard ratio (HR) with 95% confidence interval (CI).
[a] A constant of 1 was added to the data before log-transformation.

3.6. Method comparison between HPLC and GC-MS

Whereas excellent agreement was found for the concentrations of NO_3^- in 24-h urine between HPLC and GC-MS, absolute levels of NO_2^- measured by GC-MS were considerably higher than those determined by HPLC for urinary concentrations lower than 1.28 μmol/L. The reason for

Table 3
Causal path analyses of associations of log_2-transformed urinary NOx excretion with long-term outcomes.

	All-cause mortality		CVD mortality		Graft failure	
	HR [95% CI]	P	HR [95% CI]	P	HR [95% CI]	P
Crude	0.84 [0.76–0.92]	<0.001	0.80 [0.69–0.92]	0.002	0.81 [0.71–0.91]	<0.001
Model 1	0.81 [0.72–0.91]	<0.001	0.73 [0.61–0.88]	<0.001	0.89 [0.76–1.05]	0.17
Model 2	0.82 [0.72–0.94]	0.003	0.74 [0.60–0.90]	0.003	0.92 [0.77–1.10]	0.35
Model 3	0.82 [0.72–0.94]	0.004	0.75 [0.60–0.93]	0.01	0.89 [0.75–1.06]	0.19
Model 4	0.84 [0.74–0.95]	0.01	0.76 [0.62–0.93]	0.01	0.92 [0.78–1.09]	0.33
Model 5	0.88 [0.77–1.00]	0.04	0.77 [0.64–0.94]	0.01	0.91 [0.77–1.07]	0.26
Model 6	0.84 [0.74–0.96]	0.01	0.78 [0.64–0.95]	0.01	0.90 [0.77–1.07]	0.23

Model 1	Crude + adjustments for age, sex, weight, proteinuria, total dialysis time, smoking behavior, alcohol intake, and eGFR.
Model 2	Model 1 + CRP and serum albumin
Model 3	Model 1 + NT-proBNP, SBP, total cholesterol, HDL cholesterol, antihypertensive use, and cerebrovascular events before baseline.
Model 4	Model 1 + medical history of diabetes, and HbA1c
Model 5	Model 1 + urinary creatinine excretion
Model 6	Model 1 + urinary ADMA excretion

Abbreviations: ADMA: asymmetric dimethylarginine; CI: confidence interval; CNI: calcineurin inhibitor; CRP: c-reactive protein; CVD: cardiovascular disease; eGFR: estimated glomerular filtration rate; HR: hazard ratio; NT-proBNP: N-terminal pro B-type natriuretic peptide; SBP: systolic blood pressure.
Data are presented as hazard ratio (HR) with 95% confidence interval (CI).

Table 5
Competing risk analyses of associations of log_2-transformed urinary NOx excretion with cardiovascular mortality and graft failure.

	Cardiovascular mortality		Graft failure	
	Subhazard ratio [95% CI]	P	Subhazard ratio [95% CI]	P
Crude	0.81 [0.70–0.92]	0.001	0.82 [0.74–0.92]	0.001
Model 1	0.75 [0.65–0.87]	<0.001	0.93 [0.81–1.07]	0.34
Model 2	0.73 [0.62–0.86]	<0.001	0.94 [0.80–1.11]	0.48
Model 3	0.75 [0.61–0.91]	0.004	0.93 [0.79–1.10]	0.41
Model 4	0.76 [0.65–0.89]	0.001	0.94 [0.81–1.09]	0.40
Model 5	0.78 [0.66–0.91]	0.002	0.95 [0.82–1.10]	0.47
Model 6	0.80 [0.69–0.92]	0.002	0.94 [0.81–1.09]	0.39
Model 1	Crude + adjustments for age, sex, weight, proteinuria, total dialysis time, smoking behavior, alcohol intake, and eGFR.			
Model 2	Model 1 + CRP and serum albumin			
Model 3	Model 1 + NT-proBNP, SBP, total cholesterol, HDL cholesterol, antihypertensive use, and cerebrovascular events before baseline.			
Model 4	Model 1 + medical history of diabetes, and HbA1c			
Model 5	Model 1 + urinary creatinine excretion			
Model 6	Model 1 + urinary ADMA excretion			

Abbreviations: ADMA: asymmetric dimethylarginine; CI: confidence interval; CNI: calcineurin inhibitor; CRP: c-reactive protein; CVD: cardiovascular disease; eGFR: estimated glomerular filtration rate; NT-proBNP: N-terminal pro B-type natriuretic peptide; SBP: systolic blood pressure.
Data are presented as hazard ratio or subhazard ratio with 95% confidence interval (CI).

