Dietary protein intake and long-term outcomes after kidney transplantation

Said, M.Yusof

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Meat intake and risk of mortality and graft failure in kidney transplant recipients

Said MY*, Rodriguez-Niño A*, Post A, Schutten JC, Kieneker LM, Gomes-Neto AW, van Londen M, Osté MCJ, Borgonjen-van den Berg KJ, Nolte IM, van den Berg E, Heiner-Fokkema MR, de Blaauw P, van der Krogt J, Navis G, Yard BA, Bakker SJL
* These authors share first authorship.

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Abstract

It is unknown whether meat intake is beneficial for long-term patient and graft survival in kidney transplant recipients (KTR). To investigate this, we first studied the association of previously described meat intake biomarkers 1-methylhistidine and 3-methylhistidine with intake of white and red meat as estimated from a validated food-frequency-questionnaire. Secondly, we investigated the association of the meat intake biomarkers with long-term outcomes in KTR. We measured 24h urinary excretion of 1-methylhistidine and 3-methylhistidine by validated assays in a cohort of 678 clinically stable KTR. Cross-sectional associations were assessed by linear regression. We used Cox regression analyses to prospectively study associations of $\log_2$-transformed biomarkers with mortality and graft failure. Median urinary 1-methylhistidine and 3-methylhistidine excretion were 282 [132–598] µmol/24h and 231 [175–306] µmol/24h, respectively. Urinary 1-methylhistidine was associated with white meat intake (standardized beta (st. β): 0.20, 95% confidence interval (95%CI): 0.12, 0.28; *P*<0.001), while urinary 3-methylhistidine was associated with red meat intake (st.β: 0.30, 95%CI: 0.23, 0.38; *P*<0.001). During median follow-up for 5.4 [Interquartile range 4.9–6.1] years, 145 (21.4%) died and 83 (12.2%) developed graft failure. Urinary 3-methylhistidine was inversely associated with mortality independent of potential confounders (HR per doubling: 0.55, 95%CI: 0.42, 0.72; *P*<0.001). Both urinary 1-methylhistidine and urinary 3-methylhistidine were inversely associated with graft failure independent of potential confounders (HR per doubling: 0.84, 95%CI: 0.73, 0.96; *P*=0.01 and 0.59, 95%CI: 0.41, 0.85; *P*=0.004, respectively). In conclusion, high urinary 3-methylhistidine, reflecting higher red meat intake, is independently associated with lower risk of mortality. Both high urinary 1- and 3-methylhistidine, of which the former is reflecting higher white meat intake, are independently associated with lower risk of graft failure in KTR. Future intervention studies are warranted to study the effect of high meat intake on mortality and graft failure in KTR, using these biomarkers.

Introduction

Kidney transplant recipients (KTR) are at high risk of premature mortality and decline of renal function (1,2). In KTR, high dietary protein intake has been associated with lower risk of premature mortality and graft failure through a yet unknown mechanism (3,4). Whether the source of dietary protein is relevant to outcome in KTR is unknown. Meat is an important source of dietary protein. Two types of meat have been studied extensively in the literature: white and red meat. Several large cohort studies in the general population have found that high red meat intake is associated with increased risk of developing chronic kidney disease, kidney failure, and death (5–7). Conversely, white meat intake has been associated with lower risk of mortality in the general population (5). Currently, it is unknown whether white meat, red meat, or both are associated with long-term outcomes in KTR.

One of the challenges of estimating meat intake through Food Frequency Questionnaires (FFQs), is that the estimations are prone to limitations, including under- and over-reporting, illiteracy, motivation requirements, recall bias, errors in portion size estimation, and socially desirable answers (8,9). The use of meat-specific biomarkers might be a more accurate approach in estimating true meat intake. A proposed marker for white meat intake is 1-methylhistidine, which results from the metabolism of the dipeptide anserine (10,11). Up to 90% of dietary anserine is hydrolyzed to 1-methylhistidine and excreted via urine (12). Previous studies found that plasma and urinary 1-methylhistidine are associated with white meat intake, being predominantly poultry intake (13,14). A proposed biomarker for red meat intake is 3-methylhistidine, which is found in myosin and actin (15). It is formed after methylation of histidine moieties and released after catabolism of proteins (11,15,16). Hereafter, it is not further reutilized nor metabolized and thus excreted as 3-methylhistidine via urine (17). Skeletal muscle, being the main source of actin and myosin, is regarded as the predominant source of urinary 3-methylhistidine.

In a controlled dietary intervention study of 33 adult men and 17 adult women, Altorf-van der Kuil *et al*. found that urinary excretion of 1-methylhistidine (uex1MH) and 3-methylhistidine (uex3MH) respectively explained 69% and 72% of variation in total meat intake (18), making these urinary metabolites putative biomarkers of meat intake.

In the present study, we aim to investigate the potential association of uex1MH) and uex3MH with FFQ-derived estimates of meat intake, in a large cohort of clinically stable KTR who are not subjected to dietary protein intake restrictions. Secondly, we aim to prospectively study the association of uex1MH and uex3MH with long-term outcomes i.e., mortality and graft failure in KTR.

Methods and materials

Study population

From November 2008 to March 2011, adult KTR who were transplanted at least one year before and had a functioning graft (i.e., not on renal replacement therapy) were invited to participate to this study, as a part of a larger prospective cohort study of KTR (TransplantLines Food and Nutrition cohort, Clinicaltrials.gov № NCT02811835). At time of inclusion, all KTR were at clinical follow-up at the University Medical Center of Groningen, the Netherlands. Subjects with overt congestive heart failure (NYHA class 3-4), medical history of cancer other than cured skin cancer, alcohol or drug abuse, or insufficient understanding of Dutch language were excluded. KTR who signed written informed consent and had frozen urine samples available for analysis were consecutively included in the study (see supplementary Figure S1 for a flow diagram of participant inclusion). At measurement times, subjects were at steady state, i.e., biochemically stable and without an acute illness (e.g., infection). The study protocol was approved by the institutional ethical review board (METc 2008/186) and has been conducted in accordance with the declarations of Helsinki and Istanbul.

Data collection

Subjects were invited to the outpatient clinic for baseline measurements and collection of blood and urine samples. Blood samples were drawn after a minimal 8h fasting period. On the same day, 24h urine was collected by each participant, according to well-explained protocol. Urine collection was under oil and the antiseptic agent chlorhexidine was added to the urine. Physical measurements have been described in detail previously (19-21) and were done on the same day as blood and urine collection. Questionnaires were used to obtain information

on smoking and alcohol intake. We categorized smoking as never, ex, or current, and alcohol intake as 0-10, 10-30, or >30 g/day. Diabetes mellitus was characterized by the usage of antidiabetic medication or fulfillment of the American Diabetes Association criteria of 2017: a fasting plasma glucose concentration ≥7.0 mmol/L and/or HbA1c ≥6.5%. Physical activity was measured with the Short Questionnaire to Assess Health-enhancing physical activity (SQUASH questionnaire) (22). Delayed graft function was defined as need for dialysis in the first week following transplantation (23). In KTR with proteinuria at the time of baseline of the biobank and cohort study, we checked whether kidney biopsies had been performed between 2 years before and 2 years after baseline measurement. If time between 1 year after transplantation and baseline was shorter than two years, we included kidney biopsies if they had been performed between 1 year after transplantation and 2 years after baseline measurement. Biopsies were performed by a trained nephrologist, prepared according to local protocol, and examined by a trained kidney pathologist.

