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Original article

Urinary 3-hydroxyisovaleryl carnitine excretion, protein energy malnutrition and risk of all-cause mortality in kidney transplant recipients: Results from the TransplantLines cohort studies

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SUMMARY

Background: Leucine is an essential amino acid and a potent stimulator of muscle protein synthesis. Since muscle wasting is a major risk factor for mortality in kidney transplant recipients (KTR), dietary leucine intake might be linked to long-term mortality. Urinary 3-hydroxyisovaleryl carnitine (3-HIC) excretion, a functional marker of marginal biotin deficiency, may also serve as a marker for dietary leucine intake.
Objective: In this study we aimed to investigate the cross-sectional determinants of urinary 3-HIC excretion and to prospectively investigate the association of urinary 3-HIC excretion with all-cause mortality in KTR.
Design: Urinary 3-HIC excretion and plasma biotin were measured in a longitudinal cohort of 694 stable KTR. Cross-sectional and prospective analyses were performed using ordinary least squares linear regression analyses and Cox regression analyses, respectively.
Results: In KTR (57% male, 53 $\pm$ 13 years, estimated glomerular filtration rate 45 $\pm$ 19 mL/min/1.73 m^2), urinary 3-HIC excretion (0.80 [0.57−1.16] μmol/24 h) was significantly associated with plasma biotin (std. $\beta = -0.17$; P < 0.001). Subsequent adjustment for potential covariates revealed urinary creatinine excretion (std. $\beta = 0.24$; P < 0.001) and urinary urea excretion (std. $\beta = 0.53$; P < 0.001) as the primary determinant of urinary 3-HIC excretion. Whereas plasma biotin explained only 1% of the variance in urinary 3-HIC excretion, urinary urea excretion explained >45%. During median follow-up for 5.4 [4.8 −6.1] years, 150 (22%) patients died. Log$_2$-transformed urinary 3-HIC excretion was inversely associated with all-cause mortality (HR: 0.52 [0.43−0.63]; P < 0.001). This association was independent of potential confounders.
Conclusions: Urinary 3-HIC excretion more strongly serves as a marker of leucine intake than of biotin status. A higher urinary 3-HIC excretion is associated with a lower risk of all-cause mortality. Future studies are warranted to explore the underlying mechanism.
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1. Introduction

Kidney transplantation is the preferred treatment for end-stage renal disease and status after kidney transplantation becomes more prevalent each year [1]. In the past decades, advances in transplantation medicine have increased short-term survival after transplantation [2]. Unfortunately, long-term survival remains largely unchanged and kidney transplant recipients (KTR) continue

to be at increased risk of premature mortality [2–4]. A major threat throughout the course of chronic kidney disease is muscle wasting [5]. Though most prevalent and pronounced in patients on hemodialysis treatment [6,7], it can remain after transplantation, with studies suggesting that up to 15% of the KTR display signs of protein-energy malnutrition (PEM) [8,9]. The precise mechanisms of muscle wasting remain to be elucidated, but the final common pathway includes increased protein degradation and decreased protein synthesis [6,10]. Therefore, the mainstay of preventing PEM is provision of an adequate protein supply, as a higher protein intake is associated with both improved patient survival and graft survival in KTR [11]. Amongst the amino acids deriving from protein intake, leucine may be of special interest as it is considered the most potent stimulator of muscle protein synthesis [12]. A higher dietary leucine intake, alone or along with an increased protein intake, could therefore potentially improve long-term survival in KTR.

KTR also suffer from dysbiosis, a disruption in the balance of the gut microbiome, in the years following transplantation [13]. Due to the chronic usage of immunosuppressive medications and frequent usage of antibiotics, KTR may also be susceptible to deficiencies of vitamins produced by microbiota, including biotin [14,15]. Though absolute biotin deficiency is rare, marginal biotin deficiency has been reported under varying circumstances, including malnutrition [16]. Urinary excretion of 3-hydroxyisovaleryl carnitine (3-HIC) is considered a functional marker for marginal biotin deficiency [17–19], since reduced activity of one of the biotin-dependent enzymes, 3-methylcrotonyl-CoA carboxylase (MCC), increases the plasma concentration and urinary excretion of 3-hydroxyisovaleryl carnitine (Fig. 1) [20,21]. However, since 3-HIC production is inseparably linked to leucine metabolism, we hypothesize that 3-HIC excretion is determined not only by biotin insufficiency, but also by dietary leucine intake, especially in cases where biotin status is adequate.

In the current study, we have measured urinary 3-HIC excretion and plasma biotin in a large cohort of stable KTR and in healthy controls, allowing us 1) to compare urinary 3-HIC excretion and plasma biotin between kidney transplant recipients and healthy controls, 2) to investigate the cross-sectional determinants of 3-HIC excretion in KTR and 3) to prospectively investigate the association of urinary 3-HIC excretion with all-cause mortality in KTR. Secondarily, we performed survival analyses for plasma biotin.

2. Materials and methods

2.1. Study population

This observational prospective study was conducted in a large single-center KTR cohort, as previously described [22,23]. In short, all adult (≥18 years old) KTR without known or apparent systemic illnesses (i.e., malignancies, opportunistic infections) who visited the outpatient clinic of the University Medical Center Groningen between November 2008 and June 2011 were invited to participate in this prospective cohort study. KTR were all transplanted at the University Medical Center Groningen and had no history of drug or alcohol addiction. Of 817 initially invited KTR, 706 (87%) signed written informed consent to participate in this study. We excluded subjects with missing data on plasma biotin or urinary 3-hydroxyisovaleryl carnitine excretion, i.e. 12 cases, from the statistical analyses, which resulted in 694 cases eligible for analyses.