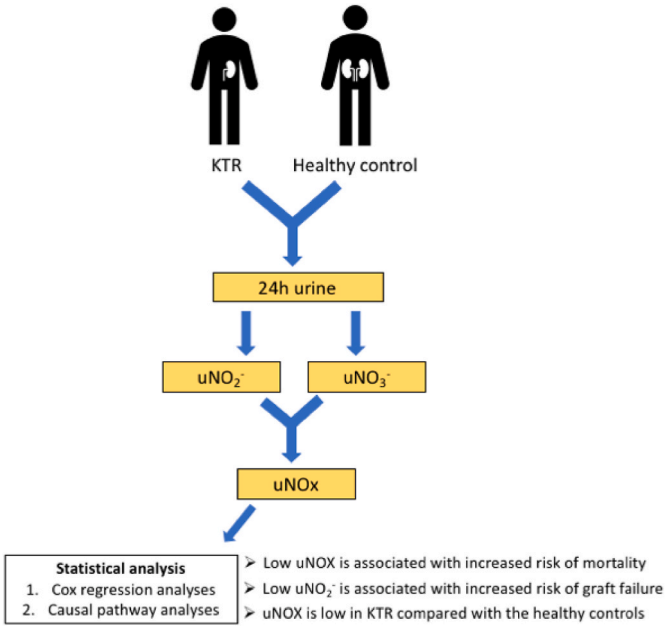

Fig. 3. Overview of the influence of uNOx on transplantation outcome.

this discrepancy remains unknown and warrants further investigation. Passing-Bablok regression analyses for urinary NO_3^- demonstrated both proportional bias (slope 1.40 [95% CI: 1.34; 1.47]) and systematic bias (intercept −3.78 [−4.38; −3.26]) between the HPLC method and the GC-MS method. Passing-Bablok and Bland-Altman plots for urinary NO_3^- analyses are displayed in Fig. S1, Table S6 and Fig. S3, respectively.

Passing-Bablok regression analyses for urinary NO_2^- demonstrated both proportional bias (slope 14.3 [11.1; 19.4]) and systematic bias (intercept −138 [−189; −105]) between the HPLC method and GC-MS method. Passing-Bablok and Bland-Altman plots for urinary NO_2^- analyses are displayed in Fig. S2, Table S6 and Fig. S4, respectively.

3.7. Associations of RXNO with mortality and graft failure

The association of urinary RXNO excretion with all-cause and cardiovascular mortality and graft failure was tested with the same Cox regression analysis as for urinary NOx, NO_2^-, and NO_3^- excretions. Urinary RXNO excretion showed no association with mortality or graft failure (Table S5).

3.8. Associations of plasma levels of NOx with mortality and graft failure

The associations of plasma NOx with all-cause and cardiovascular mortality and graft failure were analyzed using the same Cox regression analyses as for uNOx. There were no significant associations of plasma NOx with mortality or graft failure (Table S8).

4. Discussion

The main finding or our paper is that lower uNOx is associated with decreased patient survival in stable KTR, independent of any adjustment for potential confounders. This is mostly explained by reduced NO_3^- excretion. We found a protective association of uNOx with graft failure, but this was strongly dependent on baseline renal function. However, there is a separate, significant association of NO_2^- excretion with lower risk of premature graft failure. Another finding of this study is the markedly lower NOx excretion and clearance in KTR when compared to healthy controls. To the best of our knowledge, it is the first time that urinary NOx, NO_2^-, and NO_3^- excretions have been reported to have a long-term prospective association with mortality and graft failure in KTR.