Dietary assessment

We used validated semiquantitative FFQs that were developed at Wageningen University and have been described in detail before (24,25). The FFQs were distributed before visit to the outpatient clinic for baseline measurements and were filled out at home by the KTR. Household units were used to express the number of serving sizes consumed (e.g. bowls or pieces) or in weight. Frequency was expressed per day, week, or month. The FFQs were afterwards checked by trained researchers and patients were consulted to verify answers that seemed inconsistent or if FFQ were incomplete. The questionnaire data were analyzed using the 2006 Dutch Food Composition Table (NEVO), as distributed by the Dutch Ministry of Health, Welfare, and Sport (26), to calculate intakes of energy and macro- and micronutrients. FFQs reporting energy intakes of <500 or >5000 kcal per day were regarded as unreliable and therefore excluded. Certain food items were combined to produce a composite measurement of specific meat intake, such as red meat or white meat. Red meat intake was calculated by combining the daily intakes of beef, pork, lamb, liver/kidney, and processed meat products (sausages, blind finch (a type of Dutch roulade), minced meat, bacon, and luncheon meat). White meat intake was calculated by combining the daily intakes of chicken and turkey meats. In supplementary Table S1, an overview of the specific meat intakes derived from the FFQ can be found.

In addition to the FFQ measurement of total protein intake, we also calculated total protein intake with 24h urea excretion and protein excretion using the Maroni equation (27):

Protein intake (g/day) based on the Maroni equation

$$= 0.18 \times \textit{urinary urea excretion}\ (\textit{mmol/day}) + 0.19 \times \textit{body weight}\ (\textit{kg}) + \textit{urinary protein excretion (g/day)}$$

Laboratory measurements

We measured concentrations of 1-methylhistidine and 3-methylhistidine from thawed 24h urine samples using a validated ultra-high performance liquid chromatography tandem mass spectrometry analysis (UHPLC-MS/MS). The urine samples were derivatized with AccQ•Tag derivatization reagent according to the manufacturer's protocol (Waters Corporation, Milford, MA, USA). The derivates of 1-methylhistidine and 3-methylhistidine were separated using a Phenomenex Synergi™ column (4 µm Polar-RP 80 Å, 150 x 3 mm) and were detected using positive-ion electrospray ionization in multiple reaction monitoring mode using the following transitions: m/z 340.0 -> 171.0 for 1-methylhistidine and 3-methylhistidine and 335.0 -> 171.0 for the internal standard (13C6-, 15N3-histidine). Data were analyzed using MultiQuant MD 3.0.2 (Sciex). Two urine samples were used for assessment of intra-assay precision, and two others for assessment of inter-assay precision. The intra-assay coefficients of variation (CVs) for 1-methylhistidine were 3.1% at 155 µmol/L and 4.4% at 1450 µmol/L, with inter-assay CVs of 12.1% at 53 µmol/L and 8.6% at 118 µmol/L. For 3-methylhistidine, the intra-assay CVs were 4.3% at 402 µmol/L and 5.4% at 604 µmol/L, and the inter-assay CVs were 8.4% at 99 µmol/L and 8.7% at 141 µmol/L. The accuracy was 112% for 1-methylhistidine and 109% for 3-methylhistidine compared to our reference method for amino acids on a Biochrom 30 analyser (Pharmacia Biotech, Cambridge, UK). The detection, and quantification limits for 1-methylhistidine were 4.3 and 18.6 µmol/L respectively, and for 3-methylhistidine 4.5 and 6.5 µmol/L respectively, with a linear range up to 1000 µmol/L. Samples above this range were reported as >1000 µmol//L. Urine sample concentrations below or above the detection threshold of a specific compound were registered as at the lower or upper detection threshold, respectively. Two KTR had a 1-methylhistidine concentration below the lower detection threshold and 8 above

the upper detection threshold. All KTR had 3-methylhistidine concentrations within the limits of detection. Routine laboratory methods were used for other blood and urine analyses, as described earlier (19-21). Venous pH and HCO_3^- were measured as described earlier (24). Urinary taurine was measured by UHPLC-MS as previously described (28). Serum iron was measured using photometry (Modular P800, Roche Diagnostics, Mannheim, Germany).

We calculated the estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula with serum creatinine and cystatin C (29). Proteinuria was defined as urinary protein excretion ≥0.5 g/24h.

Study outcomes

Outcomes were all-cause mortality and death-censored graft failure. Graft failure was defined as return to dialysis or re-transplantation. Follow-up was up to October 2015. No patients were lost to follow-up.

Statistical analysis

Baseline data are presented as means ± standard deviation for normally distributed data, as medians [interquartile range (IQR)] for non-normally distributed data, and as numbers (percentages) for nominal data. Since uex1MH and uex3MH had a skewed distribution, these variables were $\log_2$-transformed for all analyses.

We first cross-sectionally studied the separate associations of uex1MH and uex3MH (dependent variables) with basic characteristics and transplantation-related characteristics (independent variables) by performing univariable linear regression. Categorical variables were recoded into dummy dichotomous variables and analyzed together by means of multivariable linear regression.

We also cross-sectionally analyzed the associations of uex1MH and uex3MH with dietary intake estimates by first performing univariable linear regression and consecutively multivariable linear regression. In the multivariable analyses, we adjusted the associations of uex1MH and uex3MH with food intake estimates for age, sex, total caloric intake, body mass index (BMI), and eGFR. Regression coefficients are presented as standardized beta values (St.β) referring to the number of standard deviations the dependent variable changes per standard deviation increase of the independent variable, allowing the comparison of association strengths among

different variables. Paired t-test was employed to assess differences between FFQ-derived protein intake and Maroni-calculated protein intake.

Secondly, we studied prospective associations of uex1MH and uex3MH with mortality and death-censored graft failure during follow-up by performing Cox proportional hazard analyses. We used $\log_2$-transformed uex1MH and uex3MH to allow for interpretation of hazard ratio (HR) values per doubling of uex1MH and per doubling of uex3MH, respectively. We adjusted the associations of uex1MH and uex3MH with outcomes for potential confounders. Baseline characteristics that were significantly associated with uex1Mh and uex3MH were considered potential confounders. Model 1 included adjustments for several potential confounders, including age, sex, BMI, eGFR, proteinuria, time from transplantation to baseline visit, and FFQ-estimated energy intake. Adjustments of all subsequent models were additions to model 1 in order to prevent including too many variables per number of events. In model 2 we additionally adjusted for transplantation-related factors (postmortem donation, cold ischemia time, total dialysis time, number of previous transplantations, and primary renal disease), in model 3 for post-transplantation complications (delayed graft function, rejection after transplantation (up to baseline), CMV infection (primary or secondary), in model 4 for immunosuppressive medication (prednisolone dosage, usage of calcineurin inhibitors, and/or proliferation inhibitors), in model 5 for alcohol intake, in model 6 for potential cardiovascular risk factors and parameters (C-reactive protein (CRP), high-density lipoprotein (HDL) cholesterol, diastolic blood pressure, smoking behavior, diabetes mellitus, post-transplantation diabetes mellitus (PTDM, a.k.a. new-onset diabetes mellitus after transplantation), and SQUASH score), in model 7 for metabolic acidosis (venous pH and HCO_3^-), in model 8 for serum iron ,and finally in model 9 for 24h urinary taurine excretion. Potential interactions for age, sex, BMI, eGFR, and alcohol intake, were investigated by assessing interaction terms. We performed linear spline analyses to demonstrate linearity of the prospective associations of uex1MH and uex3MH with mortality and graft failure. All data for the spline analyses were fit by a Cox proportional hazard model adjusted for age, sex, BMI, eGFR, proteinuria, time from transplantation to baseline visit to the outpatient clinic, and FFQ-estimated energy intake.

Analyses were performed with IBM SPSS statistics version 23 (2015, IBM corp., Armonk, N.Y., USA) and R statistics version 3.5.1 (2018, R Foundation for Statistical Computing, Vienna, Austria). P values ≤0.05 were considered statistically significant.