Leucine

↓

2-Ketoisocaproate

↓

Isovaleryl CoA

↓

3-Methylcrotonyl CoA ⟶ **3-hydroxyisovaleryl CoA**

❌ *Biotin dependent carboxylase*   ↓

↓   **3-hydroxyisovaleryl carnitine**

3-Methylglutaconyl CoA

↓

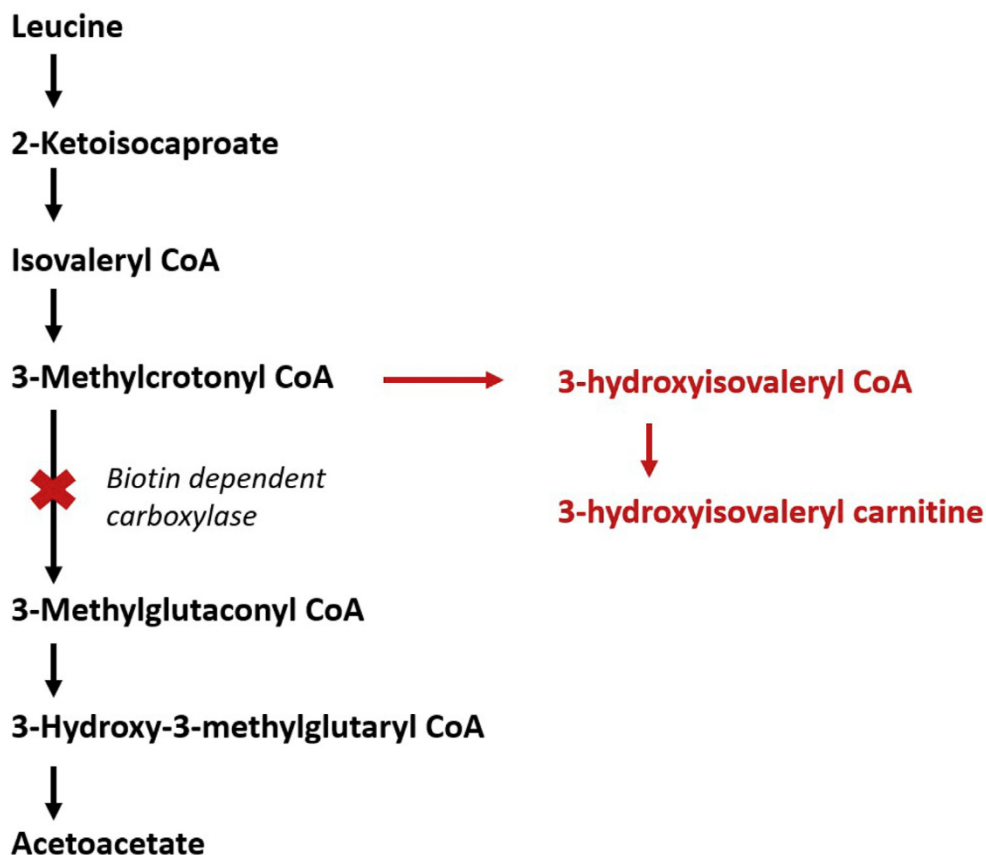**3-Hydroxy-3-methylglutaryl CoA**

↓

Acetoacetate

Fig. 1. Simplified schematic of leucine metabolism. In a state of biotin deficiency, improper functioning of the biotin-dependent carboxylase 3-methylcrotonyl CoA (MCC) leads to accumulation of the metabolite 3-methylcrotonyl CoA. Subsequently, flux through the alternative pathway, leading to production of 3-hydroxyisovaleryl carnitine, is increased.

To compare urinary 3-hydroxyisovaleryl carnitine excretion and plasma biotin of KTR with healthy controls, we included 236 kidney donors of whom biomaterial was collected before kidney donation. The study protocol was approved by the University Medical Center Groningen institutional ethical review board (Medical ethical committee 2008/186) and adhered to the Declarations of Helsinki.

2.2. Clinical parameters

All measurements were performed during a morning visit to the outpatient clinic after an 8- to 12-h overnight fasting period. Blood pressure was measured (in millimeters of mercury) with a semiautomatic device (Dinamap 1846; Critikon, Tampa, FL) according to a strict protocol as previously described [23]. Information on health status, medical history, and medication use was obtained from patient records. Information on smoking behavior and alcohol intake was obtained from a questionnaire. Participants were classified as current, former, or never smokers. Alcohol intake was split into 0–10 g/24 h, 10–30 g/24 h and >30 g/24 h. Body weight and height were measured with participants wearing indoor clothing without shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared and body surface area (BSA) was calculated using the formula of Du Bois and Du Bois [24]. Diabetes mellitus was diagnosed according to American Diabetes Association criteria (2017) as having a fasting plasma glucose concentration ≥7.0 mmol/L or the use of antidiabetic medication [25]. Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg.

2.3. Laboratory measurements

All participants were instructed to collect a 24-h urine sample according to a strict protocol at the day before their visit to the outpatient clinic. Urine was collected under oil and chlorhexidine was added as an antiseptic agent. Upon completion of the 24-h urine collection, fasting venous blood samples anti-coagulated with lithium-heparin, sodium-fluoride and potassium-EDTA were obtained the following morning. Urinary 3-HIC concentrations were analyzed by hydrophilic interaction chromatography coupled with a triple quadrupole mass spectrometry analyzer (HPLC-MS/MS). Urine samples were diluted in acetonitrile (1:10) and separated using a using a Luna HILIC (3 μm pore size, 100 × 2.0 mm (Phenomenex, Utrecht, the Netherlands)) analytical column. 3-HIC was detected using an Sciex API3200 TripleQuad mass spectrometer with positive-ion electrospray ionization (Sciex, Nieuwerkerk aan den Ijssel, the Netherlands) in multiple reaction monitoring mode, using the following transitions: m/z 262.2 -> 85.0 for 3-hydroxyisovaleryl carnitine and m/z 265.2 -> 85.0 for the deuterium labeled internal standard of 3-hydroxyisovaleryl carnitine (N-methyl-D3). The inter-assay precisions were 4.4% at 0.2 μmol/L and 3.4% at 12.8 μmol/L. The lower limit of quantitation was 62 nmol/L. All measurements were above the lower limit of quantitation. Plasma biotin was measured using an enzyme linked immunoassay (ELISA), according to the manufacturers protocol (K8141; Immundiagnostik, Bensheim, Germany). The inter-assay precisions were 4.8% at 492 ng/L and 10.9% at 149 ng/L.

Urinary protein concentration was determined by means of the Biuret reaction (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany). Proteinuria was defined as urinary protein excretion ≥0.5 g/24 h. For routine clinical chemistry assays, including lipid, inflammation, and glucose homeostasis, measurements were performed using automated and validated routine spectrophotometric-based methods (Roche Diagnostics, Basel, Switzerland). Serum cystatin C was measured with the Gentian

Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Roche Modular analyzer and was calibrated directly with the standard supplied by the manufacturer. HLA-I and HLA-II antibodies were quantified using an ELISA (LATM205, One Lambda, Canoga Park, CA). Renal function was assessed by the estimated glomerular filtration rate (eGFR) based on the Chronic Kidney Disease Epidemiology Collaboration Creatinine Cystatin C (CKD-EPI-sCr-CysC) equation [26,27].

2.4. Statistical analysis

Data analyses and computations were performed with SPSS 24.0 software (IBM, Armonk, NY, USA), Stata SE version 15 (StataCorp, College Station, TX, USA), R version 3.6.2 software (The R-Foundation for Statistical Computing), and GraphPad Prism version 7 (GraphPad Software). Baseline data are presented as means ± standard deviation for normally distributed data, as medians [interquartile range] for non-normally distributed data, and as numbers (percentages) for nominal data. A two-sided $P < 0.05$ was considered to indicate statistical significance. Differences between KTR and healthy controls were tested with a t-test for independent samples, the Mann–Whitney U test, or the chi-squared test. Difference in baseline variables amongst sex-stratified tertiles of urinary 3-HIC excretion were studied using ANOVA, Kruskal–Wallis or chi-squared tests. To investigate the association between urinary 3-HIC excretion and plasma biotin, ordinary least squares linear regression analyses were employed. In the following model we adjusted for the a priori selected potential confounders age, sex and BMI (model 2). Subsequently, the model was further adjusted for eGFR (model 3), urinary urea excretion (model 4) and urinary creatinine excretion (model 5). The improvement in fit for the data after addition of new covariates was reported using the Bayesian information criterion (BIC) and tested using a likelihood-ratio test. Lower BIC values indicate a better model fit for the data. For all models, the adjusted R^2 was calculated to determine the proportion of the variance of urinary 3-HIC excretion explained by the covariates. In addition, partial R^2 values were reported for each covariate to demonstrate the proportion explained by the individual covariates. Regression coefficients were given as standardized beta values, the latter referring to the number of standard deviations a dependent variable changes per standard deviation increase of the independent variable, thereby allowing for comparison of the strength of the associations of different variables.

Primary prospective analyses were performed for all-cause mortality. Secondary prospective analyses were performed for cause-specific mortality. Cardiovascular mortality was defined as death that was due to cerebrovascular disease, ischemic heart disease, heart failure, or sudden cardiac death according to ICD-9 codes 410–447 [28]. Cancer mortality was defined according to a previously specified list of International Classification of Diseases, Ninth Revision (ICD-9) codes [29], whereas infectious disease mortality was identified according to ICD-9 codes 1–139 [30]. Unless stated otherwise, mortality data were not censored for occurrence of graft failure. The continuous surveillance system of the outpatient program ensured that there was up-to-date information on patient status. Endpoints were recorded until September 2015 by a qualified physician. There was no loss that was due to follow-up for the primary endpoints. Initial prospective analyses were performed by means of a Kaplan–Meier curves plot of sex-stratified tertiles of urinary 3-HIC excretion for all-cause mortality, and significance was assessed using the log-rank test. To study whether $\log_2$-transformed urinary 3-HIC excretion was prospectively and independently associated with all-cause mortality, multivariable-adjusted Cox proportional

hazards regression models were fitted to the data. Secondarily, analyses were also performed for sex-stratified tertiles of plasma biotin. The Schoenfeld residuals were inspected to determine that the proportionality of hazards assumption was not violated. Adjustments were made for a priori selected variables and for potentially relevant variables identified from the baseline table by a $P < 0.05$. A priori selected variables were basic potential confounders, including age, sex, BMI and estimated GFR (model 3). To avoid overfitting and inclusion of too many variables for the number of events, additional models were created using additive adjustments to model 3 [31]. Potentially relevant variables identified from the baseline table included NT-ProBNP and HDL-cholesterol (model 4), warm ischemia time, proteinuria and hs-CRP (model 5), plasma albumin (model 6), urinary urea excretion (model 7) and urinary creatinine excretion (model 8). The percentage change in hazard ratio was calculated as: (HR before adjustment − HR after adjustment)/(HR before adjustment − 1)* 100 [32].

