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Endogenous urinary glucocorticoid metabolites and mortality in prednisolone-treated renal transplant recipients

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Abstract

Background: Chronic corticosteroid treatment suppresses HPA-axis activity and might alter activity of 11β hydroxysteroid dehydrogenases (11β-HSD). We aimed to investigate whether the endogenous glucocorticoid production and 11β-HSD activities are altered in prednisolone-treated renal transplant recipients (RTR) compared with healthy controls and whether this has implications for long-term survival in RTR.

Methods: In a longitudinal cohort of 693 stable RTR and 275 healthy controls, 24-hour urinary cortisol, cortisone, tetrahydrocorisol (THF), allotetrahydrocortisol (alloTHF), and tetrahydrocortisone (THE) were measured using liquid chromatography tandem-mass spectrometry. Twenty-four-hour urinary excretion of cortisol and metabolites were used as measures of endogenous glucocorticoid production; (THF + alloTHF)/THE and cortisol/cortisone ratios were used as measures of 11β-HSD activity.

Results: Urinary cortisol and metabolite excretion were significantly lower in RTR compared with healthy controls ($P < .001$), whereas (THF + alloTHF)/THE and cortisol/cortisone ratios were significantly higher ($P < .001$ and $P = .002$). Lower total urinary metabolite excretion and higher urinary (THF + alloTHF)/THE ratios were associated with increased risk of mortality, independent of age, sex, estimated glomerular filtration rate, C-reactive protein, body surface area, and daily prednisolone dose, respectively.

Conclusions: Endogenous glucocorticoid production and 11β-HSD activities are altered in prednisolone-treated RTR. Decreased total urinary endogenous glucocorticoid metabolite excretion and increased urinary (THF + alloTHF)/THE ratios are associated with increased risk of mortality.

KEYWORDS
11βHSD, cortisol, mortality, prednisolone, renal transplant recipients

Trial notification: The study is registered at clinicaltrials.gov as "TransplantLines Food and Nutrition Biobank and Cohort study (TxL-FN)," number NCT02811835.

1 | INTRODUCTION

Kidney transplantation is the treatment of choice for most patients with end-stage kidney disease. Over the past decades, advances in immunosuppressive treatment regimens have greatly reduced the risk of acute rejection. However, only modest improvement in long-term outcomes has been achieved. One of the major challenges that remain is the application of current immunosuppressive agents, with a narrow therapeutic window between efficacy and toxicity.

Corticosteroids were among the first drugs used to prevent and treat rejection after kidney transplantation.[1,2] However, their use is known to cause a wide range of side effects, including metabolic abnormalities, muscle atrophy, and increased susceptibility to infections.[1,2] Therefore, there has been a great effort to get rid of corticosteroids as part of maintenance immunosuppressive regimens after kidney transplantation.[2-4] Nevertheless, it has recently been concluded that corticosteroids have to remain part of the immunosuppressive regimen in order to maintain current low rates of acute rejection and optimal long-term graft survival.[5,6] Unfortunately, however, corticosteroid dosing regimens remain empiric to date, usually with fixed doses, independent of either body size and/or steroid sensitivity.[7]

The most often used corticosteroids after kidney transplantation are prednisone and prednisolone. A well-known effect of chronic treatment with these drugs is suppression of the hypothalamus-pituitary-adrenal (HPA) axis, leading to reduced endogenous cortisol synthesis by the adrenal gland[8-10] (Figure 1). The HPA axis is the central stress response system. The hypothalamus releases corticotropin-releasing hormone, causing the pituitary to release adrenocorticotropic hormone. This hormone activates the adrenal cortex to produce cortisol. In addition to its synthesis by the adrenal gland, cortisol can be regenerated from biologically inactive cortisone by the enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which is expressed in the liver and various other tissues.[11,12] Its counterpart, 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2), converts active cortisol to inactive cortisone and is highly expressed in mineralocorticoid target tissues such as the kidney.[11,12] Recent studies suggest that exogenous corticosteroids, in addition to their direct effect of suppression on the HPA axis, may stimulate cortisol regeneration from cortisone by induction of 11β-HSD1.[13-15]

Thus, while treatment with prednisolone is known to reduce adrenal production of cortisol via a feed-back mechanism suppressing adrenal cortisol production, it might at the same time paradoxically enhance systemic exposure to cortisol via a feedforward mechanism, which involves induction of 11β-HSD1 (Figure 1). However, this has not been studied in renal transplant recipients (RTR) to date. Hence, we aimed to investigate whether endogenous glucocorticoid production and 11β-HSD enzyme activities are altered in RTR who are chronically treated with prednisolone, compared with healthy controls, and whether this has implications for long-term survival in RTR.