Inadequate NO production plays a key role in the development and progression of kidney disease. Experimentally induced chronic NOS inhibition causes systemic and glomerular hypertension, proteinuria, and renal damage [28,48]. Furthermore, experimental models of renal mass reduction, chronic glomerulonephritis, and aging decreased total NOx excretion [49–51]. NO deficiency also develops as a consequence of CKD, with a decreased excretion of NOx in these patients [28,52,53]. Patients with moderate and severe renal failure had significantly lower uNOx excretion when compared to mild renal failure and healthy controls [54], and the same holds true for patients with end stage renal disease that are on dialysis [55]. NO is generated from L-arginine which is converted to L-citrulline by nitric oxide synthase (NOS). In healthy subjects, the majority of the circulating L-arginine pool is synthesized by the kidney and reabsorbed in the proximal tubuli [56–58]. In KTR, renal dysfunction may be the cause of a decreased production and failed tubular reabsorption of L-arginine [59,60]. L-arginine formation is known to be decreased in patients with CKD and remains low directly after renal transplantation [61,62]. Furthermore, an increased expression and activity of arginase and thus use of L-arginine for the production of ornithine and urea (instead of citrulline and NO) may further compromise NO production [63]; to this end, animal experimental studies have unmasked a reciprocal relationship between circulating nitrate levels and arginase expression/L-arginine availability [64]. We found in our study that, compared to healthy donors, KTR have lower uNOx and also lower fractional excretion of NOx, possibly explained by higher tubular reabsorption or lower GFR in KTR. Since plasma NOx values are higher in KTR compared to healthy controls, the lower excretion of NOx could be the result of accumulation in the circulation due to the impaired renal function, which is supported by the observation that post-nephrectomy NO3- and NOx levels increase in healthy donors to those comparable to KTR. These findings may well suggest a decreased production of NOx and possibly an attempt to increase renal retention. The relative NO deficiency in the KTR could also be an effect of CNI usage, since it has been demonstrated that they affect vascular dysfunction and long term administration could decrease endothelium-derived NO synthesis [65]. Although trough levels were available and used in the analysis, data on cumulative or toxic CNI usage

was not available and could therefore not be used in the statistical analysis. Although CNI usage could bias our observations, the addition to the statistical model had no effect on patient and graft outcome. In addition to the potential role of CNI usage on NO deficiency, the role of hypomagnesaemia in renal function and ischemic injury has been suggested via down regulation of eNOS [66]. Although we found a positive association in the current study between magnesium intake and uNOx excretion, it did not materially changed the association of uNOx excretion with transplantation outcome (Supplemental Table S7).

Alternative to the hypothesis of decreased NO production through the L-arginine-NOS pathway, there may also be a decreased NO production through the xanthine oxidase reductase pathway that is believed to reduce nitrite to NO [67]. Previously, it has been suggested that decreased total NO production in CKD patients likely reflects systemic endothelial dysfunction, rather than a decreased NO production in the kidneys alone, because the latter makes only a minor contribution to whole body NO production [48]. In secondary analyses, we studied several mechanistic pathways, among which those that have been associated with cardiovascular disease before, that could explain the association of uNOx and long-term outcomes. However, all associations remained materially unchanged, suggesting that the beneficial association is mediated through (a combination of) different pathways that have yet to be elucidated. Nevertheless, our findings of reduced uNOx in KTR are consistent with the notion that systemic NO production is impaired.

Inhibitors of endogenous NOS, like ADMA, can play a role in decreased (systemic) NO production in KTR. It has been demonstrated that plasma ADMA levels are increased in patients with CKD [68–70] as well as in patients after kidney transplantation [71]. In addition, ADMA is found to be an important risk factor for increased cardiovascular diseases in CKD [72] and is associated with all-cause mortality in KTR [41]. Previously, we have suggested that urinary ADMA excretion may be a relevant marker of ADMA homeostasis and associated with decreased risk of premature mortality [42,43]. The association of urinary NOx with mortality, however, was not explained by differences in ADMA homeostasis as the association remained independent of adjustment for urinary ADMA excretion.

An interesting finding of the current study is the significant and protective association of high NO_2^- excretion with graft failure. This may be a renal protective consequence of (local) NO production of the kidneys that expresses itself in higher NO_2^- excretion in individuals with higher NOS activity. There is no significant difference between urinary NO_2^- excretion in KTR compared to healthy donors. In the current study, we did not find a significant association of urinary NO_2^- excretion with inflammatory parameters or diabetes markers.