In separate analyses, we also adjusted for relevant comorbidities, including hypertension, diabetes, medical history of coronary intervention, myocardial infarction, cerebrovascular accident (CVA) and/or transient ischemic attack and primary renal disease.

Mediation analyses was performed using the mediation package in R, according to the method as described by Preacher and Hayes [33], which allowed for testing the significance and magnitude of (potential) mediation. In these analyses, mediation was assessed upon running 2000 Monte Carlo draws for quasi-Bayesian approximation. The proportion of mediation was obtained by dividing the standardized indirect effect coefficient by the standardized total effect coefficient, which were in each model adjusted for age, sex, eGFR and BMI.

Potential interactions for covariates were assessed by calculating interaction term, P-interaction<0.05 was considered to indicate significant effect-modification. Stratified Cox proportional hazards regression analyses were performed to assess the association of urinary 3-HIC excretion with risk of all-cause according to significant effect-modifiers.

To visualize the continuous associations of urinary 3-HIC excretion with all-cause mortality, log_2-transformed urinary 3-HIC excretion, as a continuous variable, was plotted against the risk of all-cause mortality.

As sensitivity analyses, we also investigated the association of urinary 3-HIC excretion with the long-term outcomes graft failure censored all-cause mortality, death-censored graft failure and graft loss (defined as graft failure or mortality). Graft failure was defined as return to dialysis or need for a retransplantation and graft loss was defined as a combined endpoint of death with a functioning graft and graft failure.

3. Results

3.1. KTR and healthy controls

A total of 694 patients (53 ± 13 years, 57% male, eGFR 45 ± 19 ml/min/1.73 m^2) were included at a median 5.3 [1.8−11.9] years after kidney transplantation. A group of 236 kidney donors were used as healthy controls (54 ± 11 years, 46% male, eGFR 93 ± 16 ml/min/1.73 m^2). In KTR, urinary 3-HIC concentration was 1.90 [1.39−2.61] μmol/24 h. Compared to KTR, urinary 3-HIC excretion was significantly higher ($P < 0.001$) in healthy controls, being 2.57 [1.75−3.87] μmol/24 h. Plasma biotin was 451 [346−609] ng/L in KTR, whereas it was 239 [171−343] ng/L in healthy controls ($P < 0.001$). Characteristics of KTR versus healthy controls are shown in Supplementary Table S1.

3.2. Baseline characteristics

Differences in baseline characteristics amongst sex-stratified tertiles of urinary 3-HIC excretion in KTR are shown in Table 1. Patients in the lowest sex-stratified tertile of urinary 3-HIC excretion had higher plasma biotin, NT-ProBNP, serum creatinine, serum cystatin C, proteinuria and plasma hs-CRP (all $P < 0.05$) compared to those in the higher sex-stratified tertiles. In contrast, patients in the lowest sex-stratified tertile of urinary 3-HIC excretion had lower weight, BMI, BSA, eGFR and lower urinary excretions of urea and creatinine (all $P < 0.05$). Sex-stratified tertiles of urinary 3-HIC excretion also differed in warm ischemia time and HDL-cholesterol. There were no significant differences in age, primary renal disease, smoking status, alcohol consumption, height, hemodynamics, glucose homeostasis and in transplantation-related factors, apart from warm ischemia time. A comparison of baseline characteristics between survivors and non-survivors is shown in Supplementary Table S2.

3.3. Linear regression analyses

Linear regression analyses of urinary 3-HIC excretion are shown in Table 2. Univariably, there was an inverse association between urinary 3-HIC excretion and plasma biotin (std. $\beta = -0.17$; $P < 0.001$; $R^2 = 0.026$). Addition of age, sex and BMI to the model significantly improved the model fit ($R^2 = 0.12$; BIC: 1938 versus 1887; $P < 0.001$). Besides plasma biotin, sex (std. β for male sex = 0.26; $P < 0.001$) and BMI (std. $\beta = 0.18$; $P < 0.001$) were strongly associated with urinary 3-HIC excretion. Subsequent adjustment for eGFR further improved the model fit ($R^2 = 0.20$; BIC: 1887 versus 1826; $P < 0.001$) with eGFR positively associating with urinary 3-HIC excretion (std. $\beta = 0.31$; $P < 0.001$). The adjustment for eGFR weakened the association of plasma biotin with urinary 3-HIC excretion (std. $\beta = -0.07$; $P = 0.011$). Adjustment for urinary urea excretion greatly increased the model fit ($R^2 = 0.56$; BIC: 1826 versus 1416; $P < 0.001$) and urinary urea excretion was strongly associated with urinary 3-HIC excretion (std. $\beta = 0.66$; $P < 0.001$) with a partial R^2 of 0.46. In the same model, the partial R^2 of plasma biotin was 0.01. To investigate whether urinary 3-HIC excretion is associated with muscle mass, additional adjustments were made for urinary creatinine excretion. Urinary creatinine excretion was positively associated with urinary 3-HIC excretion (std. $\beta = 0.24$; $P < 0.001$) and moderately increased the model fit ($R^2 = 0.58$; BIC: 1416 versus 1313; $P < 0.001$). A significant interaction between plasma biotin and urea excretion in the association with urinary 3-HIC excretion was found ($P < 0.001$).

3.4. Urinary 3-HIC excretion and mortality

During a median follow-up of 5.4 [4.8−6.1] years, 150 (22%) KTR died, of whom 60 (40%) died from a cardiovascular cause, 25 died from cancer (17%), 42 (28%) died from infectious disease and 23 (15%) died from other (miscellaneous) causes. Patients who died had lower urinary 3-HIC excretion compared to those who survived (1.52 [1.08−2.19] μmol/24 h versus 2.07 [1.47−2.75] μmol/24 h; $P < 0.001$). Over sex-stratified tertiles of urinary 3-HIC excretion, 79 (34%) patients died in the lowest tertile, 42 (18%) in the middle tertile and 29 (13%) in the highest tertile, log-rank test $P < 0.001$. Kaplan Meier curves for sex-stratified tertiles of urinary 3-HIC excretion are shown in Fig. 2. Cox regression analyses for sex-stratified tertiles of urinary 3-HIC excretion and for log_2-transformed urinary 3-HIC excretion as a continuous variable are shown in Table 3. Compared to the lowest sex-stratified tertiles, patients in the middle tertile (HR: 0.46 [0.31−0.66]; $P < 0.001$) and highest tertile (HR: 0.32 [0.21−0.48]; $P < 0.001$) had lower risk of all-cause

Table 1
Kidney transplant recipients characteristics according to sex-stratified tertiles of urinary 3-hydroxyisovaleryl carnitine excretion (μmol/24 h).