2 | MATERIALS AND METHODS

2.1 | Research design and subjects

We performed a post hoc analysis of an existing prospective cohort study on RTR, which has been previously described.[16] Briefly, we invited all RTR (aged ≥18 years) who visited the outpatient clinic of the University Medical Center Groningen between November 2008 and June 2011, who had a functioning graft for ≥1 year, and no history of alcohol and/or drug abuse. Written, informed consent was obtained from 707 (87%) of 817 initially invited RTR. In RTR transplanted between 1989 and 1996, standard immunosuppressive regime was cyclosporine combined with prednisolone. After 1996, standard regime was cyclosporine, mycophenolate mofetil, and prednisolone, with slow withdrawal of cyclosporine beyond one year after transplantation if no rejection had occurred in the first year after transplantation. In 2012, routine use of cyclosporine was replaced by routine use of tacrolimus.[17] For the present study, biomaterial for analysis of urinary cortisol metabolites was available in 693 RTR (98.0%). As a control group reflecting the general population, we included 275 healthy subjects who participated in a screening program before kidney donation. None of these healthy controls were treated with prednisolone or any other exogenous corticosteroid. In addition, none of the healthy controls had a history of kidney disease, diabetes, or cardiovascular events. Hypertension, if present, was treated with a maximum of one class of antihypertensive drugs. The study protocol was approved by the

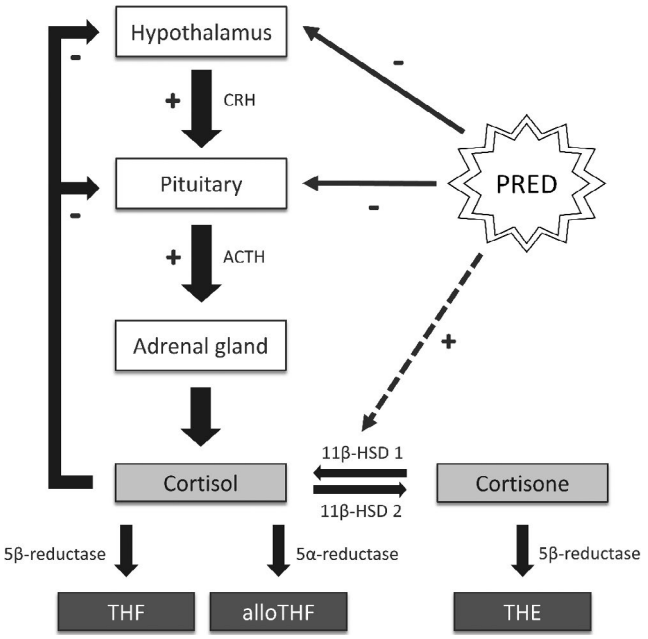

FIGURE 1 Supposed effects of prednisolone on cortisol production and metabolism. 11β-HSD1, 11beta-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11beta-hydroxysteroid dehydrogenase type 2; ACTH, adrenocorticotropic hormone; alloTHF, allotetrahydrocortisol; and PRED, prednisolone; CRH, corticotropin-releasing hormone; THE, tetrahydrocortisone; THF, tetrahydrocortisol. Continuous lines represent known effects; dashed line represents hypothesized effects

University Medical Center Groningen institutional review board (METc 2008/186) and adhered to the Declarations of Helsinki and Istanbul.