Results

General baseline characteristics, transplantation-related baseline characteristics, and urinary excretion of biomarkers

Out of 817 adult KTR, 706 signed written informed consent and 678 had frozen urine samples available for analyses. These 678 KTR were included in this study. Assessments for establishing the baseline of the prospective cohort study were performed at a median time of 5.3 [IQR 1.8-11.5] years after transplantation. Median age was 55 [IQR 45–63] years and 58% was male. The associations of urinary excretion biomarkers with general baseline characteristics are depicted in Table 1. Urinary excretion of 1-methylhistidine was 282 [IQR 132–598] µmol/24h and of 3-methylhistidine was 231 [IQR 175–306] µmol/24h. Uex1MH and uex3MH shared positive associations with male sex, BMI, body weight, SQUASH score, and urinary taurine excretion, and they shared inverse associations with age. Uex1MH was inversely associated with past smoking behavior, medical history of diabetes mellitus, antidiabetic medication usage, PTDM, and CRP concentrations. Uex3MH was positively associated with diastolic blood pressure and inversely with time since transplantation to baseline, total cholesterol, and HDL cholesterol concentrations (Table 1).

Table 1. Associations of meat intake biomarkers with basic general characteristics

	n	RTR (*n*=678)	Association with log_2-transformed biomarkers			
			uex1MH		uex3MH	
			St. β	*P*	*St. β*	*P*
General characteristics						
Age of patient, years	678	54.5 [44.8–62.9]	-0.18	*<0.001*	-0.18	*<0.001*
Male sex, n (%)	678	390 (57.5)	0.09	*0.01*	0.47	*<0.001*
Weight, kg	678	80.4 ± 16.6	0.17	*<0.001*	0.43	*<0.001*
BMI, kg/m^2	678	26.6 ± 4.8	0.12	*0.002*	0.22	*<0.001*
Time since transplantation, years	678	5.3 [1.8–11.5]	-0.06	0.12	-0.12	*0.001*
Urinary protein intake biomarkers						
uex1MH, µmol/24h	678	281.7 [132.0–597.7]	N/A		0.36	*<0.001*
uex3MH, µmol/24h	678	231.0 [175.4–306.3]	0.42	*<0.001*	N/A	
Smoking behavior, n (%)[1]	639					
Never		267 (39.4)	Ref.		Ref.	
Ex		290 (42.8)	-0.11	*0.01*	-0.05	0.26
Current		82 (12.1)	-0.02	0.65	0.04	0.31
Cardiovascular parameters						
Systolic pressure, mmHg	676	136 ± 17	-0.07	0.08	0.04	0.30
Diastolic pressure, mmHg	676	83 ± 11	0.04	0.33	0.14	*<0.001*
Total cholesterol, mmol/l	678	5.11 ± 1.12	-0.002	0.96	-0.10	*0.01*
HDL cholesterol, mmol/l	669	1.30 [1.10–1.60]	-0.05	0.18	-0.17	*<0.001*
LDL cholesterol, mmol/l	669	2.90 [2.30–3.50]	0.02	0.57	-0.03	0.44
Triglycerides, mmol/l	670	1.68 [1.25–2.29]	-0.04	0.35	-0.01	0.82
History of cardiovascular event, n (%)[2]	678	101 (14.9)	-0.04	0.27	-0.02	0.59
Diabetes						
Diabetes, n (%)[3]	678	162 (23.9)	-0.09	*0.02*	-0.04	0.28
Antidiabetics usage, n (%)	678	107 (15.8)	-0.09	*0.02*	-0.07	0.06
Acidosis						
Venous pH	626	7.37 ± 0.04	0.06	0.13	0.04	0.32
Venous HCO_3^-	626	24.6 ± 3.1	-0.04	0.27	-0.01	0.83
Inflammation						
CRP, mg/l	638	1.6 [0.7–4.5]	-0.08	*0.04*	0.02	0.71
Blood leucocyte, x10^9/l	677	8.1 ± 2.6	0.01	0.89	0.06	0.11
Urine taurine excretion, µmol/24h	678	533 [210–946]	0.15	*<0.001*	0.48	*<0.001*
Serum iron, µmol/L	673	15.3 ± 6.1	0.06	*0.12*	0.06	*0.12*
SQUASH physical activity score	678	5160 [2040–8073]	0.12	*0.003*	0.18	*<0.001*

Table 1. *(continued)*
Data are presented as mean± SD, median [IQR] or absolute number (%)
Associations of biomarkers with variables were tested via univariable regression analyses of which standardized βeta coefficients (St. β) are given, referring to the number of SD change in the dependent variable (biomarker) per SD increment in the independent variable.
Abbreviations: BMI: body mass index; CRP: C-reactive protein; HDL: high-density lipoprotein; KTR: Kidney transplant recipients, LDL: low-density lipoprotein; SQUASH: short questionnaire to assess health-enhancing physical activity; uex1MH: urinary 1-methylhistidine excretion; uex3MH: urinary 3-methylhistidine excretion.
[1] Categories do not sum up to 100% because of missing data (*n*=44 (6.5%))
[2] Defined as myocardial infarction, coronary intervention (including percutaneous coronary intervention and coronary artery bypass grafting), and cerebral ischemic event (including cerebrovascular accident and transient ischemic attack).
[3] Defined as blood glucose ≥7 mmol/L, HbA1c ≥6.5%, and/or use of antidiabetics.

From the transplantation-related characteristics described in Table 2, uex1MH and uex3MH shared positive associations with living donor transplantation, and high prednisolone dosage. Uex1MH was positively associated with proliferator inhibitor usage. Uex3MH was positively associated with calcineurin inhibitor usage, eGFR, delayed graft function, and it was inversely associated with cold ischemia time.

Of note, 17 KTR (2.6%) had a baseline eGFR <15 mL/min/1.73m^2. Kidney biopsies were performed in 19 (2.8%) subjects and they were mainly performed because of unexpected renal function decline. From these biopsies, 4 (21%) showed signs of cellular rejection, 2 (11%) showed signs of humoral rejection, 2 (11%) had extensive arteriolar hyalinosis suggestive of CNI toxicity, 1(5%) had signs of BK infection, and 1(5%) showed signs of focal segmental sclerosis. Some biopsies showed two or more of these abnormalities at the same time, while there were 9 (47%) biopsies in which no abnormalities were found.

Dietary intakes

Information on the association of protein intake biomarkers with dietary intake patterns is shown in Table 3. Of 678 KTR, 58 (8.6%) had missing FFQ data. Maroni-calculated protein intake was 86 ± 22 g/24h, which was close to the FFQ-derived total protein intake: 82 ± 20 g/24h, yet significantly different (P<0.001). Maroni-calculated protein intake and FFQ-derived total protein intake were significantly associated (st. β: 0.35, 95%CI: 0.28, 0.43; P<0.001).

Table 2. Associations of meat intake biomarkers with transplantation-related characteristics

	n	KTR (*n*=678)	Association with log_2-transformed biomarkers			
			uex1MH		uex3MH	
			St. β	*P*	*St.* β	*P*
Primary renal disease, n (%)	678					
Primary glomerular disease		194 (28.6)	0.04	0.46	0.15	*0.01*
Glomerulonephritis		49 (7.2)	0.03	0.51	0.08	0.08
Tubular interstitial disease		83 (12.2)	-0.01	0.92	0.07	0.16
Polycystic renal disease		139 (20.5)	0.01	0.82	0.02	0.70
Dysplasia and hypoplasia		28 (4.1)	-0.004	0.93	0.04	0.41
Renovascular disease		38 (5.6)	-0.01	0.79	0.03	0.44
Diabetes mellitus		34 (5.0)	-0.05	0.29	0.06	0.17
Other/unknown cause		113 (16.7)	Ref.		Ref.	
Transplantation-related characteristics						
Total dialysis time, months	669	27 [10–52]	-0.07	0.07	-0.02	0.61
HLA mismatch, n (%)[1]	634					
0		122 (18)	Ref.		Ref.	
1		85 (12.5)	-0.06	0.18	-0.03	0.55
2		165 (24.3)	-0.02	0.77	0.03	0.55
≥3		262 (38.6)	-0.07	0.18	0.10	0.08
Living donor transplantation, n (%)	678	232 (34.2)	0.08	*0.04*	0.08	*0.03*
Cold ischemia times (h)	670	15.3 [2.8–21.0]	-0.07	0.09	-0.10	*0.01*
2 or more transplantations, n (%)	678	66 (9.7)	-0.07	*0.05*	-0.08	*0.05*
Induction immunosuppression at transplantation, n (%)[2]	672					
Azathioprine		26 (3.8)	0.08	0.13	-0.04	0.44
Ciclosporin A		189 (27.9)	0.03	0.77	-0.11	0.25
Tacrolimus		14 (2.1)	0.06	0.21	0.05	0.25
ATG		60 (8.8)	0.02	0.76	-0.01	0.83
OKT3 monoclonal AB[3]		16 (2.4)	-0.01	0.81	-0.03	0.51
Anti-IL2R monoclonal AB		338 (49.9)	0.12	0.22	0.01	0.90
Rituximab		2 (0.3)	0.003	0.94	-0.03	0.40
Other		27 (4.0)	Ref.		Ref.	
Immunosuppressive medication at baseline						
Prednisolone dosage, mg/24-h	678	10.0 [7.5–10.0]	0.09	*0.02*	0.12	*0.002*