	KTR cohort n = 694 0.16–1.80 μmol/24 h	Tertile 1 n = 230 ♂: <1.80 ♀: <1.36 μmol/24 h	Tertile 2 n = 232 ♂: 1.80–2.60 ♀: 1.36–1.92 μmol/24 h	Tertile 3 n = 232 ♂: >2.60 ♀: >1.92 μmol/24 h	P value[a]
Biotin					
Plasma biotin, ng/L	451 [346–609]	496 [352–655]	447 [344–609]	430 [343–558]	**<0.001**
Plasma biotin <200 ng/L	30 (4)	7 (3)	11 (5)	12 (5)	0.49
Plasma biotin <100 ng/L	4 (0.6)	1 (0.4)	1 (0.4)	2 (0.9)	0.78
Demographics					
Age, years	53 ± 13	52 ± 13	54 ± 13	53 ± 12	0.22
Sex, n (%) male	395 (57)	131 (57)	132 (57)	132 (57)	1.00
Smokers, n (%)					
Never	273 (42)	92 (42)	96 (44)	85 (40)	0.56
Past	294 (45)	93 (43)	98 (45)	103 (47)	
Current	83 (13)	32 (15)	22 (10)	29 (13)	
Alcohol					
0–10 g/24 h	468 (74)	163 (79)	152 (73)	153 (70)	0.16
10–30 g/24 h	135 (21)	37 (18)	46 (22)	52 (24)	
>30 g/24 h	30 (5)	6 (3)	9 (4)	15 (7)	
Body composition					
Weight, kg	80 ± 17	77 ± 16	81 ± 17	83 ± 16	**<0.001**
Height, cm	174 ± 10	173 ± 10	174 ± 10	174 ± 10	0.14
BMI, kg/m²	26.6 ± 4.8	25.7 ± 4.5	26.8 ± 4.9	27.4 ± 4.8	**<0.001**
BSA, m²	1.94 ± 0.22	1.90 ± 0.22	1.95 ± 0.22	1.98 ± 0.21	**<0.001**
Primary renal disease, n (%)					
Primary glomerulosclerosis	196 (28)	62 (27)	66 (28)	68 (29)	0.85
Glomerulonephritis	53 (8)	16 (7)	18 (8)	19 (8)	0.88
Tubulointerstitial nephritis	83 (12)	28 (12)	30 (13)	25 (11)	0.77
Polycystic kidney disease	144 (21)	48 (21)	50 (22)	46 (20)	0.90
Hypo- or dysplasia	27 (4)	10 (4)	8 (3)	9 (4)	0.88
Renovascular disease	39 (6)	15 (7)	13 (6)	11 (5)	0.71
Diabetes	35 (5)	15 (7)	13 (6)	7 (3)	0.20
Cardiovascular parameters					
Systolic blood pressure, mmHg	136 ± 17	138 ± 18	136 ± 17	134 ± 17	0.09
Diastolic blood pressure, mmHg	83 ± 11	83 ± 11	83 ± 11	82 ± 11	0.81
Mean arterial pressure, mmHg	107 ± 15	108 ± 14	107 ± 15	106 ± 14	0.29
Pulse pressure, mmHg	53 ± 13	55 ± 14	54 ± 13	52 ± 12	0.06
Heart rate, bpm	69 ± 12	70 ± 12	68 ± 12	68 ± 12	0.40
Hypertension[b], n (%)	283 (41)	105 (46)	96 (42)	82 (35)	0.07
Antihypertensive drugs, n (%)	612 (88)	209 (91)	200 (86)	203 (88)	0.28
NT-proBNP, ng/L	254 [108–623]	364 [146–907]	239 [111–618]	177 [75–423]	**<0.001**
Lipids					
Total cholesterol, mmol/L	5.1 ± 1.1	5.2 ± 1.2	5.1 ± 1.1	5.1 ± 1.0	0.84
HDL cholesterol, mmol/L	1.4 ± 0.5	1.3 ± 0.5	1.4 ± 0.5	1.4 ± 0.5	**0.04**
LDL cholesterol, mmol/L	2.9 ± 0.9	3.0 ± 1.0	3.0 ± 0.9	3.0 ± 0.9	0.97
Triglycerides, mmol/L	1.68 [1.24–2.29]	1.80 [1.27–2.48]	1.61 [1.19–2.22]	1.67 [1.26–2.24]	0.14
Statins, n (%)	367 (53)	121 (53)	126 (54)	120 (52)	0.85
Glucose homeostasis					
Glucose, mmol/L	5.3 [4.8–6.0]	5.3 [4.8–6.2]	5.2 [4.7–5.8]	5.3 [4.8–6.2]	0.19
HbA₁c, %	5.8 [5.5–6.2]	5.8 [5.5–6.3]	5.8 [5.5–6.2]	5.8 [5.5–6.2]	0.74
Diabetes, n (%)	166 (24)	61 (27)	52 (22)	53 (23)	0.52
Antidiabetic drugs, n (%)	107 (15)	43 (17)	36 (16)	28 (12)	0.28
Cardiovascular history					
Coronary intervention	55 (8)	21 (9)	22 (9)	12 (5)	0.16
Myocardial infarction	35 (5)	14 (6)	11 (5)	10 (4)	0.66
CVA and/or TIA	41 (6)	11 (5)	19 (8)	11 (5)	0.20
Transplantation-related					
Dialysis vintage, months	27 [9–52]	26 [8–60]	25 [10–48]	28 [10–53]	0.72
Time since transplantation, years	5.3 [1.8–11.9]	5.3 [1.7–12.0]	5.9 [2.3–13.2]	5.1 [1.5–10.9]	0.36
Deceased donor, n (%)	457 (66)	165 (72)	145 (63)	147 (63)	0.07
Cold ischemia time, hours	15.3 [2.8–21.1]	16.0 [3.3–22.0]	14.2 [2.6–21.0]	15.6 [2.7–21.5]	0.16
Warm ischemia time, minutes	43 ± 15	45 ± 16	41 ± 15	43 ± 14	**0.03**
Transplantations up to baseline, n (%)					
1 transplantation	625 (90)	203 (88)	210 (91)	212 (91)	0.48
≥2 transplantations	68 (10)	27 (12)	21 (9)	20 (9)	
Calcineurin inhibitors, n (%)	397 (57)	139 (60)	132 (57)	126 (54)	0.41
Proliferation inhibitors, n (%)	578 (83)	192 (83)	184 (79)	202 (87)	0.08
HLA antibodies, n (%)					
HLA-I	106 (15)	34 (15)	35 (15)	37 (16)	0.94
HLA-II	120 (17)	43 (19)	41 (18)	36 (16)	0.65
Renal function					
Serum creatinine, μmol/L	112 [97–141]	141 [115–188]	123 [97–155]	112 [97–141]	**<0.001**
Serum cystatin C, mg/L	1.7 [1.3–2.2]	2.1 [1.6–2.8]	1.7 [1.3–2.1]	1.5 [1.2–1.9]	**<0.001**

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Table 1 (*continued*)

	KTR cohort n = 694 0.16–1.80 µmol/24 h	Tertile 1 n = 230 ♂: <1.80 ♀: <1.36 µmol/24 h	Tertile 2 n = 232 ♂: 1.80–2.60 ♀: 1.36–1.92 µmol/24 h	Tertile 3 n = 232 ♂: >2.60 ♀: >1.92 µmol/24 h	P value[a]
eGFR, mL/min/1.73 m^{2c}	45 ± 19	38 ± 18	46 ± 18	51 ± 18	**<0.001**
Proteinuria, n (%)	156 (22)	70 (30)	54 (23)	32 (14)	**<0.001**
Venous parameters					
Sodium, mmol/L	141 ± 3	141 ± 3	141 ± 3	141 ± 3	0.42
Chloride, mmol/L	105 ± 3	106 ± 4	105 ± 3	105 ± 3	0.29
Albumin, g/L	43.0 ± 3.0	42.3 ± 3.4	43.2 ± 2.7	43.5 ± 2.7	**<0.001**
hs-CRP, mg/L	1.5 [0.6–3.8]	1.9 [0.8–5.8]	1.4 [0.6–4.0]	1.5 [0.6–3.8]	**0.03**
Urinary excretion					
Creatinine, mmol/24 h	11.6 ± 3.5	9.8 ± 2.9	11.9 ± 3.4	13.2 ± 3.2	**<0.001**
Urea, mmol/24 h	388 ± 115	304 ± 86	386 ± 81	473 ± 106	**<0.001**

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

[a] Evidence against the null hypothesis of no differences amongst sex-stratified tertiles of 3-hydroxyisovaleryl carnitine excretion, tested using ANOVA, Kruskal–Wallis or chi-squared tests. Bold indicates statistical significance (P value < 0.05).