2.2 | Study procedures and measurements

All measurements were obtained during a morning visit to the outpatient clinic. Participants were instructed to collect a 24-hour urine sample the day before the visit. Twenty-four-hour collection was performed according to a strict protocol. Patients were instructed to void the bladder empty in the toilet and note the time that this was performed and to subsequently start collecting urine in a dedicated container until at the next day the time was reached at which the collection was started. It was requested to cool the urine at approximately 4 degrees Centigrade in a refrigerator during collection.[18] Upon completion of the 24-hours urine collection, fasting blood samples were obtained the following morning, and venous blood samples were analyzed immediately thereafter. Blood electrolytes, lipids, proteins, and urinary electrolytes were measured with automated and validated spectrophotometric routine methods (Modular, Roche Diagnostics). Urinary albumin was quantified by using nephelometry (Dade Behring Diagnostic), with a lower limit of detection of 3 mg/L. Total urinary protein concentration was determined by means of the Biuret reaction (MEGA AU 510; Merck Diagnostica), with a lower limit of detection of 0.1 g/L. Kidney function was assessed using estimated glomerular filtration rate (eGFR), which was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[19] Body weight and height were measured with participants wearing indoor clothing without shoes. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Body surface area (BSA) was calculated according to the formula of Dubois and Dubois as (weigh t$^{0.425}$ × height$^{0.725}$) × 0.007184.[20] Blood pressure was measured according to a strict protocol as previously described.[21] Diabetes mellitus was diagnosed if the fasting plasma glucose was at least 7.0 mmol/L (≥ 126 mg/dL) or if antidiabetic medication was used. Information on patient health status, medical history, and medication use was obtained from patient records. Pregnancy and other health conditions potentially affecting glucocorticoid metabolism were excluded.

2.3 | Endogenous glucocorticoid production and 11β-HSD enzyme activity

Cortisol is converted to biologically inactive cortisone by the 11β-HSD2 enzyme and can be converted back from cortisone to biologically active cortisol by the counter-enzyme 11β-HSD1 (Figure 1). In addition, cortisol and cortisone are metabolized by 5α- and 5β-reductases to form tetrahydrocortisol (THF), allotetrahydrocortisol (alloTHF), and tetrahydrocortisone (THE; Figure 1).[11,15,22] For the current study, endogenous glucocorticoid production was assessed by measurement of 24-hour urinary cortisol excretion and 24-hour total urinary endogenous glucocorticoid metabolite excretion, which was calculated by summation of urinary excretion of cortisol + cortisone +THF + alloTHF +THE (all in μmol/24 h). Urinary ratios of (THF + alloTHF)/THF and cortisol/cortisone are widely used to assess the combined enzymatic activities of both 11β-HSD1 and 11β-HSD2. In a refinement, urinary cortisol/cortisone ratio has been advocated to reflect activity of 11β-HSD2 activity, while urinary (THF + alloTHF)/THE has been advocated to reflect activity of 11β-HSD1.[11,15]

2.4 | Measurement of urinary cortisol metabolites

Total urinary cortisol, cortisone, THF, alloTHF, and THE were measured by using a validated high-performance liquid chromatography tandem-mass spectrometry (LC-MS/MS) assay.[23] For THE, THF, and a-THF, we compared the LC-MS/MS method with a GC-MS/MS method and found the following correlations: THE r^2 = .99, THF r^2 = .97, and a-THF r^2 = .94 for 40 urines.[24]

For all components, appropriate internal standards were added and the mixtures were incubated with an enzyme solution consisting of sulfatases and β-glucuronidases, to ensure hydrolysis of cortisol and the metabolites from their sulfated and glucuronidated forms. Internal standards that were used were cortisol-^{13}C3, cortisone-D_7, THE-D_5, THF-D_5, and alloTHF-D_5. Subsequently, the analytes were extracted using a Supported Liquid Extraction technique. Finally, separation and detection were performed by use of a Phenomenex Luna Phenyl-Hexyl column (particle size 3 μm, 2.0 mm internal diameter by 150 mm; Waters) and a Quattro Premier® tandem mass spectrometer operated in negative electrospray ionization mode (Waters). Intra- and inter-assay variation coefficients were <5.7% and <9.8%, respectively.

