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ORIGINAL ARTICLE

High urinary homoarginine excretion is associated with low rates of all-cause mortality and graft failure in renal transplant recipients

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Abstract Renal transplant recipients (RTR) have an increased cardiovascular risk profile. Low levels of circulating homoarginine (hArg) are a novel risk factor for mortality and the progression of atherosclerosis. The kidney is known as a major source of hArg, suggesting that urinary excretion of hArg (UhArg) might be associated with mortality and graft failure in RTR. hArg was quantified by mass spectrometry in 24-h urine samples of 704 RTR (functioning graft ≥1 year) and 103 healthy subjects. UhArg determinants were identified with multivariable linear regression models. Associations of UhArg with all-cause mortality and graft failure were assessed using multivariable Cox regression analyses. UhArg excretion was significantly lower in RTR compared to healthy controls [1.62 (1.09–2.61) vs. 2.46 (1.65–4.06) μmol/24 h, $P < 0.001$]. In multivariable linear regression models, body surface area, diastolic blood pressure, eGFR, pre-emptive transplantation, serum albumin, albuminuria, urinary excretion of urea and uric acid and use of sirolimus were positively associated with UhArg, while donor age and serum phosphate were inversely associated (model $R^2 = 0.43$). During follow-up for 3.1 (2.7–3.9) years, 83 (12 %) patients died and 45 (7 %) developed graft failure. UhArg was inversely associated with all-cause mortality [hazard risk (HR) 0.52 (95 % CI 0.40–0.66), $P < 0.001$] and graft failure [HR 0.58 (0.42–0.81), $P = 0.001$]. These associations remained independent of potential confounders. High UhArg levels are associated with reduced all-cause mortality and graft failure in RTR. Kidney-derived hArg is likely to be of particular importance for proper maintenance of cardiovascular and renal systems.

Keywords Cardiovascular risk · Transplantation · Graft survival · Kidney

Abbreviations

ADMA	Asymmetric dimethylarginine
BSA	Body surface area
CI	Confidence interval
DBP	Diastolic blood pressure
eGFR	Estimated glomerular filtration rate
FFQ	Food frequency questionnaire
hArg	Homoarginine
HbA_{1c}	Glycated hemoglobin
HDL	High-density lipoprotein
HLA	Human leukocyte antigen
HR	Hazard risk
hsCRP	High-sensitivity C-reactive protein
IQR	Interquartile range
KTx	Kidney transplantation
LDL	Low-density lipoprotein
LOD	Lower limit of detection
NO	Nitric oxide
NOS	Nitric oxide synthase

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NT-pro-BNP	N-terminal pro-hormone of brain natriuretic peptide
PTH	Parathyroid hormone
QC	Quality control
RTR	Renal transplant recipients
SDMA	Symmetric dimethylarginine
UhArg	Urinary homoarginine

Introduction

Nitric oxide (NO) is a signaling molecule which plays numerous important roles in the renal and cardiovascular systems (Moncada and Higgs 1993; Passauer et al. 2005; Zoccali 2006; O'Connor and Cowley 2010). NO is produced from L-arginine (Arg) by the catalytic action of constitutive and inducible NO synthase (NOS) isoforms virtually in all types of cell. Although Arg is abundantly involved in many pathways (Wu et al. 2009), its function as a precursor of NO is of particular importance. Homoarginine (hArg) is an Arg homolog; it contains an additional methylene group (CH_2) in its main chain. hArg may also be converted by NOS isoforms to NO (Hecker et al. 1991; Moali et al. 1998; Bretscher et al. 2003), thus contributing to NO-related functions.

Low circulating hArg concentrations emerged as a crucial risk factor for cardiovascular diseases and mortality (März et al. 2010; Pilz et al. 2011a, b, 2014; Drechsler et al. 2011; Atzler et al. 2013; Kayacelebi et al. 2014a). Higher circulating hArg concentrations were measured in pregnancy (Valtonen et al. 2008; Khalil et al. 2013) and found to relate to increased flow-mediated vasodilatation (Valtonen et al. 2008). These observations suggest that circulating hArg reflects endothelial function in the cardiovascular system, presumably by modulating NO synthesis and/or bioavailability. In patients with impaired renal function, low circulating hArg concentration emerged as a cardiovascular risk factor (Tomaschitz et al. 2014), suggesting that the kidney may play a decisive role in hArg's function in the renal and cardiovascular systems.

Renal transplantation is the preferred treatment option for individuals with end-stage renal disease. Many risk factors including cardiovascular disease (Kasiske 2000; Ojo et al. 2000; see also Smith et al. 2006), low-grade inflammation (Israni et al. 2013) and impaired graft function (Mallon et al. 2013) are highly prevalent after renal transplantation. Improvement of patient and graft survival demands deeper insights into the complex mechanisms underlying post-transplant morbidity and mortality.

hArg is excreted in the urine (Marescau et al. 1997; Kayacelebi et al. 2014b), yet the significance of urinary hArg (UhArg) in the renal and cardiovascular systems is unknown. Therefore, the aim was to evaluate the role of UhArg in cardiovascular risk and outcome after kidney transplantation (KTx). For this, we measured the concentration of hArg in 24-h collected urine samples of a large cohort of stable RTR and analyzed the relationship of 24-h UhArg excretion with all-cause mortality and graft failure in this cohort.

Methods

Design and study population

We conducted cross-sectional and longitudinal analyses in a single-center RTR cohort. All stable, adult RTR (age $\geq$18 years, $n = 817$) with a functioning graft for at least 1 year, who visited the outpatient department of the University Medical Center Groningen (UMCG), the Netherlands, between November 2008 and June 2011 were invited to participate in our study. A total of 707 RTR signed for informed consent and participated in the present study. All transplantations were conducted in our center. Twenty-four hours urine of 704 RTR in total was collected for analysis of hArg and other parameters. Further details of the study population have been published previously (van den Berg et al. 2012a, 2014). We also included 103 healthy control subjects. The Institutional Review Board approved the study protocol (METc 2008/186), which was in adherence to the Declaration of Helsinki. The primary outcome measures of the study were all-cause mortality and death-censored graft failure; the latter was defined as restart of dialysis or re-transplantation. Participants were followed-up till the end of May 2013.