[b] Hypertension defined as systolic blood pressure >140 and/or diastolic blood pressure >90.

[c] Assessed using the CKD-EPI formula based on creatinine and cystatin C.

mortality. In continuous analyses, $\log_2$-transformed urinary 3-HIC excretion was inversely associated with all-cause mortality (HR: 0.52 [0.43–0.63]; P < 0.001). These associations were independent of potential confounders, including age, sex, BMI, eGFR, NT-ProBNP, HDL-cholesterol, warm ischemia time, proteinuria, hs-CRP, urinary sodium excretion and plasma albumin. Adjustments for urinary urea excretion and urinary creatinine excretion weakened the hazard ratios by 20.8 and 43.8%, respectively. Nonetheless, the association of urinary 3-HIC excretion with all-cause mortality remained significant throughout adjustments.

The association of urinary 3-HIC excretion with all-cause mortality also remained significant throughout adjustments for comorbidities, including hypertension, diabetes, cardiovascular history, and primary renal disease (Supplementary Table S3).

To further explore the association with mortality, we performed cause-specific Cox regression analyses, of which results are shown in Supplementary Table S4. Higher urinary 3-HIC excretion was significantly associated with a reduced risk of cardiovascular

mortality (HR: 0.57 [0.42–0.77]; P < 0.001), infectious mortality (HR: 0.48 [0.34–0.69]; P < 0.001) and mortality from miscellaneous causes (HR: 0.41 [0.26–0.64]; P < 0.001), but not significantly with cancer mortality (HR: 0.64 [0.39–1.03]; P = 0.07). The significant associations with cardiovascular, infectious mortality and mortality from miscellaneous causes remained materially unchanged after adjustment for potential confounders, while the non-significant associations with cancer mortality remained non-significant.

Mediation analyses demonstrated that urinary creatinine excretion explained 45% of the association found between urinary 3-HIC excretion and all-cause mortality (Table 4). Alternatively, urinary 3-HIC excretion itself explained 61% of the association between urinary urea excretion and all-cause mortality.

Significant interactions in the association of urinary 3-HIC excretion with all-cause mortality were found for sex (P = 0.02), eGFR (P = 0.05), proteinuria (P = 0.04) and NT-ProBNP (P = 0.04). Further stratified analyses by subgroup of patients according to sex, eGFR (cut-off 36 ml/min/1.73 m^2), proteinuria and NT-ProBNP (cut-

Table 2
Multivariable linear regression analyses of nested models for the determinants of urinary 3-hydroxyisovalerylcarnitine excretion.

Model	Variables	Std. β	95% CI of Std. β	P value	Partial R^2	Adjusted R^2	BIC	P$_{comparison}$[a]
1	Plasma biotin	−0.17	−0.24−−0.91	<0.001	0.03	0.03	1938	—
2	Plasma biotin	−0.18	−0.25−−0.11	<0.001	0.04	0.12	1887	<0.001
	Sex, male	0.26	0.33−0.19	<0.001	0.07			
	Age	0.01	−0.06−0.08	0.74	0.01			
	BMI	0.17	0.10−0.24	<0.001	0.03			
3	Plasma biotin	−0.07	−0.14−0.01	0.07	0.01	0.20	1826	<0.001
	Sex	0.25	0.19−0.32	<0.001	0.07			
	Age	0.04	−0.03−0.10	0.31	0.01			
	BMI	0.18	0.11−0.25	<0.001	0.04			
	eGFR	0.31	0.24−0.38	<0.001	0.09			
4	Plasma biotin	−0.07	−0.13−−0.02	0.007	0.01	0.56	1416	<0.001
	Sex	0.04	−0.01−0.10	0.10	0.01			
	Age	0.03	−0.02−0.08	0.27	0.01			
	BMI	0.05	−0.01−0.10	0.07	0.01			
	eGFR	0.20	0.14−0.25	<0.001	0.07			
	Urinary urea excretion	0.66	0.61−0.71	<0.001	0.46			
5	Plasma biotin	−0.06	−0.11−−0.01	0.03	0.01	0.58	1390	<0.001
	Sex	−0.05	−0.11−0.01	0.12	0.01			
	Age	0.09	0.04−0.15	0.001	0.02			
	BMI	0.01	−0.05−0.05	0.95	0.01			
	eGFR	0.20	0.14−0.25	<0.001	0.07			
	Urinary urea excretion	0.53	0.46−0.60	<0.001	0.25			
	Urinary creatinine excretion	0.24	0.16−0.33	<0.001	0.05			

Urinary 3-HIC excretion and plasma biotin were $\log_2$ transformed before analysis. Abbreviations: BIC, Bayesian information criterion; std. β, standardized regression coefficient; eGFR, estimated glomerular filtration rate; BMI, body mass index.

[a] Evidence against the null hypothesis of no improvement in fit for the data by the current model, compared to the previous model, based upon a likelihood-ratio test.
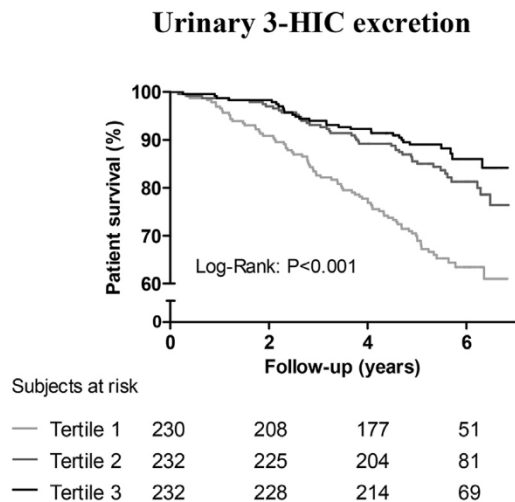
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Urinary 3-HIC excretion

Fig. 2. Kaplan–Meier curves for all-cause mortality according to sex-stratified tertiles of urinary 3-HIC excretion.

off 620 ng/L) showed that the association of urinary 3-HIC excretion with all-cause mortality was stronger within females, patients with a high eGFR, patients without proteinuria and patients with a low NT-ProBNP (Fig. 3). The associations of log$_2$-transformed urinary 3-HIC excretion with all-cause mortality, crude and adjusted for age, sex, BMI, eGFR and plasma albumin, are visualized in Fig. 4.

Sensitivity analyses in which we investigated the association of urinary 3-HIC excretion with graft failure censored all-cause mortality, death-censored graft failure and graft loss are shown in Supplementary Table S5. Urinary 3-HIC excretion was associated with graft failure censored all-cause mortality (HR: 0.50 [0.40–0.62]; P < 0.001), death-censored graft failure (HR: 0.44 [0.34–0.56]; P < 0.001) and graft loss (HR: 0.47 [0.40–0.58]; P < 0.001). The associations with graft failure censored all-cause mortality and graft loss remained independent of potential confounders (all models P < 0.001), while the association with death-censored graft failure lost statistical significance in models 4 to 6.