Table 2. *(continued)*

	n	KTR (*n*=678)	Association with log_2-transformed biomarkers uex1MH		uex3MH	
			St. β	*P*	*St. β*	*P*
CNI usage[4], n (%)	678	381 (56.2)	0.07	0.09	0.09	*0.02*
Proliferation inhibitor usage[5], n (%)	678	567 (83.6)	0.08	*0.03*	0.07	0.08
Rejection after transplantation (up to baseline), n (%)	678	177 (26.1)	0.03	0.52	0.04	0.31
PTDM, n (%)	678	128 (18.9)	-0.08	*0.05*	-0.02	0.57
Delayed graft function, n (%)	678	49 (7.2)	0.02	0.56	0.11	*0.01*
Cytomegalovirus infection[6], n (%)	622	173 (25.5)	-0.03	0.47	0.01	0.82
BK viral load, copies/ml[7]	641					
Undetectable		611 (90.1)	Ref.		Ref.	
<5000		27 (4.0)	0.08	0.06	0.02	0.59
5000-10,000		1 (0.1)	-0.003	0.94	0.04	0.30
>10,000		2 (0.3)	-0.05	0.24	-0.02	0.62
Renal allograft function						
Serum urea, mmol/l	676	9.4 [7.2–13.3]	-0.05	0.22	-0.06	*0.15*
Serum creatinine, µmol/l	676	124 [99–160]	0.01	0.85	0.04	0.28
eGFR, ml/min/1.73m^2[8]	663	45.4 ± 18.8	0.05	0.17	0.10	*0.01*
Protein excretion, g/24 h	678	0.20 [0.02–0.37]	-0.03	0.44	0.01	0.87
Proteinuria (>0.5 g/24-h), n (%)	678	152 (22.4)	-0.02	0.66	-0.03	0.44

Data are presented as mean± SD, median [IQR] or absolute number (%)
Associations of biomarkers with variables were tested via univariable regression analyses of which standardized βeta coefficients (St. β) are given, referring to the number of SD change in the dependent variable (biomarker) per SD increment in the independent variable.
Abbreviations: CNI: calcineurin inhibitor; eGFR: estimated glomerular filtration rate; HLA: human leukocyte antigens; KTR: Kidney transplant recipients; PTDM: Post-transplant diabetes mellitus; uex1MH: urinary 1-methylhistidine excretion; uex3MH: urinary 3-methylhistidine excretion.
[1] Categories do not sum up to 100% because of missing data (*n*=44 (6.5%))
[2] Categories do not sum up to 100% because of missing data (*n*=6 (0.9%)). All induction immunosuppression protocols included corticosteroids.
[3] Muromonab-CD3
[4] e.g. tacrolimus
[5] e.g. mycophenolate mofetil
[6] primary or secondary cytomegalovirus infection
[7] categories do not sum up to 100% because of missing data (*n*=37 (5.5%))
[8] calculated by the CKD-EPI creatinine-cystatin C

In the univariable model (Table 3: models 1), both uex1MH and uex3MH were significantly associated with urinary urea excretion, Maroni-calculated protein intake, FFQ-derived total protein intake, animal protein intake, and total meat intake. Uex1MH was also associated with white meat and fish intake, while uex3MH was associated with red meat intake, plant protein intake, total fat intake, energy intake in men, alcohol intake, and total carbohydrate intake (Table 3, models 1). Additionally, uex3MH was inversely associated with fruit intake.

In the multivariable models (Table 3: models 2), adjustments for age, sex, energy intake, BMI, and eGFR strengthened the association of uex1MH with the Maroni-calculated protein intake, FFQ-derived total protein intake, animal protein intake, and fish intake, but weakened the association of uex1MH with total meat intake (st. β: 0.13, 95%CI: 0.05, 0.21; *P*=0.002 vs. st. β: 0.11, 95%CI: 0.02, 0.19; *P*=0.01). For uex3MH, the adjustments of model 2 weakened the associations with urea excretion, Maroni calculated protein intake, FFQ-derived total protein intake and animal protein intake, total meat intake, and red meat intake. Interestingly, the multivariable model unveiled a positive association of uex3MH with fish intake (st. β: 0.07, 95%CI: 0.01, 0.14; *P*=0.03), while the associations of uex3MH with plant protein intake and total carbohydrate intake became inverse (st. β: -0.17, 95%CI: -0.28, -0.05; *P*=0.004 and st. β: -0.19, 95%CI: -0.33, -0.05; *P*=0.01, respectively). The associations of uex3MH with fruit, total fat, and alcohol intakes were no longer significant after the adjustments in the multivariable analysis (Table 3, model 2).

Association of meat intake biomarkers with mortality and graft failure

During median follow-up of 5.4 [IQR 4.9 - 6.1] years, 145 (21%) KTR died. Of these, 60 (41%) died of cardiovascular disease, 40 (28%) of infectious causes, 23 (16%) of malignancy, 20 (14%) of miscellaneous causes, and 2 (1%) of unknown causes. KTR who died had lower uex1MH (226 [IQR 97–407] µmol/24h vs. 299 [IQR 139–654] µmol/24h, *P*<0.001) and lower uex3MH (204 [IQR 158–262] µmol/24h vs. 239 [IQR 182–322] µmol/24h, *P<0.001*). Prospective analyses of the associations of $\log_2$ transformed uex1MH and $\log_2$ transformed uex3MH with mortality and death-censored graft failure are described in Table 4. The proportionality of hazards assumption was checked with the Schoenfeld residual test and was not violated for the associations (P>0.05).

Table 3. Meat intake biomarkers and their associations with nutritional and lifestyle variables

			Association with log_2-transformed biomarkers							
			uex1MH				uex3MH			
	n	RTR (*n*=678)	Model 1		Model 2		Model 1		Model 2	
			St. β	*P*	*St. β*	*P*	*St. β*	*P*	*St. β*	*P*
Urea excretion, mmol/24h	678	388 [309–458]	0.31	*<0.001*	0.31	*<0.001*	0.65	*<0.001*	0.51	*<0.001*
Maroni-formula protein intake, g/24h[1]	678	86 ± 22	0.32	*<0.001*	0.33	*<0.001*	0.67	*<0.001*	0.53	*<0.001*
Alcohol intake, n (%)[2]	620									
0-10 g/24h		454 (67.0)	Ref.		Ref.		Ref.		Ref.	
10-30 g/24h		138 (20.4)	0.05	0.19	0.04	0.38	0.17	*<0.001*	0.06	0.08
>30 g/24h		28 (4.1)	0.05	0.05	0.04	0.30	0.10	*0.01*	0.05	0.18
Dietary intake estimates										
Energy, kcal/day	620	2172 ± 619	0.06	0.14	N/A		0.24	*<0.001*	N/A	
Women	270	1917 ± 475	-0.04	0.47	N/A		0.02	0.74	N/A	
Men	350	2368 ± 646	0.07	0.21	N/A		0.12	*0.03*	N/A	
Fat, g/day	620	84 [64–105]	0.04	0.27	-0.08	0.39	0.21	*<0.001*	0.01	0.91
Saturated fat	620	30 [23–38]	0.02	0.59	-0.11	0.16	0.20	*<0.001*	0.07	0.25
Monounsaturated fat	620	28 [21–35]	0.07	0.10	0.02	0.78	0.21	*<0.001*	<0.001	1.00
Polyunsaturated fat	620	17 [13–23]	0.03	0.53	-0.05	0.41	0.16	*<0.001*	-0.07	0.18
Total carbohydrate intake, g/day	620	243 [194–290]	0.04	0.35	-0.07	0.43	0.17	*<0.001*	-0.19	*0.01*
Protein intake, g/day	620									
Total protein	620	82 ± 20	0.08	*0.05*	0.17	*0.02*	0.21	*<0.001*	0.16	*0.01*
Plant protein	620	31 ± 10	0.03	0.45	-0.05	0.44	0.13	*0.001*	-0.17	*0.004*
Animal protein	620	51 ± 15	0.09	*0.03*	0.13	*0.01*	0.19	*<0.001*	0.16	*<0.001*