Clinical and biochemical analyses, assessment of dietary intake

Prior to their visit to the outpatient clinic, participants collected 24-h urine in chlorhexidine-containing containers to prevent bacterial growth. All RTR visited the outpatient clinic in the morning, after an overnight fasting period. As previously described (van den Berg et al. 2012b), a strict protocol was followed to measure both blood pressure (mmHg) and heart rate with a semi-automatic device (Dinamap® 1846, Critikon, Tampa, FL, USA).

Body weight and height were measured and BSA was calculated. Blood was drawn and subsequently venous blood gas analyses were performed spectrophotometrically. Plasma and urinary concentrations of electrolytes including phosphate, creatinine, albumin, uric acid, urea, serum cholesterol, HbA_{1c}, NT-pro-BNP and hsCRP were measured using routine clinical laboratory methods. Urea excretion was used as a measure of protein intake. Serum calcium was corrected for hypoalbuminemia [if <40 g/L; corrected

calcium = serum calcium (mM) + 0.02 × (40 − serum albumin (g/L))]. As a measure for renal function, the estimated glomerular filtration rate (eGFR) was calculated using the CKD Epidemiology Collaboration (CKD-EPI) equation (Levey et al. 2009).

Participants registered their dietary intake using a validated semi-quantitative FFQ. More detailed information about the procedure has been published elsewhere (van den Berg et al. 2013).

In brief, intake was recorded in times per day, week or month, depending on the food item. A trained researcher checked inconsistencies and validity of the food frequency questionnaire (FFQ) for RTR assessed by comparing excretion of several urinary components with intake of e.g. sodium, potassium and proteins. Dietary data was converted into daily nutrient intake using the Dutch food composition table of 2006 (The Hague 2006).

All information on participants' medical history, medication use and health status was obtained from patient records. Relevant transplant information was extracted from the UMCG renal transplant database. Information on smoking behavior was obtained by using a questionnaire, with classification as current, former or never smokers.

The concentration of hArg was determined in 10-µL aliquots of urine samples by stable-isotope dilution gas chromatography-tandem mass spectrometry (GC–MS/MS) as described elsewhere (Kayacelebi et al. 2014b). Urine samples were analyzed within 16 runs along with quality control (QC) samples which were analyzed in duplicate. A pooled urine sample of a healthy volunteer served as QC sample. QC1 samples were analyzed without addition of synthetic hArg. QC2 samples were spiked with 1 µM hArg. The hArg concentration in the QC1 and QC2 samples was determined to be 0.551 (0.039) µM and 1.554 (0.111) µM [mean (SD)], respectively. The mean imprecision (relative standard deviation) was each 7.1 % in the QC1 and QC2 samples. The concentration of hArg in the QC2 samples was determined with an accuracy (recovery, %) of 100.3 (9.1) %. These data indicate that the hArg concentration in the patients' urine samples was determined with high accuracy and precision.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Data in text and tables are presented as mean (SD) or median [interquartile range (IQR)], unless stated otherwise. Histograms and probability plots were displayed to test the distribution of all parameters. For the same purpose, the Kolmogorov–Smirnov test was performed. Skewed data were normalized by logarithmic transformation, for instance for UhArg, hsCRP, triglycerides, albuminuria.

To study possible associations of UhArg excretion in our RTR, the study population was subdivided into tertiles. For normally distributed continuous data, ANOVA was used. The Kruskal–Wallis test was performed for non-normally distributed data and the Chi square test for nominal data. We investigated possible associations of UhArg excretion with mortality and death-censored graft failure. Kaplan–Meier analysis was performed and significance was tested using the log-rank test.

The Kruskal–Wallis test was performed for non-normally distributed data and the Chi square test for nominal data. First we performed univariable linear regression analyses. All variables that appeared to be significantly associated with UhArg excretion in the univariable regression analysis were included in the multivariable linear regression analysis and stepwise excluded using backward selection ($P_{out} > 0.05$).

Cox regression analysis was conducted for both, all-cause mortality and graft failure. Models were constructed with inclusion of all parameters that significantly associated with UhArg excretion in multivariable analysis ($P < 0.05$). Thereby, we divided these parameters into two groups, i.e., one group for potential determinants and another group for potential consequences of UhArg excretion. First, crude Cox analysis was performed (model 1), followed by adjustment for age and gender (model 2). Additionally, significant determinants of UhArg excretion were added (BSA, pre-emptive KTx, age of the donor, and use of sirolimus; model 3). In model 4, excretion of urea as a marker of total protein intake was included. Parameters that significantly associated with UhArg, but were more likely to be consequences of UhArg excretion rather than determinants, were added (model 5: DBP, eGFR, serum phosphate, serum albumin, albuminuria and uric acid excretion). Additional adjustment was performed for BMI, hemoglobin, NT-proBNP and CRP in Cox regression analysis. These potential confounders were added to model 4, thus resulting in model 6.

HR values are presented per standard deviation (SD) increase. Within all statistical analyses, a two-sided P value less than 0.05 was considered statistically significant.

Results

Cohort characteristics

hArg excretion in the urine (µmol/24 h) had a median value of 1.62 and an IQR of 1.09–2.61, and was log-transformed to meet normal distribution criteria (Fig. 1). Baseline patient characteristics are displayed per tertile in

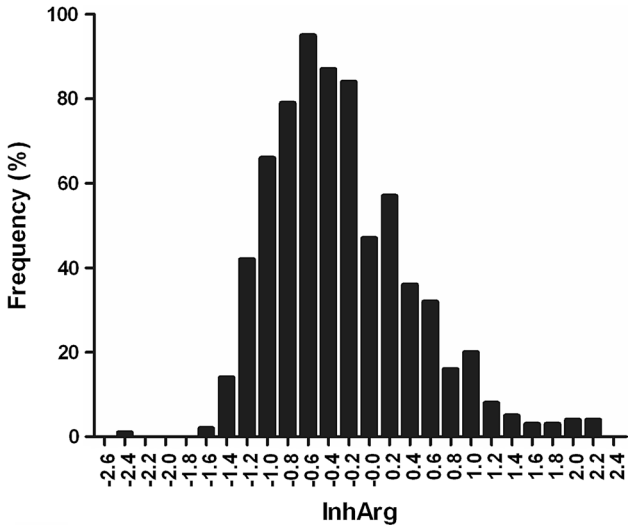

Fig. 1 *Histogram* of urinary homoarginine excretion. After log-transformation, urinary excretion of hArg measured in 704 renal transplant recipients was normally distributed