3.5. Plasma biotin and mortality

Patients who died did not have a significantly higher concentration of plasma biotin compared to those who survived (478 [334–637] ng/L versus 447 [351–592] ng/L; P = 0.37). Over sex-

stratified tertiles of plasma biotin, 35 (15%) patients died in the middle tertile, while 55 (24%) and 60 (26%) died in the lowest and highest tertiles, respectively (log-rank test P = 0.003). Kaplan Meier curves for sex-stratified tertiles of plasma biotin are shown in Fig. 5. Cox regression analyses for sex-stratified tertiles of plasma biotin are shown in Table 5. Compared to the middle tertile, patients in the lower tertile (HR: 1.65 [1.08–2.52]; P = 0.02) and higher tertile (1.92 [1.26–2.91]; P = 0.002) had increased risk of all-cause mortality. Adjustment for age, sex, BMI and eGFR increased the risk of all-cause mortality for patients in the lowest tertile (HR: 2.23 [1.44–3.44]; P < 0.001), but decreased the risk of all-cause mortality for patients in the highest tertile (HR: 1.54 [1.01–2.36]; P = 0.05). After further adjustment for potential confounders, the association of highest tertile lost significance. Patients in the lower tertile had increased risk of all-cause mortality, independent of further adjustments for potential confounders, including NT-ProBNP, HDL-cholesterol, warm ischemia time, proteinuria, hs-CRP and plasma albumin.

4. Discussion

In a large cohort of stable KTR, we demonstrated that urinary 3-HIC excretion is lower and plasma biotin concentration is higher in KTR compared to healthy controls. Subsequent analyses demonstrated that renal function, urinary creatinine excretion and, most notably, urinary urea excretion are the primary determinants of 3-HIC excretion in KTR. In prospective analyses, higher urinary 3-HIC excretion was associated with a lower risk of all-cause mortality, independent of potential confounders. Mediation analyses implicated a role of protein intake and muscle mass in the protective association between urinary 3-HIC excretion and long-term mortality. Secondary analyses for plasma biotin demonstrated that patients in the lowest sex-stratified tertile of plasma biotin had an increased risk of mortality.

Improvements in surgical techniques, immunosuppressant drugs and postoperative care have steadily improved the one-year survival rate. Yet, the long-term survival of KTR has disappointingly remained almost unchanged over the years [34]. One of the more important risk factors for poor long-term survival in KTR is muscle wasting [35–37], which is believed to be the result of a state of both increased protein degradation and decreased protein synthesis [10]. Thus, maintaining an adequate supply of protein may be of great importance. Previous studies in KTR have demonstrated that a higher protein intake is associated with improved long-term outcomes [11]. In this respect, leucine, one of the essential amino acids, is of special interest as it is a potent stimulator of muscle protein synthesis [12,38–40]. Both under the circumstance of high

Table 3
Cox regression analyses of the associations of log$_2$-transformed urinary 3-hydroxyisovalerylcarnitine excretion with all-cause mortality.

	Sex stratified tertiles of urinary 3-HIC excretion					Continuous analyses of urinary 3-HIC excretion	
	I	II		III			
		HR [95% CI]	P-value	HR [95% CI]	P-value	HR [95% CI]	P-value
Model 1	Ref	0.46 [0.31–0.66]	<0.001	0.32 [0.21–0.48]	<0.001	0.52 [0.43–0.63]	<0.001
Model 2	Ref	0.38 [0.26–0.55]	<0.001	0.29 [0.19–0.44]	<0.001	0.44 [0.36–0.53]	<0.001
Model 3	Ref	0.46 [0.31–0.68]	<0.001	0.40 [0.25–0.63]	<0.001	0.52 [0.41–0.65]	<0.001
Model 4	Ref	0.53 [0.35–0.80]	0.002	0.45 [0.28–0.72]	0.001	0.55 [0.43–0.70]	<0.001
Model 5	Ref	0.51 [0.34–0.77]	0.001	0.45 [0.28–0.72]	0.001	0.55 [0.43–0.70]	<0.001
Model 6	Ref	0.52 [0.35–0.78]	0.001	0.46 [0.29–0.72]	0.001	0.56 [0.44–0.70]	<0.001
Model 7	Ref	0.56 [0.37–0.85]	0.007	0.62 [0.36–1.04]	0.07	0.62 [0.46–0.85]	0.002
Model 8	Ref	0.63 [0.42–0.96]	0.03	0.70 [0.42–1.17]	0.17	0.73 [0.55–0.97]	0.03

Urinary 3-hydroxyisovalerylcarnitine excretion was log$_2$ transformed for analyses. Model 1: crude. Model 2: adjusted for age, sex and BMI. Model 3; as model 2, additionally adjusted for eGFR. Model 4, as model 3, additionally adjusted for NT-ProBNP and HDL-cholesterol. Model 5, as model 3, additionally adjusted for warm ischemia time, proteinuria and hs-CRP. Model 6, as model 3, additionally adjusted for plasma albumin. Model 7, as model 3, additionally adjusted for urea excretion. Model 8, as model 3, additionally adjusted for creatinine excretion.

Table 4
Mediation analyses of the associations with all-cause mortality.

Predictor	Potential mediator	Effect (path)[a]	Multivariable model[b]		Proportion mediated[c]
			Coefficient (95% CI)	P-value	
3-HIC excretion	Creatinine excretion	Indirect	−0.03 (−0.05−−0.01)	0.002	45%
		Total	−0.07 (−0.10−−0.05)	<0.001	
3-HIC excretion	Urea excretion	Indirect	−0.02 (−0.04−0.01)	0.24	Not mediated
		Total	−0.07 (−0.10−−0.05)	<0.001	
Urea excretion	3-HIC excretion	Indirect	−0.04 (−0.07−−0.01)	0.004	61%
		Total	−0.06 (−0.09−−0.04)	<0.001	
Urea excretion	Creatinine excretion	Indirect	−0.05 (−0.07−−0.02)	<0.001	73%
		Total	−0.06 (−0.09−−0.04)	<0.001	

Excretion of 3-HIC, urea and creatinine each refer to excretion in 24 h urinary collections.
[a] For each path, the outcome is all-cause mortality.
[b] All coefficients are standardized coefficients with adjustments for age, sex, eGFR and BMI.
[c] The size of the significant mediated effect is calculated as the standardized indirect effect divided by the total effect multiplied by 100.
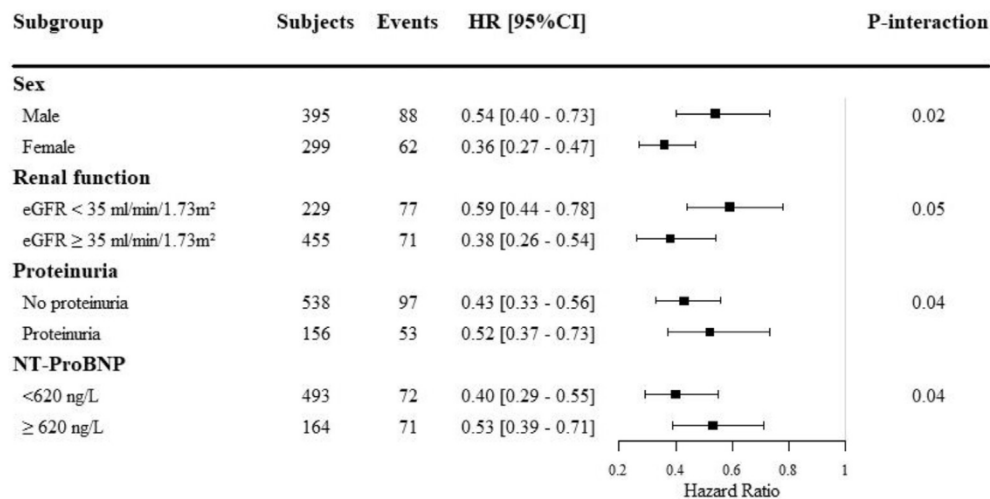