The current study shows absence of an association of urinary RXNO excretion with long-term outcomes. Not much is known about the origin of urinary RXNO species; however, it is conceivable that their formation scales not only with total-body NO production but also with the degree of oxidative stress. Urinary nitroso species alone are likely not a reflection of systemic NO-bioavailability, since we also did not find a significant difference in excretion between KTR and healthy individuals despite evidence that KTR have impaired NO production.

As previously mentioned, our data support the notion that inadequate NO production plays a role in the development and progression of kidney disease. We furthermore found that uNOx production is impaired in KTR and that low uNOx is associated with decreased patient survival. In our opinion, associations are too weak to position uNOx as a biomarker for prediction of future development of renal failure or poor patient survival. In addition, there is a discrepancy in the literature regarding associations of NO_2^- and NO_3^- with acute rejection. Albrecht et al. showed a reduction in glomerular eNOS expression combined with an interstitial and glomerular increase in iNOS protein expression in chronic renal transplant failure [18]. Similar results were shown by others for iNOS expression and NOS activity in human renal transplant rejection [74]. On the other hand, another study demonstrated a lack of differentiation between renal transplant rejection or stable graft function by urinary nitrate levels [75]. In all these cohorts, there is a difference in measurement techniques and a difference in how the associations were calculated (i.e. corrected for creatinine or not). Although these analyses focus on acute transplant rejection, whilst ours focus on the long-term survival of patient and graft, it emphasizes the difficulty and limitations of generalizing a single NO measurement as indicator for rejection or graft failure. Nevertheless, the pathways that could underlie our findings deserve further investigation and could reveal new pathophysiological insights in the development of graft failure and poor patient outcome in KTR. Unraveling these could provide as a base for development of new strategies or therapies to improve long-term outcome after kidney transplantation. For example, the reduction of uNOx in allograft rejection may be caused by insufficient availability of the NOS cofactor tetrahydrobiopterin (BH4) since reperfusion injury and inflammatory changes can limit BH4 activity. NOS can become uncoupled after which the heme group in the enzyme reduces oxygen and subsequently releases superoxide instead of nitric oxide. Administration of BH4 may improve NO-availability in conditions associated with inflammation and oxidative stress as shown in the experimental setting [73].

The current study has some limitations: our study design does not allow to establish causality. Another limitation is the single-center set-up and the fact that almost all KTR were of Caucasian origin, which might limit extrapolation to the general population. The comparison made between healthy kidney donors that have not donated yet and transplant recipients demonstrates an interesting difference in NO bioavailability, but the risk factors (e.g. cardiovascular risk profile) and eGFR are not similar between both groups. Although we adjusted for the eGFR and cardiovascular risk profile in the multivariable linear regression models, persistence of residual confounding cannot be excluded and it would be relevant to also compare our results to a population with similar risk factors. In addition, the multiple statistical tests that were performed on the same cohort could have induced potential false positive results. The healthy controls had an interval between donor surgery and the follow-up visit with a median time of 1.64 months. Although surgery is a potential oxidative stress inducing event which may interfere with the NOS levels, previous studies on levels of the NO precursors arginine and citrulline showed a trend towards normalization within days after surgery [76]. We believe, therefore, that one and a half months after surgery is an appropriate time interval for measurements to take place. Strengths of this study are the large population size, the use of 24-h urine samples to determine NOx excretion and extensive data collection that allowed adjustment for potential confounders and a comparison between KTR and healthy subjects. Moreover, we measured NOx using two independent analytical techniques. Although the Passing-Bablok and Bland-Altman plots reveals a discrepancy between the results of both methods, the association of NOx, measured with GC-MS, with all-cause mortality, cardiovascular mortality, and graft failure was consistent with the HPLC outcomes. This shows that our findings of the association of uNOx with transplantation outcome are not related to the peculiarity of one specific analytical technique.

5. Conclusion

Reduced uNOx in KTR is associated with an adverse cardiovascular risk profile and higher all-cause mortality in KTR. Further studies should to be conducted to determine the mechanism behind this finding.

Data availability

Data is available on request from the corresponding author.

Authors' contributions

Hanno Maassen and M. Yusof Said contributed equally to this work.

Declaration of competing interest

The authors of this manuscript have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2022.05.005.

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