Table 1. Mean (SD) age was 53.0 (12.8) years, 400 RTR (57 %) were male. Time between renal transplant procedure and baseline measurement was 5.4 (1.9–12.1) years. RTR with the highest UhArg excretion were younger and had significant higher body surface area values. Diastolic blood pressure (DBP) was higher in RTR with the highest hArg excretion. In RTR, transplant vintage was lower in the highest tertile of UhArg excretion. More RTR received a renal transplantation from a living donor and less RTR received dialysis prior to transplantation when compared to the lowest UhArg tertile. Renal function, expressed as eGFR, and hemoglobin were significantly higher in patients with high UhArg excretion, whereas serum phosphate, uric acid, HDL-cholesterol and NT-pro-BNP levels were lower. Excretion of uric acid and urea was significantly higher within the highest UhArg tertiles. Vitamin D supplements were less frequently used in patients within the highest UhArg tertiles, whereas use of proliferation inhibitors was significantly higher.

UhArg excretion was significantly lower in the 704 RTR than in the 103 healthy controls [1.62 (1.09–2.61) vs. 2.46 (1.65–4.06) µmol/24 h, $P < 0.001$]. With regard to dietary habits, there were no differences between RTR and controls after correction for differences in caloric intake (2175 ± 637 kcal in RTR vs. 2309 ± 743 kcal in controls, $P = 0.02$). RTR had a significantly higher systolic blood pressure compared to controls (136 vs. 125 mmHg, $P < 0.001$) and DBP (83 vs. 75 mmHg, $P < 0.001$). eGFR was significantly ($P < 0.001$) lower in RTR [52.2 (20.1)] compared to controls [93 (13)]. Urinary albumin excretion was significantly higher in RTR than in controls [41 (11–179) vs. 5.5 (3.1–8.3), $P < 0.001$]. NT-pro-BNP, HbA_{1c}, triglycerides and hsCRP were all significantly higher in RTR compared to healthy controls ($P < 0.001$).

An overview of tested associations of UhArg excretion with different parameters in univariable and multivariable regression analyses is given in Table 2. BSA, DBP, preemptive kidney transplantation, donor age, eGFR, serum phosphate, serum albumin, albuminuria, urinary excretion of urea and uric acid, and use of sirolimus showed the strongest associations with UhArg (model $R^2 = 0.43$). Furthermore, UhArg was positively associated with intake of lysine ($P = 0.001$, $R^2 = 0.018$), a precursor of hArg, but did not show significant association with plant, animal or total protein intake (data not shown).

Association of urinary homoarginine with mortality and graft failure

During a follow-up period of 3.1 (2.7–3.9) years, 83 (12 %) RTR died in total. In the highest gender-stratified tertile of UhArg, 12 out of 234 patients (5 %) died; in the middle tertile, 36 out of 238 (15 %) patients died; and in the lowest tertile, 35 out of 232 (15 %) patients died (log-rank test, $P = 0.001$, Fig. 2).

Cox regression analyses for the association with all-cause mortality are shown in Table 3. The crude Cox regression analysis (model 1) demonstrates that higher levels of UhArg are significantly associated with a lower risk of mortality (HR 0.52; 95 % CI 0.40–0.66, $P < 0.001$). This significant association held through after adjustment for age and gender (model 2; HR 0.52; 95 % CI 0.40–0.67, $P < 0.001$), as well as after adjustment for significant determinants of UhArg excretion (model 3; HR 0.50; 95 % CI 0.38–0.66, $P < 0.001$). Upon consideration of urea excretion, to adjust for total protein intake, higher UhArg remained significantly associated with lower mortality (model 4; HR 0.63; 95 % CI 0.47–0.85, $P = 0.002$). After adjusting for potential consequences of UhArg, UhArg excretion remained significantly associated with all-cause mortality (model 5; HR 0.70; 95 % CI 0.50–0.98, $P = 0.04$). In model 6, UhArg remained independently associated with mortality (adjusted HR 0.66; 95 % CI 0.48–0.91, $P = 0.01$).

In total, 45 out of 704 (7 %) RTR developed graft failure. In the highest UhArg excretion tertile, the lowest frequency of graft failure was observed, i.e., 6 out of 234 (3 %) RTR suffered from graft failure, while in the middle tertile of UhArg excretion, the highest risk of developing graft failure, with 23 out of 238 (10 %) RTR developing graft failure, and 16 out of 232 (7 %) patients developed graft failure in the lowest tertile of UhArg (log-rank test, $P = 0.004$, Fig. 3).

Table 1 Renal transplant recipients characteristics presented as tertiles of homoarginine urinary excretion