Table 3. *(continued)*

			Association with log_2-transformed biomarkers							
			uex1MH				uex3MH			
	n	RTR (*n*=678)	Model 1		Model 2		Model 1		Model 2	
			St. β	*P*	*St. β*	*P*	*St. β*	*P*	*St. β*	*P*
Meat products, g/day										
Total meat and meat products	612	94 [72–117]	0.13	*0.002*	0.11	*0.01*	0.29	*<0.001*	0.18	*<0.001*
Red meat	612	82 [59–106]	0.06	0.13	0.03	0.52	0.30	*<0.001*	0.19	*<0.001*
White meat	613	11 [0–16]	0.20	*<0.001*	0.20	*<0.001*	-0.02	0.57	-0.01	0.73
Fish intake	612	11 [4–18]	0.13	*0.001*	0.16	*<0.001*	0.05	0.20	0.07	*0.03*
Dairy, g/day	613	333 [205–480]	-0.06	0.14	-0.03	0.45	-0.06	0.16	-0.06	0.09
Of which cheese	612	30 [15–46]	-0.02	0.66	-0.02	0.71	0.05	0.24	0.03	0.35
Legumes and nuts, g/day	612	11 [4–23]	-0.04	0.32	-0.05	0.26	0.02	0.69	-0.02	0.55
Vegetable, g/day	612	106 [69–149]	-0.02	0.64	0.03	0.48	-0.06	0.14	0.004	0.91
Fruit, g/day	611	123 [66–232]	-0.04	0.30	0.01	0.87	-0.08	*0.04*	0.002	0.96

Data are presented as mean± SD, median [IQR] or absolute number (%)

Associations of biomarkers with variables were tested via univariable (Model1) and multivariable (Model 2) regression analyses of which standardized βeta coefficients (St. β) are given, referring to the number of SD change in the dependent variable (biomarker) per SD increment in the independent variable.

Model 1: crude model. Model 2: model 1 + adjustment for age, sex, total caloric intake, BMI, and eGFR.

Abbreviations: kcal: kilocalories; KTR: Kidney transplant recipients; uex1MH: urinary 1-methylhistidine excretion; uex3MH: urinary 3-methylhistidine excretion.

[1] Maroni equation (g/day) = *0.18 * urinary urea excretion in mmol/24h + 0.19 * body weight in kg + urinary protein excretion in g/day)*

[2] data do not sum up to 100% because of missing data (n=58 (8.6%))

In univariable Cox regression analyses, uex1MH and uex3MH were both associated with significantly lower risk of mortality (HR per doubling: uex1MH: 0.82, 95%CI 0.74,0.91; P<0.001 and uex3MH: 0.55, 95%CI: 0.42,0.72; P<0.001). The inverse association of uex1MH with mortality was lost after adjustment for potential confounders. The inverse association of uex3MH with mortality remained independent of further adjustments (Model 1 to 9).

Of 678 KTR, 83 (12%) subjects developed graft failure. Most of these patients developed chronic rejection (*n*=61, 74%). Other causes include vascular problems, infections, and other miscellaneous causes of graft failure. Compared to patients with a preserved renal graft during the follow up, patients that experienced graft failure had lower uex1MH (248 [IQR 87 - 506] µmol/24h vs. 288 [IQR 138 - 625] µmol/24h; *P*=0.04) and lower uex3MH (206 [IQR 149 - 275] µmol/24h vs. 235 [IQR 178 - 311] µmol/24h; *P* =0.02). Univariable Cox regression analyses revealed an inverse association of uex1MH and uex3MH with graft failure (HR per doubling: uex1MH: 0.84, 95%CI: 0.73,0.96; *P*=0.01, and uex3MH: 0.59 ,95%CI: 0.41,0.85; *P*=0.004). The association of uex1MH with lower risk of graft failure was independent of adjustments for potential confounders (models 1-6). However, when adjusted for metabolic acidosis markers, the association became borderline significant: HR per doubling: uex1MH: 0.84, 95%CI: 0.70,1.01; *P*=0.06 (Model 7). The association of uex3MH with lower risk of graft failure was independent of adjustments for potential confounders including transplantation complications (models 1-7).

We additionally adjusted for other elements that are also abundantly found in meat. Adjusting for iron did not change the associations of uex1MH and uex3MH with outcomes (Table 4, Model 8). Adjusting for urinary taurine did not materially change the association of uex1MH and uex3MH with mortality (Table 4; Model 9). Also, after adjustment for taurine the association of uex1MH with graft failure did not materially change (HR per doubling: 0.82, 95%CI: 0.69, 0.98; *P*=0.03), but did slightly weaken the association of uex3MH with graft failure (HR per doubling: 0.59, 95%CI: 0.35, 1.00; *P*=0.05 (Model 9).

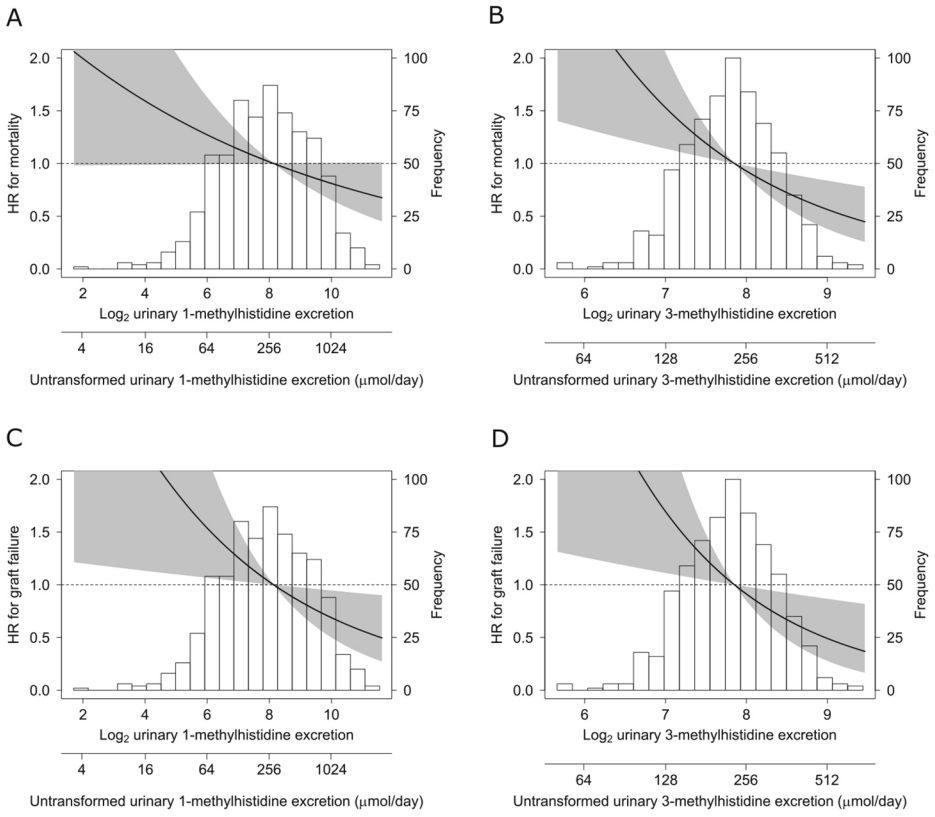

Figure 1. Linear splines of the associations of log_2-transformed 24h urinary 1-methylhistidine and 3-methylhistidine excretions with mortality and graft failure.