2.5 | End points

The primary end point of the study was all-cause mortality in RTR. Secondary end points were mortality from cardiovascular causes and mortality from infectious causes. Mortality from cardiovascular causes was defined as death due to cerebrovascular disease, ischemic heart disease, heart failure, or sudden cardiac death according to the International Classification of Diseases, ninth revision, codes 410-447. Mortality from infectious cause was defined as death due to infectious disease as defined by the International Classification of Diseases, ninth revision, codes 001-139. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status and cause of death. General practitioners or referring nephrologists were contacted in case the status of a patient was unknown. End points were recorded until September 2015. There was no exclusion due to loss of follow-up.

2.6 | Statistical analysis

Data were analyzed with SPSS statistics version 22.0 (SPSS Inc) and GraphPad Prism version 5.01 for Windows (GraphPad Software

TABLE 1 Baseline characteristics of 693 stable renal transplant recipients compared with 275 healthy controls

Variable	RTR (n = 693)	Controls (n = 275)	*P*-value
Recipient demographics			
Age (y)	53 ± 13	53 ± 11	.7
Male sex, n (%)	394 (57)	132 (48)	.01
Weight (kg)	80 ± 17	80 ± 14	.5
Waist (cm)	99 ± 15	91 ± 10	<.001
BMI (kg/m^2)	26.6 ± 4.8	25.9 ± 3.5	.01
BSA (m^2)	1.94 ± 0.22	1.95 ± 0.21	.7
Muscle mass			
Creatinine excretion (mmol/24 h)	11.3 [9.2-14.0]	12.4 [10.2-15.8]	<.001
Blood pressure			
SBP (mm Hg)	136 ± 18	125 ± 14	<.001
DBP (mm Hg)	83 ± 11	76 ± 9	<.001
No. of antihypertensive drugs (n)	1.8 ± 1.0	0.2 ± 0.5	<.001
Sodium & potassium homeostasis			
Serum Na (mmol/l)	141 ± 2.8	142 ± 1.9	.01
Serum K (mmol/l)	3.9 ± 0.5	3.9 ± 0.3	.2
Urinary Na excretion (mmol/24 h)	146 [112-190]	187 [144-235]	<.001
Urinary K excretion (mmol/24 h)	70 [55-97]	82 [68-100]	<.001
Fractional Na excretion (%)	1.2 [0.85-1.6]	0.74 [0.58-0.88]	<.001
Fractional K excretion (%)	20 [15-26]	12 [10-15]	<.001
Urinary Na/K ratio (mmol/mmol)	2.2 [1.6-2.9]	2.3 [1.8-2.9]	.12
Lipids			
Total cholesterol (mmol/l)	5.1 ± 1.1	5.4 ± 1.0	.001
HDL cholesterol (mmol/l)	1.4 ± 0.5	n.m.	
LDL cholesterol (mmol/l)	3.0 ± 0.9	n.m.	
Triglycerides (mmol/l)	1.7 [1.3-2.3]	1.2 [0.9-1.7]	<.001
Statin use, n (%)	366 (53)	8 (3)	<.001
Diabetes			
Glucose (mmol/l)	5.3 [4.8-6.0]	5.3 [5.0-5.7]	.3
HbA_{1c} (%)	6.0 ± 0.8	5.6 ± 0.3	<.001
Diabetes mellitus, n (%)	166 (24)	0 (0)	<.001
Antidiabetic drug use, n (%)	107 (15)	0 (0)	<.001
Inflammation			
hsCRP (mg/l)	1.6 [0.7-4.6]	1.1 [0.6-2.3]	<.001
Serum total protein (g/l)	72 [68-74]	74 [71-77]	<.001
Serum albumin (g/l)	43 [41-45]	45 [44-47]	<.001
Kidney function			
Serum creatinine (µmol/l)	124 [99-160]	73 [65-82]	<.001
eGFR (ml/min*1.73)	52 ± 20	91 ± 14	<.001
Creatinine clearance (ml/min)	66 ± 26	125 ± 37	<.001
Urinary protein excretion (g/24 h)	0.2 [0.0-0.4]	0.0 [0.0-0.1]	<.001
Urinary albumin excretion (mg/24 h)	41 [11-182]	5 [3-9]	<.001
Transplantation			
Transplant vintage (y)	5.3 [1.8-12.0]	n/a	
Previous dialysis duration (y)	2.6 [1.3-4.7]	n/a	

(Continues)