Sex-stratified tertiles of homoarginine					
	Overall ($n = 704$)	Tertile 1 ($n = 232$)	Tertile 2 ($n = 238$)	Tertile 3 ($n = 234$)	*P* value
Homoarginine excretion (μmol/24 h)	1.62 (1.09–2.61)	1.01 (0.78–1.28)	1.63 (1.24–2.04)	3.32 (2.29–4.64)	**<0.001**
Demographics					
Age (years)	53.0 (12.8)	56.6 (11.2)	52.4 (12.9)	50.0 (13.5)	**<0.001**
Current smoker, *n* (%)	83 (13)	28 (13)	33 (15)	22 (10)	0.34
Current diabetes, *n* (%)	171 (24)	62 (27)	56 (23)	53 (23)	0.55
BSA (m^2)	1.94 (0.22)	1.89 (0.21)	1.93 (0.22)	2.00 (0.22)	**<0.001**
Systolic blood pressure (mmHg)	136 (18)	136 (19)	136 (17)	135 (17)	0.82
Diastolic blood pressure (mmHg)	83 (11)	81 (11)	83 (10)	83 (12)	**0.03**
Heart rate (bpm)	68.6 (11.9)	68.6 (12.5)	69.1 (11.9)	68.0 (11.4)	0.65
Renal transplantation					
Transplant vintage (years)	5.4 (1.9–12.1)	7.1 (2.9–14.8)	5.0 (1.5–10.8)	5.0 (1.7–10.0)	**0.001**
Living donor, *n* (%)	236 (34)	49 (21)	96 (41)	91 (40)	**<0.001**
Pre-emptive KTx, *n* (%)	112 (16)	20 (9)	44 (19)	48 (21)	**0.001**
HLA mismatches, *n*	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–3)	0.26
Age donor (years)	42.8 (15.5)	43.6 (15.8)	43.3 (15.5)	41.5 (15.4)	0.32
Acute rejection, *n* (%)	188 (27)	73 (32)	64 (27)	51 (22)	0.06
Laboratory measurements					
Hemoglobin (mM)	8.2 (1.1)	8.1 (1.0)	8.1 (1.1)	8.4 (1.1)	**<0.001**
HbA_{1C} (%)	6.0 (0.8)	6.0 (0.7)	6.0 (0.9)	6.0 (0.9)	0.84
eGFR, CKD-EPI (mL/min/1.73 m^2)	52.2 (20.1)	46.8 (17.8)	50.8 (19.7)	59.0 (20.9)	**<0.001**
Corrected calcium (mM)	2.34 (0.15)	2.35 (0.14)	2.34 (0.15)	2.34 (0.15)	0.61
Phosphate (mM)	0.96 (0.21)	1.02 (0.23)	0.97 (0.20)	0.91 (0.20)	**<0.001**
Magnesium (mM)	0.95 (0.12)	0.97 (0.12)	0.94 (0.12)	0.96 (0.12)	0.07
PTH (pM)	8.9 (5.9–14.7)	8.7 (5.9–15.1)	8.5 (5.3–15.4)	9.3 (6.3–14.2)	0.69
Venous pH	7.37 (0.04)	7.37 (0.04)	7.37 (0.05)	7.37 (0.04)	0.94
Venous HCO_3^- (mM)	24.6 (3.1)	25.0 (3.1)	24.3 (3.2)	24.6 (2.9)	0.07
Uric acid (mM)	0.43 (0.11)	0.45 (0.12)	0.43 (0.11)	0.42 (0.11)	**0.004**
hsCRP (mg/L)	1.6 (0.7–4.5)	1.6 (0.8–4.7)	1.4 (0.5–4.5)	1.9 (0.8–4.4)	0.07
Albumin (g/L)	43.0 (3.0)	42.8 (2.9)	42.8 (3.2)	43.4 (2.8)	0.05
Alkaline phosphatase (U/L)	67 (54–83)	66 (51–79)	67 (54–86)	68 (55–82)	0.20
Total cholesterol (mM)	5.0 (4.3–5.8)	5.1 (4.3–5.8)	5.1 (4.3–5.8)	5.0 (4.4–5.7)	0.97
HDL cholesterol (mM)	1.3 (1.1–1.6)	1.3 (1.1–1.8)	1.3 (1.0–1.6)	1.3 (1.0–1.6)	**0.02**
LDL cholesterol (mM)	2.9 (2.3–3.5)	2.8 (2.2–3.4)	2.9 (2.4–3.5)	2.9 (2.4–3.7)	0.35
Triglycerides (mM)	1.68 (1.25–2.30)	1.65 (1.28–2.35)	1.70 (1.25–2.38)	1.65 (1.21–2.22)	0.79
NT-pro-BNP (ng/L)	254 (109–621)	363 (129–974)	269 (126–568)	164 (70–398)	**<0.001**
Albuminuria (mg/24 h)	41 (11–179)	31 (8–131)	42 (12–179)	46 (13–213)	0.20
Uric acid excretion (mmol/24 h)	2.58 (0.98)	2.17 (0.86)	2.60 (0.87)	2.99 (1.03)	**<0.001**
Urea excretion (mmol/24 h)	389 (114)	320 (94)	408 (102)	438 (113)	**<0.001**
Medication					
Anti-hypertensives, *n* (%)	622 (88)	210 (91)	206 (86)	206 (88)	0.34
Statins, *n* (%)	371 (53)	132 (57)	133 (57)	106 (46)	0.05
Calcium supplements, *n* (%)	151 (21)	57 (25)	55 (23)	39 (17)	0.09
Vitamin D supplements, *n* (%)	174 (25)	73 (32)	53 (22)	48 (21)	**0.01**
Vitamin K antagonists, *n* (%)	78 (11)	33 (14)	28 (12)	17 (7)	0.05
Prednisone (mg/day)	10 (7.5–10)	10 (7.5–10)	10 (7.5–10)	10 (7.5–10)	0.19
Calcineurin inhibitors, *n* (%)	405 (57)	140 (60)	142 (59)	123 (53)	0.18
Proliferation inhibitor, *n* (%)	585 (83)	177 (76)	207 (87)	201 (86)	**0.004**

Table 1 continued

Sex-stratified tertiles of homoarginine					
	Overall ($n = 704$)	Tertile 1 ($n = 232$)	Tertile 2 ($n = 238$)	Tertile 3 ($n = 234$)	P value
Sirolimus, n (%)	13 (2)	2 (1)	4 (2)	7 (3)	0.21

Data are presented as mean (SD), percentage or median (IQR)

Statistical analysis was performed using ANOVA, Kruskal–Wallis or χ^2 test when appropriate

Bold indicates statistical significance (P value < 0.05)

Cox regression analyses revealed that higher UhArg is associated with a decreased risk of graft failure (crude model; HR 0.58; 95 % CI 0.42–0.81, $P = 0.001$, Table 4). This association remained statistically significant after adjusting for age and gender (model 2; HR 0.54; 95 % CI 0.39–0.76, $P < 0.001$). After inclusion of BSA, pre-emptive KTx, age of the donor and use of sirolimus in the regression analysis, the association of UhArg with graft survival was still present (model 3: HR 0.60; 95 % CI 0.41–0.88, $P = 0.009$). However, additional adjustment for urea excretion (model 4; HR 0.75; 95 % CI 0.50–1.12, $P = 0.16$) and DBP, eGFR, serum phosphate, serum albumin, albuminuria and uric acid excretion (model 5; HR 0.79; 95 % CI 0.45–1.36, $P = 0.39$) revealed loss of statistical significance for the association of UhArg excretion with graft survival. As expected, adding BMI, hemoglobin, NT-proBNP and CRP to Cox regression model 4 (i.e., model 6) did not materially change the results from model 4, namely that UhArg was no longer significantly associated with graft failure [0.96 (0.61–1.51), $P = 0.85$].