Fig. 3. Stratified analysis of the association of urinary 3-HIC excretion with risk of all-cause mortality in kidney transplant recipients. P-interaction<0.05 was considered to indicate significant effect-modification. Stratified Cox proportional hazards regression analyses were performed to assess the association of urinary 3-HIC excretion with risk of all-cause according to significant effect-modifiers. Coefficient estimates are shown with adjustment for age, sex and BMI.
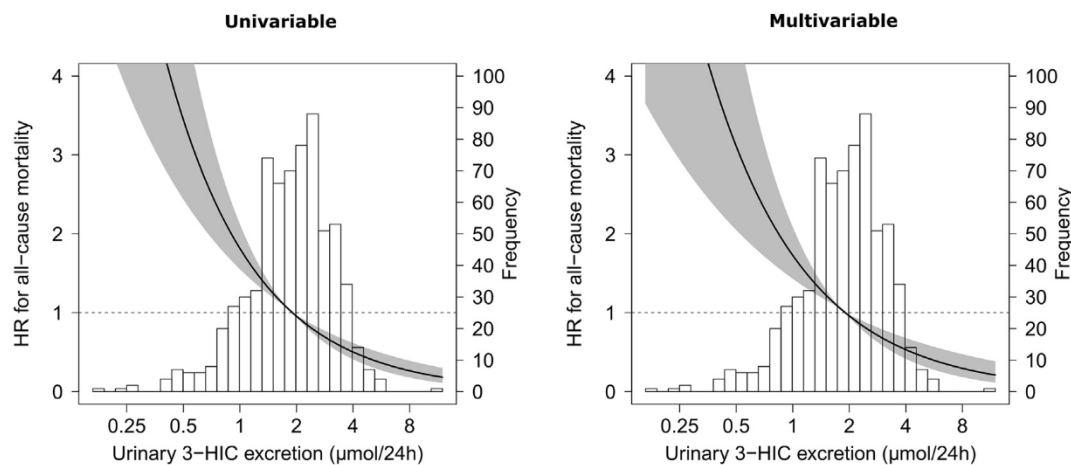

Fig. 4. Univariable (left) and multivariable (right) association of urinary 3-HIC excretion with all-cause mortality. The Urinary 3-HIC excretion was log$_2$-transformed for prospective analysis. The histograms depict the distribution of urinary 3-HIC excretion. The black line shows the adjusted hazard ratio (HR) and the gray areas correspond to the 95% confidence interval (CI). The multivariable association was adjusted for age, sex, BMI, eGFR and plasma albumin. P-effect <0.001 for both.

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protein intake or protein catabolism due to muscle wasting, 3-HIC can be increased due to increased leucine oxidation. The finding of an association between higher urinary 3-HIC excretion and lower risk of all-cause mortality, and the positive association between urinary 3-HIC excretion and urine creatinine excretion as a measure of muscle mass, may indicate that protein intake, and not muscle wasting, is the underlying cause of the association. Compared to the healthy controls we found lower urinary 3-HIC excretion in KTR, indicating a lower leucine intake, most likely related to a lower protein intake overall. Potential causes for a lower protein intake may be loss of appetite, anorexia, acidosis, and adherence to pretransplantation diets [6].

Dietary intake can be assessed using self-reported measures, however such assessments have many limitations, including under- or overreporting, motivation requirements, changes in diet due to self-reflections, socially desirable answers and errors in portion size estimates [41,42]. To overcome these potential errors and the added bias introduced by the use of food composition tables, biomarkers can be used as a reliable alternative [43,44]. Leucine, after absorption and distribution, is freely filtered in the glomeruli after which it is reabsorbed nearly completely [45—47]. Rather than being excreted unchanged, leucine is either incorporated into protein or metabolized thereby limiting the use of urinary leucine excretion as marker for dietary intake [46]. E.g. a ^{14}C-leucine absorption, distribution, metabolism and excretion study in rats demonstrated that less than 0.9% of dietary leucine was recovered in excretory routes [48]. The first step in the metabolism of leucine

Table 5
Cox regression analyses of the associations of sex-stratified tertiles of plasma biotin with all-cause mortality.

	Sex stratified tertiles of plasma biotin					
	I		II	III		
	HR [95% CI]	P-value		HR [95% CI]	P-value	
Model 1	1.65 [1.08—2.52]	0.02	Ref. (1.0)	1.92 [1.26—2.91]	0.002	
Model 2	1.65 [1.08—0.252]	0.02	Ref. (1.0)	1.96 [1.29—2.98]	0.002	
Model 3	2.23 [1.44—3.44]	<0.001	Ref. (1.0)	1.54 [1.01—2.36]	0.05	
Model 4	2.30 [1.46—3.61]	<0.001	Ref. (1.0)	1.45 [0.93—2.26]	0.10	
Model 5	2.13 [1.35—3.36]	0.001	Ref. (1.0)	1.44 [0.92—2.26]	0.11	
Model 6	1.97 [1.27—3.05]	0.003	Ref. (1.0)	1.47 [0.96—2.25]	0.08	

Model 1: crude. Model 2: adjusted for age, sex and BMI. Model 3; as model 2, additionally adjusted for eGFR. Model 4, as model 3, additionally adjusted for NT-ProBNP and HDL-cholesterol. Model 5, as model 3, additionally adjusted for warm ischemia time, proteinuria and hs-CRP. Model 6, as model 3, additionally adjusted for plasma albumin.

is the conversion into α-ketoisocaproate by the enzyme branched-chain amino-acid aminotransferase. The majority of α-ketoisocaproate is converted to isovaleryl-CoA [49,50]. Isovaleryl-CoA is subsequently metabolized via a few enzymatic conversions to acetoacetate and acetyl-CoA [49,50], which can be further processed to either ketones, or to energy equivalents in the citric acid cycle. An intermediate step in this pathway, the conversion of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA, is catalyzed by the biotin-dependent enzyme 3-methylcrotonyl-CoA carboxylase
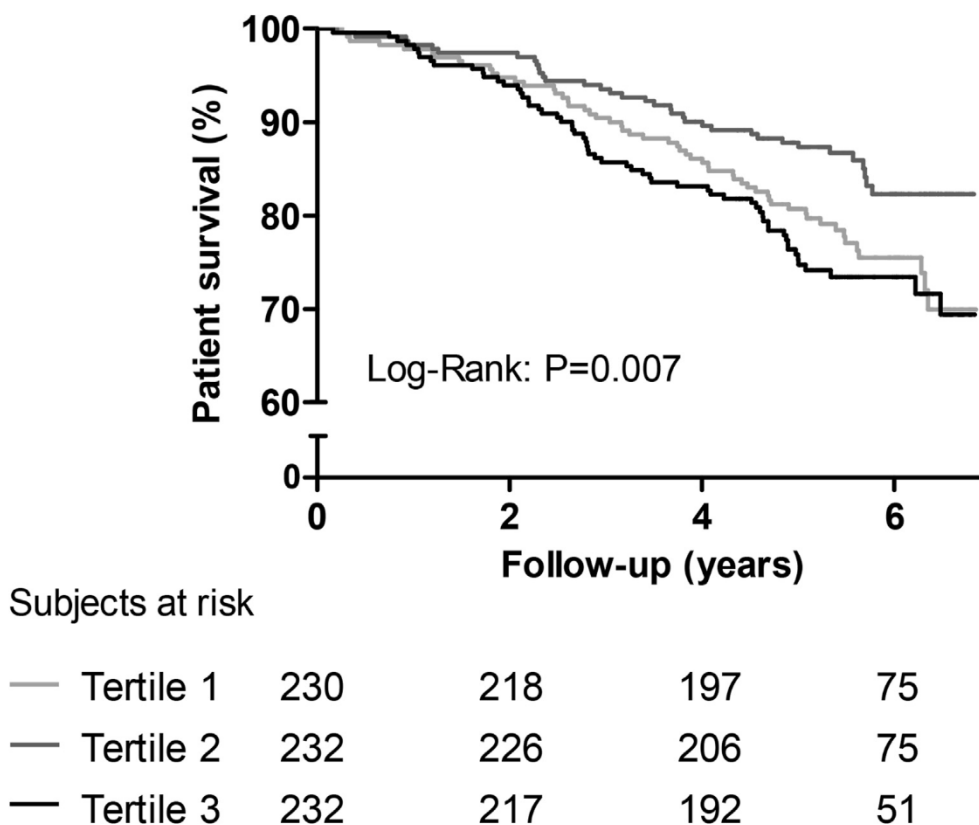

Fig. 5. Kaplan—Meier curves for all-cause mortality according to sex-stratified tertiles of plasma biotin.