Data were fit by a Cox proportional hazard model and were adjusted for age, sex, body mass index, estimated glomerular filtration rate, proteinuria, time from transplantation to baseline visit, and FFQ-estimated energy intake. N=678. The black line represents the hazard ratio (HR), while the grey area represents the 95% confidence interval. The HRs were plotted relative to a value of 1.0 for the mean value of either urinary 1-methylhistidine excretion or urinary 3-methylhistidine excretion as a reference, respectively. A histogram of each distribution is plotted in the background. A: association of urinary 1-methylhistidine excretion with mortality, B: association of urinary 3-methylhistidine excretion with mortality, C: association of urinary 1-methylhistidine excretion with graft failure, D: association of urinary 3-methylhistidine excretion with graft failure.

Table 4. Cox regression analyses for the associations of log_2-transformed urinary excretions of 1-methylhistidine and 3-methylhistidine with mortality and graft failure in KTR

	1-methylhistidine		3-methylhistidine	
	HR (95% CI)[1]	*P*	*HR (95% CI)*[1]	*P*
All-cause mortality				
Crude	0.82 (0.74, 0.91)	<0.001	0.55 (0.42, 0.72)	<0.001
Model 1	0.90 (0.80, 1.01)	0.07	0.59 (0.41, 0.83)	0.003
Model 2	0.91 (0.81, 1.03)	0.13	0.55 (0.38, 0.78)	0.001
Model 3	0.91 (0.81, 1.03)	0.14	0.59 (0.41, 0.86)	0.01
Model 4	0.89 (0.79, 1.00)	0.06	0.58 (0.41, 0.82)	0.002
Model 5	0.91 (0.80, 1.02)	0.10	0.60 (0.42, 0.87)	0.01
Model 6	0.91 (0.81, 1.04)	0.16	0.62 (0.41, 0.93)	0.02
Model 7	0.92 (0.82, 1.04)	0.19	0.65 (0.45, 0.93)	0.02
Model 8	0.90 (0.80, 1.02)	0.09	0.59 (0.41, 0.84)	0.003
Model 9	0.90 (0.80, 1.01)	0.08	0.53 (0.36, 0.79)	0.002
Graft failure				
Crude	0.84 (0.73, 0.96)	0.01	0.59 (0.41, 0.85)	0.004
Model 1	0.82 (0.69, 0.97)	0.02	0.54 (0.33, 0.88)	0.01
Model 2	0.82 (0.69, 0.99)	0.04	0.55 (0.33, 0.94)	0.03
Model 3	0.77 (0.64, 0.92)	0.01	0.50 (0.30, 0.83)	0.01
Model 4	0.81 (0.68, 0.96)	0.02	0.55 (0.34, 0.90	0.02
Model 5	0.84 (0.70, 0.99)	0.04	0.55 (0.33, 0.91)	0.02
Model 6	0.82 (0.68, 0.99)	0.04	0.54 (0.31, 0.92)	0.02
Model 7	0.84 (0.70, 1.01)	0.06	0.58 (0.35, 0.97)	0.04
Model 8	0.81 (0.69, 0.97)	0.02	0.55 (0.33, 0.90)	0.02
Model 9	0.82 (0.69, 0.98)	0.03	0.59 (0.35, 1.00)	0.05

Crude	Log_2-transformed variable
Model 1	Crude + adjustments for age, sex, BMI, eGFR, proteinuria, time from transplantation to baseline, and FFQ-estimated energy intake
Model 2	Model 1 + adjustments for postmortal donation, cold ischemia time, total dialysis time, total number of transplantations, primary renal disease pre-transplantation.
Model 3	Model 1 + delayed graft function, rejection up to baseline, and post-transplantation CMV infection
Model 4	Model 1 + adjustments for prednisolone dosage, CNI usage, and proliferation inhibitor usage.
Model 5	Model 1 + adjustments for alcohol intake.
Model 6	Model 1 + adjustments for CRP, HDL cholesterol, diastolic blood pressure, smoking behavior, diabetes, PTDM, and SQUASH score
Model 7	Model 1 + adjustment for metabolic acidosis (venous pH and venous HCO_3)
Model 8	Model 1 + adjustment for serum iron
Model 9	Model 1 + adjustment for 24h urinary taurine excretion

Abbreviations: BMI: body mass index; CNI: calcineurin inhibitors; CRP: C-reactive protein; eGFR: estimated glomerular filtration rate; FFQ: food frequency questionnaire; HDL: high density lipoprotein; KTR: Kidney transplant recipients; SQUASH: Short Questionnaire to Assess Health-enhancing physical activity.

N=678

[1] per log_2 increment = per doubling of urinary 1-methylhistidine or 3-methylhistidine excretion.

No significant interactions with age, sex, BMI, eGFR, or alcohol intake were found for the associations of uex1MH and uex3MH with outcomes (P>0.05). Spline analyses in Figure 1 depict the associations of $\log_2$ transformed uex1MH and uex3MH with mortality (A, B) and graft failure (C, D).

When excluding KTR with a baseline eGFR <15 mL/min/1.73m^2 (n=17), the associations of uex1MH and uex3MH with graft failure did not materially change (Model 1; HR per doubling of uex1MH: 0.82, 95%CI: 0.69, 0.99; *P*=0.03, and per doubling of uex3MH: 0.54, 95%CI: 0.32, 0.93; *P*=0.03).

Discussion

In the current study in KTR, we found that uex1MH is independently and significantly associated with white meat intake, while uex3MH is independently and significantly associated with red meat intake, supporting their role as biomarkers for white and red meat, respectively. We found that uex3MH is inversely associated with mortality, and that both uex1MH and uex3MH are inversely associated with graft failure, independent of adjustments for potential confounders. The unadjusted risk reduction for graft failure per doubling of uex1MH was 16% and for doubling of uex3MH 41%.

Our results are supported by clinical data studying the relationship of meat intake with uex1MH and uex3MH. Datta and Harris made the early observation that predominantly carnivores excreted methylhistidines (30). Several human studies have shown a dose-dependent increase in uex1MH and uex3MH after meat intake (11,12,31,32). We observed in the current study that uex1MH is associated with specifically white meat and fish intake, and that uex3MH is associated with red meat intake, corroborating previous findings (12,14).

When looking at meat supply in the western world, red meat supply (53.9 kg/capita/year) in the Netherlands was lower, whereas white meat (22.5/kg/capita/year) supply was higher compared to Germany. The supply of both kinds of meat was higher in the United States when compared to the Netherland at the time of the study inclusion (33).

A major finding is the inverse association of uex1MH with graft failure. This finding suggests that high intake of white meat is protective for allograft outcome in KTR. This may in part be explained by an improvement of nutritional status (34).

Earlier, we found that high protein intake is associated with improved patient and graft survival in KTR (3,4). KTR may be at risk of protein energy wasting, partially because of the constant low-grade inflammation reaction against the allograft, and partially because of corticosteroid-related protein catabolism (35,36). High intake of protein, especially white meat, may in part compensate protein energy wasting in KTR, resulting in favorable graft outcomes (4). Secondly, the inverse association of uex1MH with graft failure may be explained in part by its origin. Uex1MH largely originates from the metabolism of dietary anserine through poultry intake. Anserine is endowed with a broad spectrum of biological properties including antioxidant and quenching effects (37,38). Studies suggest that short term treatment with anserine improved vascular permeability and proteinuria in diabetic mice (39). Anserine and other histidine-containing peptides are mobile cytoplasmic buffers that facilitate the exchange of ions such as H^+, acting as biological pumps, in circumstances of acido-basic imbalances (39,40). Thus, it is plausible that these mechanisms might indirectly mediate the protective association of uex1MH with graft failure.