In view of a potential interplay between hArg and parathyroid hormone (PTH) (Tomaschitz et al. 2015), we looked for an interaction between UhArg and PTH levels for predicting mortality risk and risk of graft failure. We found significant effect modification for the association between UhArg and mortality by PTH (P interaction = 0.02). We subsequently split the cohort to subgroups above and below the median PTH level (8.9 pM). Given the relatively low numbers of events ($n = 53$ vs. $n = 30$, respectively), we performed Cox regression analysis adjusted for age and gender in both subgroups. In patients with PTH levels above the median, UhArg was strongly associated with mortality (HR 0.44; 95 % CI 0.31–0.63, $P < 0.001$). In patients with PTH levels below the median, UhArg was less strongly, but still significantly associated with mortality [0.60 (0.39–0.93), $P = 0.02$]. These findings suggest that the association between UhArg and mortality is influenced by PTH, with the association being strongest in subjects with PTH levels above 8.9 pM. We found no evidence for interaction by PTH regarding the association between UhArg and graft failure ($P = 0.82$).

Discussion

The most important finding of the present study is that high UhArg excretion is associated with reduced all-cause mortality and graft failure in stable RTR. For all-cause mortality, these associations remained significant after adjustment for potential confounders and for factors that could share the causal pathway of UhArg. This is the first study linking UhArg excretion to patient and graft survival.

One of the first studies on guanidine compounds including hArg reported mean circulating hArg levels of 1.5 (females)–2 (males) µM in 33 healthy adults, with UhArg ranging between the lower limit of detection (LLOD, not specified) of the method used (i.e., HPLC) and 12 µmol/24 h (Marescau et al. 1997). In 19 healthy subjects, we measured (by GC–MS/MS) mean circulating and excretory hArg concentrations of 1.87 µM and 0.18 µmol/mmol creatinine (Kayacelebi et al. 2014b). The latter corresponds to about 2 µmol hArg/24 h in males and 3 µmol hArg/24 h in females. These UhArg levels are comparable to those we measured in the present study, but are considerably lower than those reported by others (Marescau et al. 1997). In non-dialyzed patients with chronic kidney disease (CKD), this group reported lower UhArg levels compared to controls, which decreased with decreasing creatinine clearance to values up to LLOD 2.3 µmol/24 h (Marescau et al. 1997), supporting our observations albeit not on a quantitative basis. It is interesting to note that other circulating guanidine compounds, namely asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), behaved oppositely to hArg in CKD. Thus, the serum concentrations of ADMA and SDMA increased with decreasing creatinine clearance, while the serum hArg concentration decreased (Marescau et al. 1997). This remarkable difference may indicate that in humans, the kidney plays an important role in the biosynthesis and reabsorption of hArg, and in the elimination of SDMA and ADMA (Frenay et al. 2015).

Low circulating concentrations of hArg are a novel risk factor for cardiovascular diseases (Pilz et al. 2011b, 2014; Atzler et al. 2014). In patients with CKD, plasma levels of hArg are generally lower compared to those in healthy

Table 2 Associations of homoarginine urinary excretion with clinical parameters in RTR

	Homoarginine			
	Univariable		Multivariable	
	St. β	*P* value	St. β	*P* value
Demographics				
Age (years)	−0.146	**<0.001**		
Male gender	−0.210	**<0.001**		
Current smoker	−0.021	0.58		
Current diabetes	−0.050	0.18		
BSA	0.332	**<0.001**	0.206	**<0.001**
SBP (mmHg)	0.026	0.50		
DBP (mmHg)	0.125	**0.001**	0.071	**0.03**
Heart rate (bpm)	−0.053	0.17		
Renal transplantation				
Transplant vintage (years)	−0.123	**0.001**		
Living donor	0.106	**0.005**		
Pre-emptive KTx	0.078	**0.04**	0.082	**0.01**
HLA mismatches	0.042	0.28		
Age donor (years)	−0.102	**0.008**	−0.084	**0.01**
Acute rejection	-0.072	0.06		
Laboratory measurements				
Hemoglobin (mM)	0.310	**<0.001**		
HbA_{1C} (%)	−0.005	0.91		
eGFR, CKD-EPI (mL/min/1.73 m^2)	0.301	**<0.001**	0.136	**0.003**
Corrected calcium (mM)	−0.016	0.66		
Phosphate (mM)	−0.252	**<0.001**	−0.115	**0.001**
Magnesium (mM)	0.015	0.69		
PTH (pM)	0.004	0.92		
Venous pH	0.064	0.10		
Venous HCO_3^- (mM)	0.039	0.32		
Uric acid (mM)	−0.183	**<0.001**		
hsCRP (mg/L)	−0.048	0.22		
Albumin (g/L)	0.176	**<0.001**	0.072	**0.04**
Alkaline phosphatase (U/L)	0.000	0.99		
Total cholesterol (mM)	−0.013	0.74		
HDL cholesterol (mM)	−0.054	0.23		
LDL cholesterol (mM)	0.021	0.58		
Triglycerides (mM)	−0.027	0.47		
NT-pro-BNP (ng/L)	−0.288	**<0.001**		
Albuminuria (mg/24 h)	0.084	**0.03**	0.160	**<0.001**
Uric acid (mmol/24 h)	0.556	**<0.001**	0.366	**<0.001**
Urea excretion (mmol/24 h)	0.383	**<0.001**	0.180	**<0.001**
Medication				
Anti-hypertensives	0.013	0.73		
Statins	−0.035	0.35		
Calcium supplements	−0.137	**<0.001**		
Vitamin D supplements	−0.112	**0.003**		
Vitamin K antagonists	−0.080	**0.03**		
Prednisone (mg/day)	0.025	0.51		
Calcineurin inihibitors	−0.050	0.18		
Proliferation inhibitor	0.072	0.06		
Sirolimus	0.100	**0.01**	0.066	**0.04**