(MCC) [19]. During marginal biotin deficiency, reduced activity of 3-MCC leads to a buildup of 3-methylcrotonyl CoA which is then converted to 3-hydroxyisovaleryl CoA (Fig. 1). 3-hydroxyisovaleryl is then converted by an carnitine acyltransferase to 3-hydroxyisovaleryl carnitine (3-HIC) and shuttled out of the mitochondria [19,20,51]. For this reason, urinary excretion of 3-HIC is considered a marker for biotin status [19]. Indeed, in our linear regression analyses we demonstrated a significant, but small, inverse association between urinary 3-HIC excretion and plasma biotin. However, subsequent adjustments clearly indicated that urinary urea excretion, reflecting total protein intake, was the primary determinant of urinary 3-HIC excretion. Compared to plasma biotin, urinary urea explained up to 40 times more of the variance in urinary 3-HIC excretion. Based on these data, in combination with knowledge of the metabolic pathways, we conclude that urinary 3-HIC excretion reflects dietary leucine intake more than it reflects plasma biotin in our population. This could be explained by the relatively high plasma biotin values in KTR, which may point to a lack of biotin deficiency. Undeniably, biotin values were nearly twice as high in KTR as in healthy controls and only a few patients had plasma biotin values lower than 100 and 200 ng/L, cutoffs used for absolute and marginal biotin deficiency, respectively [52]. However, some caution is warranted here, since plasma biotin is known to be affected by renal function and renal function in KTR is less than half compared with healthy controls [53].

The influence of urea excretion on urinary 3-HIC excretion implies that urinary 3-HIC excretion is not a suitable marker for marginal biotin status when dietary leucine intake is not controlled for. However, it should be noted that changes in urinary 3-HIC excretion after a fixed dose of leucine, i.e. a leucine challenge test, can be used to determine biotin status [19].

Results from the survival analyses also corroborate that urinary 3-HIC excretion primarily reflects dietary leucine intake. A high urinary 3-HIC excretion was associated with a reduced risk of all-cause mortality. Assuming urinary 3-HIC excretion reflects dietary leucine intake, dietary leucine intake may protect against all-cause mortality. Interestingly, this association was independent of many potential confounders. Even after adjusting for urinary urea excretion, reflecting total protein intake, the association remained significant, indicating a protective role for dietary leucine separate from being a component of total protein intake. Mediation analyses demonstrated that urinary 3-HIC excretion explains 61% of the association between urinary urea excretion and all-cause mortality in kidney transplant recipients.

In further analyses, significant interactions were found for sex, renal function, proteinuria and NT-ProBNP, all of which are risk factors for mortality itself. Stratified analyses demonstrated that higher urinary 3-HIC excretion was most protective in women, patients with better renal function, patients without proteinuria and patients with low NT-ProBNP, implicating that dietary leucine intake is most protective in patients with a lower baseline risk for all-cause mortality.

A possible mechanism underlying the inverse association between dietary leucine intake and mortality might be an increase in muscle mass, as reflected by the strong positive association between urinary 3-HIC excretion and urinary creatinine excretion. This is supported by the fact that the hazard ratio for mortality was affected most by adjusting for differences in muscle mass, which resulted in 44% reduction in hazard ratio. Leucine is known to stimulate muscle protein synthesis, most likely due to either an increase in substrate availability, an increased secretion of anabolic hormones such as insulin or through direct modulation of the anabolic pathways in skeletal muscles [12,39,40]. In addition, part

of the effects of leucine on muscle might also be modulated through its metabolite β-hydroxy-β-methylbutyrate [12]. Furthermore, leucine increases the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) expression leading to mitochondrial biogenesis and improving oxidative metabolism in skeletal muscle [54,55]. However, it should be noted that the association between urinary 3-HIC excretion and mortality did not lose significance after adjustment for urinary creatinine excretion, implicating that part of the association is explained by mechanisms other than the effect on muscle mass. Indeed, in mediation analyses urinary creatinine excretion only partially explained the association of urinary 3-HIC excretion with mortality. Either way, the protective association of urinary 3-HIC excretion with all-cause mortality poses the question whether leucine supplementation, alone or preferably as total protein, might be beneficial in kidney transplant recipients, and potentially also in patients with chronic kidney disease without a transplanted kidney, because protein energy malnutrition is also highly prevalent in these groups [8,9]. At this point we can only state that future research is warranted to investigate the potential of leucine supplementation in kidney transplant recipients and in patients with chronic kidney disease without a transplanted kidney.

Secondary analyses with sex-stratified tertiles of plasma biotin demonstrated that a low plasma biotin was independently associated with an increased risk of mortality. Potential causes for a low plasma biotin may be an insufficient nutrient intake or dysbiosis of the gut microbiota, which recently was demonstrated in KTR, most likely with chronic use of immunosuppressants as an important contributor [13]. Though biotin status has not been quantified in kidney transplants recipients before, it is known that transplant recipients are prone to vitamin deficiencies, including vitamin B6, vitamin K, vitamin C and vitamin B3 [28,56–58].

Unfortunately, we were unable to further investigate the association of low plasma biotin with mortality, since we did not have data on other biotin status markers. Therefore, future studies on biotin status in KTR, including patients with biotin deficiency, are warranted, preferable using renal function independent markers, such as biotinylated PCC abundance in lymphocytes or PCC activity in lymphocytes [59].

Strengths of this study include the large sample size of this well-defined and specific patient group of KTR, the long follow-up and the presence of appropriate controls. In addition, extensive data collection of many demographic and laboratory parameters enabled adjustment for many potential confounders. However, several limitations of this study need to be addressed. Due to the observational design of this study, we were unable to investigate whether the relationship between urinary 3-HIC excretion and all-cause mortality in KTR is causal or associative. Similarly, the observational design of this study does not allow us to elucidate the biological mechanisms underlying the association of urinary 3-HIC excretion with all-cause mortality. It should also be realized that most epidemiologic studies use a single baseline measurement for studying the association of variables with outcomes, which adversely affects the strength and significance of the association of these variables with outcomes [60,61]. Furthermore, despite adjusting for many potential confounders, the possibility of residual confounding remains. Lastly, we did not have additional methods for assessing body composition, i.e. dual energy X-ray absorptiometry scans. However, assessment of the 24 h urinary creatinine excretion is a reliable marker for estimating the total-body muscle mass, which has been demonstrated in a wide variety of populations, including patients with kidney disease and in patients with wasting conditions [35,62–70].

5. Conclusion

In the current study we demonstrated that urinary 3-HIC excretion is lower in KTR compared to healthy controls, indicating a lower dietary leucine intake in KTR. Within KTR, a lower urinary 3-HIC excretion was associated with a higher risk of all-cause mortality, independent of potential confounders. A higher leucine intake might have a protective role against mortality in KTR. Future studies could further investigate the mechanisms underlying these association and explore the potential of leucine, branched chain amino acids or protein supplementation in KTR.

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Data share statement

Data described in the manuscript, code book, and analytic code will be made available upon request of the editor.

Author contributions

All authors have substantially contributed to the study and manuscript design, data analyses: data interpretation and/or revision and have approved this final version of the work. The authors have agreed to take accountability for all aspects of this study. The authors' responsibilities were as follows: S.J.L.B. designed the study; A.P., I.M., T.B. and M.R.H.-F. performed the laboratory analysis. A.P. and M.Y.S. analyzed the data and performed statistical analyses; A.P. and S.J.L.B. wrote the manuscript and had primary responsibility for the final content; A.P., M.Y.S., A.W.G.-N., I.M., D.G., J.C.S., I.P.K., M.R.H.-F., C.F.M.F. and S.J.L.B. revised and edited the manuscript.

Conflict of interest

None of the authors declare a conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2020.09.035.

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