Another major finding of this study is the inverse and independent association of uex3MH with mortality and graft failure. Also for specific transplantation-related determinants of graft loss (41), such as HLA mismatches and immunosuppression, we found minimal influence of these on the prospective association of uex1MH and uex3MH with graft failure. Our results suggest that red meat intake is protective against graft failure in this population. Meat is an important nutritional source of functional amino acids and dipeptides (42). Renoprotective properties derived from these, (43,44) might be of high relevance considering the inflammatory milieu that might take place in the kidney of KTR. Furthermore, because histidine-containing peptides and taurine also promote skeletal muscle health (45,46), it is likely that they also contribute in preventing protein energy wasting in KTR.

Of note, adjustment for urinary taurine excretion did slightly weaken the association of uex3MH with graft failure. This does not necessarily mitigate the suggestion that the association of uex3MH is fueled by dietary meat intake, as taurine excretion also reflects meat intake and was shown to be inversely associated with graft failure in the past (28).

Some studies in the general population suggest that high red meat intake is associated with adverse outcomes, including kidney disease and kidney failure

(5–7,47,48), while studies in patients with a higher likelihood of underlying chronic kidney disease, particularly patients with type 2 diabetes are more suggestive of a protective effect. As such, in the ONTARGET (Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial) study, animal protein intake was prospectively associated with lower risk of development or progression of CKD among these patients (49). In line, the American Diabetes Association does not recommend restricting protein intake in patients with diabetes or diabetic kidney disease (50), given the higher risk of malnutrition that protein-restriction might pose on these patients (51). Our study results are in line with this by suggesting that meat intake, including red meat, is beneficial for long-term kidney survival in KTR. A possible explanation is that high red meat intake may partially compensate the earlier mentioned risk of protein energy wasting in KTR (3,4). Another possible explanation is that meat intake, as a part of animal protein intake, can have specific advantages. As such, meat intake is generally of higher protein quality and digestibility, and has superior bioavailability of high physiological importance elements (42,52,53). Altogether, these properties in meat might indirectly explain the beneficial effects of meat on the graft survival in KTR.

Alexandrov *et al*. found in the large (*n*=76,633) Lifelines Cohort Study of the general population in the Netherlands that animal protein intake, in particular meat, fish, and egg intake, was positively associated with muscle mass, but plant protein intake was not (54). Interestingly, this association was strongest in elderly women (>65 years), which supports the growing belief that older individuals should increase their protein intake, possibly through increased meat intake, above the recommended daily allowance to prevent wasting (55). This may also apply for the current study, given the median age of 55 [45–63] years, implying that 25% of the study population is older than 63 years. It should be noted that, high red meat intake is associated with other adverse outcomes (e.g. colon carcinoma and hypertension) (56,57). Future intervention studies should also take these outcomes into account.

Strengths of this study are its large sample size, no loss to follow-up, minimal missing data, the ability to measure uex1MH and uex3MH in 24h urine samples to account for daily dietary changes, and the possibility to compare these meat intake biomarkers with well-established total protein intake biomarkers i.e. urea excretion and with data derived from the FFQ. It must be noted, however, that FFQ data is often biased by underreporting, especially for total protein intake (58). A limitation

of the study design is the use of a single collection moment for 24h urine, which can result in bias through day-to-day variation of specific protein intake. Another limitation is that adjustment for other trace elements, including e.g., zinc, was not possible because these data were not available.

In conclusion, we found that high excretions of uex1MH as biomarker of white meat intake and uex3MH as biomarker of red meat intake are associated with lower risk of graft failure in KTR. These associations may be explained through potential benefits of white and red meat intake and through potential compensation of protein energy wasting in KTR, although further studies are required to confirm this. Future intervention studies are warranted to study the effect of high meat intake on graft failure in KTR, using these biomarkers.

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Conflict of interest

The authors declare no conflict of interest.
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Supplementary data of chapter 3

Table S1. Food frequency questionnaire meat intake combinations

Red meat	*White meat*	*Meat and meat products*	*Fish*
Beef: beefsteak and other types of whole meat beef meat products	Chicken and chicken products	Chicken and chicken products	Raw herring and herring snack
Beef: 'blinde vink'* and other types of processed beef products	Turkey and turkey products	Turkey and turkey products	Salmon and similar fish
Pork: pork leg meat and other types of boneless whole pork meat products		Beef: beefsteak and other types of whole meat beef meat products	Flounder and similar flatfish
Pork: pork chops and other types of whole pork meat products with bone		Beef: 'blinde vink' and other types of processed beef products	Trout, plaice, gurnard, and other types of fish
Pork: smoked sausage and other types of processed pork products		Pork: pork leg meat and other types of boneless whole pork meat products	Readymade fish
Processed meat products: bacon and similar meat products		Pork: pork chops and other types of whole pork meat products with bone	Unknown type of fish
Processed meat products: liver pate and similar meat products		Pork: smoked sausage and other types of processed pork products	Shellfish
Processed meat products: ham and similar products		Processed meat products: bacon and similar meat products	
Processed meat products: bologna sausage and similar products		Processed meat products: liver pate and similar meat products	
Processed meat products: snack sausage and similar products		Processed meat products: ham and similar products	
Cooked liver		Processed meat products: bologna sausage and similar products	
Liver and kidney products		Processed meat products: snack sausage and similar products	

Table S1. *(continued)*

Red meat	*White meat*	*Meat and meat products*	*Fish*
Lamb or sheep meat		Cooked liver	
Ground meats (all sorts)		Liver and kidney products	
Other meat products: goat, horse, etc.		Lamb or sheep meat	
Unknown meat and meat products		Minced meats (all sorts)	
		Other meat products: goat, horse, etc.	
		Unknown meat and meat products	

* Blinde vink is a roulade-type of ground meat product, popular in the Netherlands.