Data are presented as standardized beta coefficient (β) with corresponding *P* value

Bold indicates statistical significance (*P* value < 0.05)

subjects and dependent on the degree of kidney failure. Furthermore, it was demonstrated that plasma hArg is also significantly lower in patients with progressing CKD, suggesting that low circulating levels of hArg may be useful in the prediction of disease progression (Drechsler et al. 2013). In dialysis patients, lower plasma hArg levels were associated with a significantly higher risk of dying compared to those patients with higher plasma hArg levels (Pilz et al. 2014). Serum hArg was shown to be inversely associated with NT-pro-BNP levels in patients at cardiovascular risk with preserved left ventricular ejection fraction (Pilz et al. 2011a). This is in line with our results which indicate an inverse association in the univariable analysis between UhArg excretion and serum NT-pro-BNP in RTR while others found an association between hsCRP (Tomaschitz et al. 2014); UhArg excretion in our study was not significantly associated with serum hsCRP.

Furthermore, low serum hArg concentration was related to a decreased creatinine-based eGFR, adverse cardiovascular events and death due to heart failure in a large cohort of patients who were hospitalized to undergo coronary angiography, having a normal or slightly decreased eGFR (Tomaschitz et al. 2014). These associations were more pronounced in patients with low serum hArg levels and an eGFR below 60 mL/min per 1.73 m^2. Additionally, a significant association between low circulating levels of hArg and sudden cardiac death or death due to cardiac failure was found in patients on hemodialysis (Atzler et al. 2013). Similar associations were also found in patients with CKD (Ravani et al. 2013), with plasma hArg predicting the risk of progression to dialysis and death in CKD. Circulating hArg concentration was also positively related to eGFR, independent of other relevant risk factors (Ravani et al. 2013). A positive association between eGFR and UhArg was also found in our study. Interestingly, UhArg excretion was demonstrated to be an independent associate of all-cause mortality, even after correction for eGFR, which by itself, is a strong predictor of mortality.

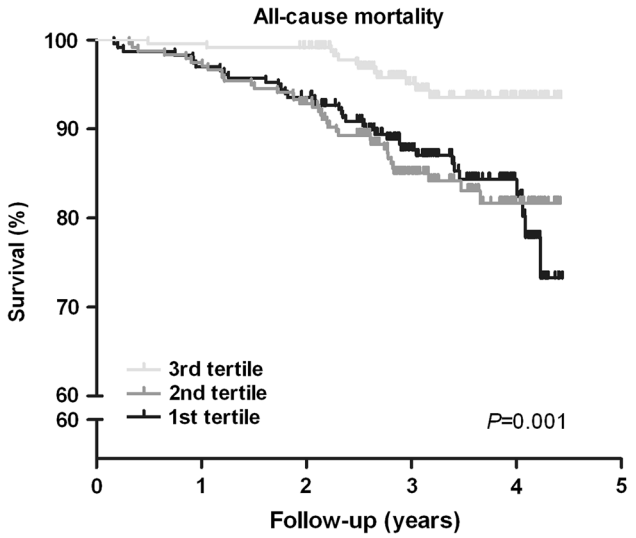

Fig. 2 Kaplan–Meier plot of the association of homoarginine excretion with all-cause mortality in RTR. Higher excretion of hArg is associated with significantly survival benefit in renal transplant recipients. Kaplan–Meier curve displayed for patient survival, with log-rank test *P* value 0.001

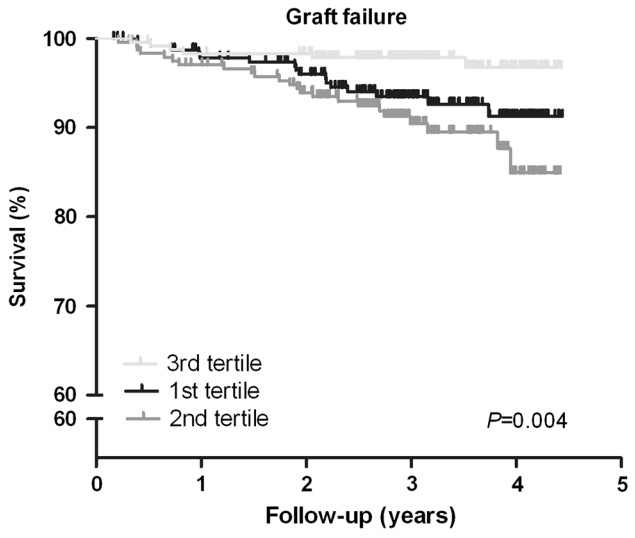

Fig. 3 Kaplan–Meier plot of the association of homoarginine excretion with graft failure in RTR. Higher excretion of hArg is associated with significantly longer graft survival in renal transplant recipients. Kaplan–Meier curve displayed for graft survival, with log-rank test *P* value 0.004

Table 3 Associations of homoarginine urinary excretion with all-cause mortality in RTR

	Homoarginine continuous	
	HR (95 % CI) per SD	*P* value
Model 1	0.52 (0.40–0.66)	**<0.001**
Model 2	0.52 (0.40–0.67)	**<0.001**
Model 3	0.50 (0.38–0.66)	**<0.001**
Model 4	0.63 (0.47–0.85)	**0.002**
Model 5	0.70 (0.50–0.98)	**0.04**
Model 6	0.66 (0.48–0.91)	**0.01**

Bold values indicate statistical significance ($P < 0.05$)

Model 1: crude

Model 2: adjusted for age and gender

Model 3: adjusted for model 2 plus BSA, pre-emptive KTx, donor age, use of sirolimus

Model 4: adjusted for model 3 plus urea excretion

Model 5: adjusted for model 4 plus DBP, eGFR, serum phosphate, serum albumin, albuminuria, uric acid excretion

Model 6: adjusted for model 4 plus BMI, hemoglobin, NT-pro-BNP and CRP

Median (IQR) 1.62 (1.09–2.61) µmol/24 h

Table 4 Associations of homoarginine urinary excretion with graft failure in RTR

	Homoarginine continuous	
	HR (95 % CI) per SD	*P* value
Model 1	0.58 (0.42–0.81)	**0.001**
Model 2	0.54 (0.39–0.76)	**<0.001**
Model 3	0.60 (0.41–0.88)	**0.009**
Model 4	0.75 (0.50–1.12)	0.16
Model 5	0.79 (0.45–1.36)	0.39
Model 6	0.96 (0.61–1.51)	0.85

Bold values indicate statistical significance ($P < 0.05$)

Model 1: crude

Model 2: adjusted for age and gender

Model 3: adjusted for model 2 plus BSA, pre-emptive KTx, donor age and use of sirolimus

Model 4: adjusted for model 3 plus urea excretion

Model 5: adjusted for model 4 plus DBP, eGFR, serum phosphate, serum albumin, albuminuria, uric acid excretion

Model 6: adjusted for model 4 plus BMI, hemoglobin, NT-pro-BNP and CRP

Median (IQR) 1.62 (1.09–2.61) µmol/24 h

L-Lysine supplementation to rats and humans resulted in enhanced excretion of hArg in the urine, suggesting that in vivo L-lysine is a precursor of hArg. These findings are supported by our study showing a significant association between dietary lysine intake and UhArg excretion in RTR. Urea excretion, a marker of total protein intake, was significantly associated with UhArg excretion. After correction for urea excretion, UhArg was still positively associated with all-cause mortality. This was not the case for graft failure, where UhArg lost its significant association after correction for urea excretion.

Associations of hArg with energy metabolism have been described (Wyss and Kaddurah-Daouk 2000). We found that UhArg is positively associated with serum albumin, BSA and urea excretion, which are all involved in protein balance and nutritional status (Kopple et al. 2000), and are important determinants of cardiovascular morbidity and mortality pre- and post-renal transplantation (Rettkowski et al. 2007; Molnar et al. 2011).

The present study and reports from other groups provide strong evidence that higher urinary or higher circulating concentrations of hArg are beneficial to humans. However, the underlying mechanisms are poorly understood. One mechanism by which hArg may exert beneficial effects in the renal and cardiovascular systems may be related to NO, because hArg, like its homolog Arg, serves for NOS-catalyzed formation of NO (Valtonen et al. 2008). NO is a potent vasodilatator and protects against cardiovascular disease (Xia and Vanhoutte 2011; Jones and Bolli 2006). Impaired NO synthesis leads to endothelial and myocardial dysfunction (Takahashi and Harris 2014; Yang and Ming 2006). Circulating hArg was found to be inversely correlated with markers of impaired endothelial function (Pilz et al. 2014). Yet, there is no strong evidence that the beneficial effects of hArg are indeed due to NO formation from hArg.

Strengths of our study are the long follow-up time and the large cohort size and comprehensive registration of the participants, which contribute to the robustness of our results. As our study has an observational design, no conclusions can be drawn regarding causal relationships of the found associations. Further potential study limitations are that our cohort is single-centered and most participants were Caucasian, and the number of incident cases is relatively low for the adjustments made in the Cox models. Extrapolation of our findings to the general population is limited.

In the present study, we demonstrated that higher UhArg is independently associated with reduced all-cause mortality in stable renal transplant recipients. Higher UhArg was also associated with better graft survival and renal function. These data suggest that kidney-derived hArg is of importance in RTR and that preservation of kidney function in these patients is crucial to preserve cardiovascular function.

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Compliance with ethical standards

Ethical statement The Institutional Review Board approved the study protocol (METc 2008/186) which was in adherence to the Declaration of Helsinki.

Conflict of interest All authors report no conflicts of interest.

References

Atzler D, Rosenberg M, Anderssohn M et al (2013) Homoarginine—an independent marker of mortality in heart failure. Int J Cardiol 168:4907–4909

Atzler D, Gore MO, Ayers CR et al (2014) Homoarginine and cardiovascular outcome in the population-based Dallas Heart Study. Arterioscler Thromb Vasc Biol 34:2501–2507

Bretscher LE, Li H, Poulos TL, Griffith OW (2003) Structural characterization and kinetics of nitric-oxide synthase inhibition by novel N5-(iminoalkyl)- and N5-(iminoalkenyl)-ornithines. J Biol Chem 278:46789–46797

Drechsler C, Meinitzer A, Pilz S et al (2011) Homoarginine, heart failure, and sudden cardiac death in haemodialysis patients. Eur J Heart Fail 13:852–859

Drechsler C, Kollerits B, Meinitzer A et al (2013) Homoarginine and progression of chronic kidney disease: results from the Mild to Moderate Kidney Disease Study. PLoS One 8:e63560

Frenay AR, van den Berg E, de Borst MH, Beckmann B, Tsikas D, Feelisch M, Navis G, Bakker SJ, van Goor H (2015) Plasma ADMA associates with all-cause mortality in renal transplant recipients. Amino Acids. doi:10.1007/s00726-015-2023-0

Hecker M, Walsh DT, Vane JR (1991) On the substrate specificity of nitric oxide synthase. FEBS Lett 294:221–224

Israni AK, Leduc R, Jacobson PA et al (2013) Inflammation in the setting of chronic allograft dysfunction post-kidney transplant: phenotype and genotype. Clin Transplant 27:348–358

Jones SP, Bolli R (2006) The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol 40:16–23

Kasiske BL (2000) Cardiovascular disease after renal transplantation. Semin Nephrol 20:176–187

Kayacelebi AA, Nguyen TH, Neil C et al (2014a) Homoarginine and 3-nitrotyrosine in patients with takotsubo cardiomyopathy. Int J Cardiol 173:546–547