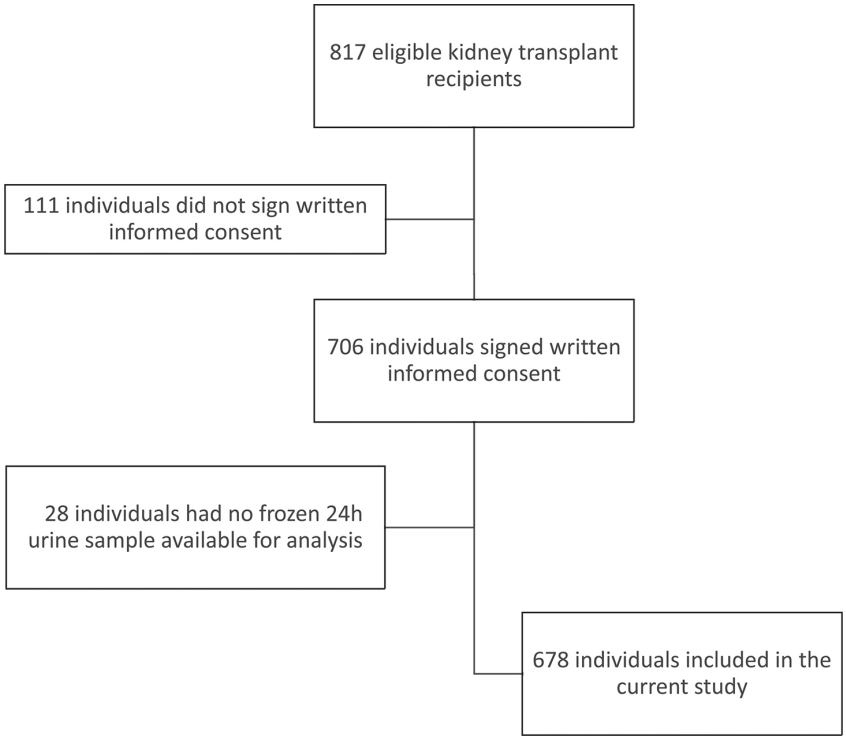
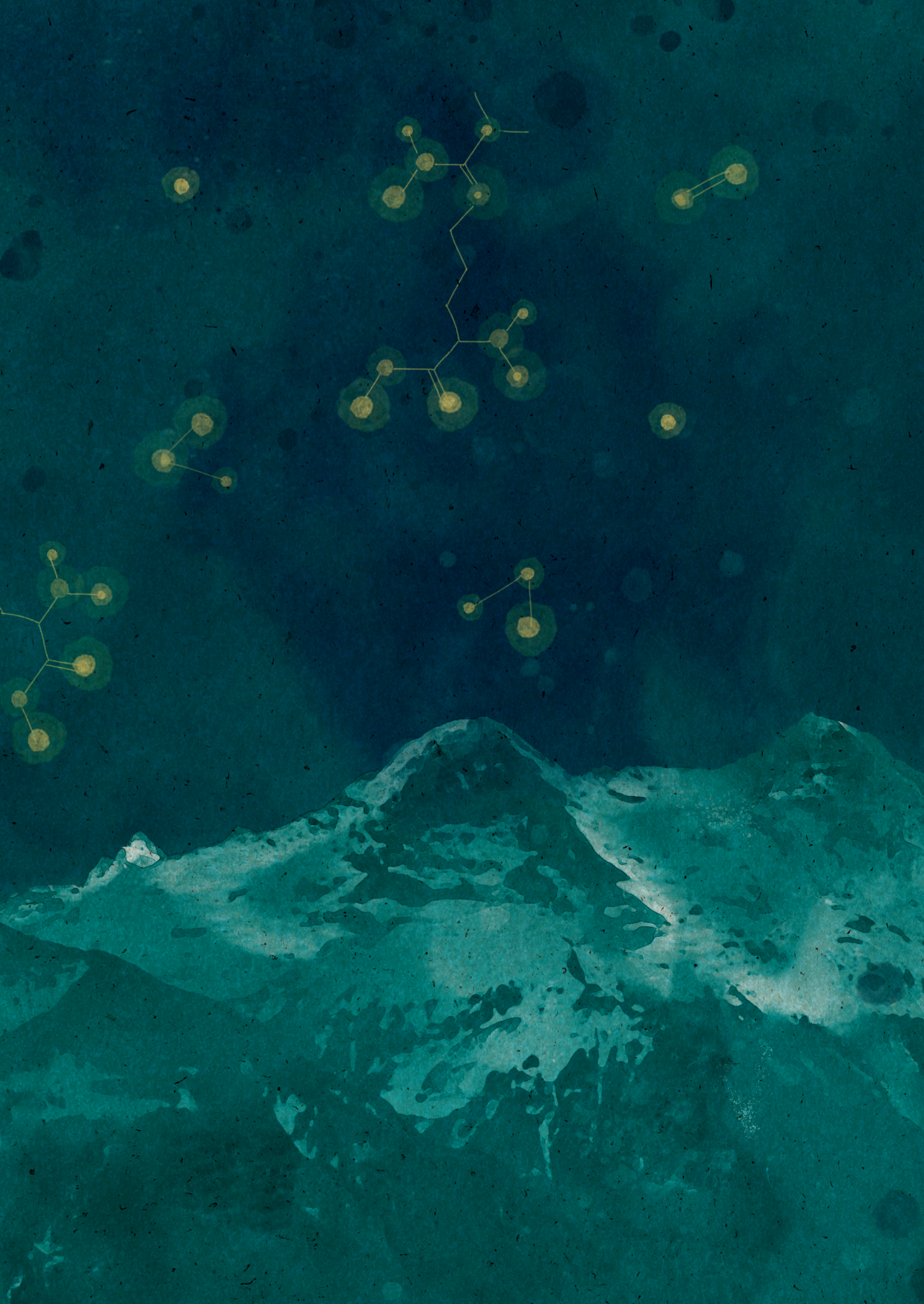
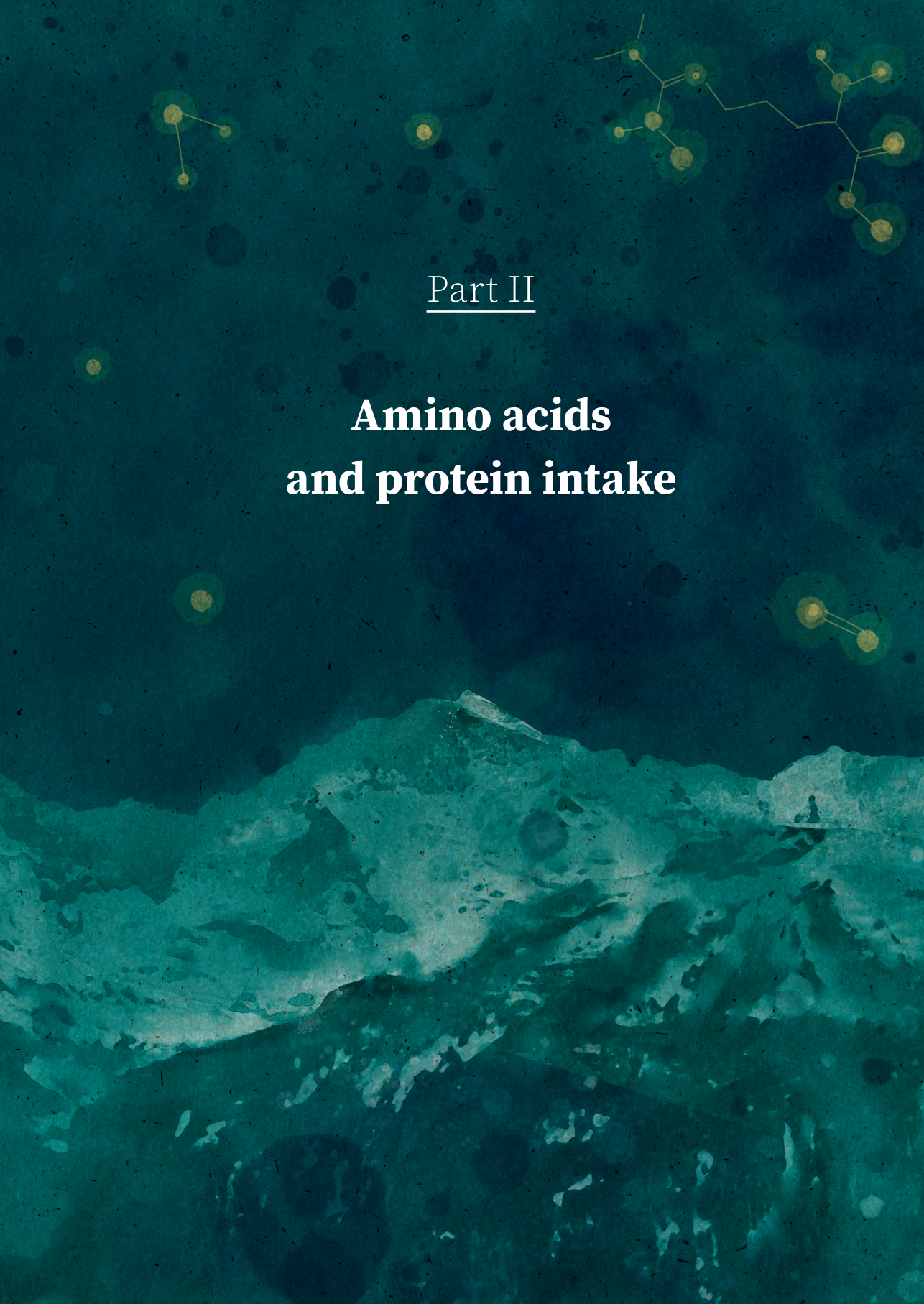


Figure S1. Flow diagram of participant inclusion.





Part II

Amino acids and protein intake