Kayacelebi AA, Beckmann B, Gutzki FM, Jordan J, Tsikas D (2014b) GC-MS and GC-MS/MS measurement of the cardiovascular risk factor homoarginine in biological samples. Amino Acids 46:2205–2217

Khalil AA, Tsikas D, Akolekar R et al (2013) Asymmetric dimethylarginine, arginine and homoarginine at 11–13 weeks' gestation and preeclampsia: a case-control study. J Hum Hypertens 27:38–43

Kopple JD, Greene T, Chumlea WC et al (2000) Relationship between nutritional status and the glomerular filtration rate: results from the MDRD study. Kidney Int 57:1688–1703

Levey AS, Stevens LA, Schmid CH et al (2009) A new equation to estimate glomerular filtration rate. Ann Intern Med 150:604–612

Mallon DH, Summers DM, Bradley JA, Pettigrew GJ (2013) Defining delayed graft function after renal transplantation: simplest is best. Transplantation 96:885–889

Marescau B, Nagels G, Possemiers I, De Broe ME, Becaus I, Billiouw JM, Lornoy W, De Deyn PP (1997) Guanidino compounds in serum and urine of nondialyzed patients with chronic renal insufficiency. Metabolism 46:1024–1031

März W, Meinitzer A, Drechsler C et al (2010) Homoarginine, cardiovascular risk, and mortality. Circulation 122:967–975

Moali C, Boucher JL, Sari MA et al (1998) Substrate specificity of NO synthases: detailed comparison of L-arginine, homo-L-arginine, their N omega-hydroxy derivatives, and N omega-hydroxynor-L-arginine. Biochemistry 37:10453–10460

Molnar MZ, Kovesdy CP, Bunnapradist S et al (2011) Associations of pretransplant serum albumin with post-transplant outcomes in kidney transplant recipients. Am J Transplant 11:1006–1015

Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012

O'Connor PM, Cowley AW Jr (2010) Modulation of pressure-natriuresis by renal medullary reactive oxygen species and nitric oxide. Curr Hypertens Rep 12:86–92

Ojo AO, Hanson JA, Wolfe RA et al (2000) Long-term survival in renal transplant recipients with graft function. Kidney Int 57:307–313

Passauer J, Pistrosch F, Bussemaker E (2005) Nitric oxide in chronic renal failure. Kidney Int 67:1665–1667

Pilz S, Meinitzer A, Tomaschitz A et al (2011a) Low homoarginine concentration is a novel risk factor for heart disease. Heart 97:1222–1227

Pilz S, Tomaschitz A, Meinitzer A et al (2011b) Low serum homoarginine is a novel risk factor for fatal strokes in patients undergoing coronary angiography. Stroke 42:1132–1134

Pilz S, Edelmann F, Meinitzer A et al (2014) Associations of methylarginines and homoarginine with diastolic dysfunction and cardiovascular risk factors in patients with preserved left ventricular ejection fraction. J Card Fail 20:923–930

Ravani P, Maas R, Malberti F et al (2013) Homoarginine and mortality in pre-dialysis chronic kidney disease (CKD) patients. PLoS One 8:e72694

Rettkowski O, Wienke A, Hamza A et al (2007) Low body mass index in kidney transplant recipients: risk or advantage for long-term graft function? Transplant Proc 39:1416–1420

Smith GL, Lichtman JH, Bracken MB, Shlipak Phillips CO, DiCapua P et al (2006) Renal impairment and outcomes in heart failure: systematic review and meta-analysis. J Am Coll Cardiol 47:1987–1996

Takahashi T, Harris RC (2014) Role of endothelial nitric oxide synthase in diabetic nephropathy: lessons from diabetic eNOS knockout mice. J Diabetes Res 2014:590541

The Hague (2006) Dutch food composition table (NEVO 2006) NEVO table 2006 (dutch nutrient databank: NEVO table 2006)

Tomaschitz A, Meinitzer A, Pilz S et al (2014) Homoarginine, kidney function and cardiovascular mortality risk. Nephrol Dial Transplant 29:663–671

Tomaschitz A, Verheyen N, Gaksch M, Meinitzer A, Pieske B, Kraigher-Krainer E, Colantonio C, März W, Schmidt A, Belyavskiy E, Rus-Machan J, van Ballegooijen AJ, Stiegler C, Amrein K, Ritz E, Fahrleitner-Pammer A, Pilz S (2015) Homoarginine in patients with primary hyperparathyroidism. Am J Med Sci 349:306–311

Valtonen P, Laitinen T, Lyyra-Laitinen T et al (2008) Serum L-homoarginine concentration is elevated during normal pregnancy and is related to flow-mediated vasodilatation. Circ J 72:1879–1884

van den Berg E, Engberink MF, Brink EJ et al (2012a) Dietary acid load and metabolic acidosis in renal transplant recipients. Clin J Am Soc Nephrol 7:1811–1818

van den Berg E, Geleijnse JM, Brink EJ et al (2012b) Sodium intake and blood pressure in renal transplant recipients. Nephrol Dial Transplant 27:3352–3359

van den Berg E, Engberink MF, Brink EJ et al (2013) Dietary protein, blood pressure and renal function in renal transplant recipients. Br J Nutr 109:1463–1470

van den Berg E, Pasch A, Westendorp WH et al (2014) Urinary sulfur metabolites associate with a favorable cardiovascular risk profile and survival benefit in renal transplant recipients. J Am Soc Nephrol 25:1303–1312

Wu G, Bazer FW, Davis TA et al (2009) Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168

Wyss M, Kaddurah-Daouk R (2000) Creatine and creatinine metabolism. Physiol Rev 80:1107–1213

Xia Z, Vanhoutte PM (2011) Nitric oxide and protection against cardiac ischemia. Curr Pharm Des 17:1774–1782

Yang Z, Ming XF (2006) Endothelial arginase: a new target in atherosclerosis. Curr Hypertens Rep 8:54–59

Zoccali C (2006) Traditional and emerging cardiovascular and renal risk factors: an epidemiologic perspective. Kidney Int 70:26–33