TABLE 1 (Continued)

Variable	RTR (n = 693)	Controls (n = 275)	*P*-value
Warm ischemia times (min)	40 [34-50]	n/a	
Cold ischemia times (h)	14 [3-21]	n/a	
Acute rejection, n (%)	184 (27)	n/a	
Medication			
Prednisolone dose (mg/24 h)	10.0 [7.5-10.0]	n/a	
<5 mg, n (%)	2 (0,4)		
5-7.4 mg, n (%)	53 (7.6)		
7.5 mg, n (%)	220 (31.7)		
8-10 mg, n (%)	412 (59.5)		
>10 mg, n (%)	5 (0.8)		
Calcineurin inhibitors			
Cyclosporine use, n (%)	271 (39)	n/a	
Tacrolimus use, n (%)	123 (18)	n/a	
Proliferation inhibitors			
Mycophenolate mofetil use, n (%)	455 (66)	n/a	
Azathioprine use, n (%)	120 (17)	n/a	
Carbamazepine use, n (%)	7 (1)	n/a	
Urinary glucocorticoid metabolites excretion			
Cortisol excretion (nmol/24 h)	30 [15-57]	332 [244-445]	<.001
Cortisone excretion (nmol/24 h)	40 [20-80]	526 nn	<.001
THF excretion (μmol/24 h)	1.2 [0.58-2.1]	6.9 [5.1-9.3]	<.001
alloTHF excretion (μmol/24 h)	0.36 [0.14-0.84]	4.2 [2.6-6.5]	<.001
THE excretion (μmol/24 h)	1.0 [0.37-2.1]	12.5 [8.5-16.8]	<.001
Total endogenous GC metabolite excretion (μmol/24 h)	2.7 [1.2-5.3]	24.6 [17.4-33.7]	<.001
(THF + alloTHF)/THE ratio (μmol/μmol)	1.6 [1.2-2.2]	0.94 [0.79-1.0]	<.001
Cortisol/cortisone ratio (nmol/nmol)	0.68 [0.52-0.95]	0.63 [0.54-0.74]	.002

Note: Nominal data are presented as absolute number (percentage), normally distributed data as mean ± standard deviation and non-normally distributed data as median [interquartile range]. Differences between RTR and controls were tested using t tests for normally distributed variables, Mann-Whitney U tests for non-normally distributed variables, and chi-square tests for nominal variables.

Abbreviations: alloTHF, allotetrahydrocortisol; BMI, body mass index; BSA, body surface area; DBP, diastolic blood pressure, eGFR, estimated glomerular filtration rate; GC, glucocorticoid; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; n/a, not applicable; n.m., not measured; SBP, systolic blood pressure; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

Inc). Normally distributed data are presented as mean ± standard deviation, non-normally distributed data as median (interquartile range [IQR]) and nominal data as number (percentage). Hazard ratios (HRs) are reported with 95% confidence intervals (CI). A two-sided *P*-value <.05 was considered to indicate statistical significance. Variable distribution was tested with histograms and probability plots. In further analyses, we first tested differences in baseline characteristics between RTR and healthy controls by using independent-samples *t* tests, Mann-Whitney *U* tests, and chi-square tests, where appropriate. Second, we performed linear regression analyses to identify age- and sex-adjusted associations of clinical and biochemical parameters with urinary cortisol excretion and total urinary endogenous glucocorticoid metabolite excretion as measures of endogenous glucocorticoid production, and urinary (THF + alloTHF)/THE ratio and urinary cortisol/cortisone ratio as measures of activities of 11β-HSD1 and 11β-HSD2, respectively, in RTR. Subsequently, we performed multivariable linear regression analyses to identify independent associates of these parameters. Multivariable regression analyses were performed using backward selection (Pout > 0.05), including variables that were significantly associated with urinary cortisol parameters in explorative analyses. Non-normally distributed variables were log-transformed to fulfill criteria for performing linear regression analyses. Finally, we assessed prospective associations of urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF/THE) ratio, and cortisol/cortisone ratio with all-cause mortality, mortality from cardiovascular causes, and mortality from infectious cause by using Cox proportional hazard regression analyses, in which we adjusted for potential confounders, including age, sex,

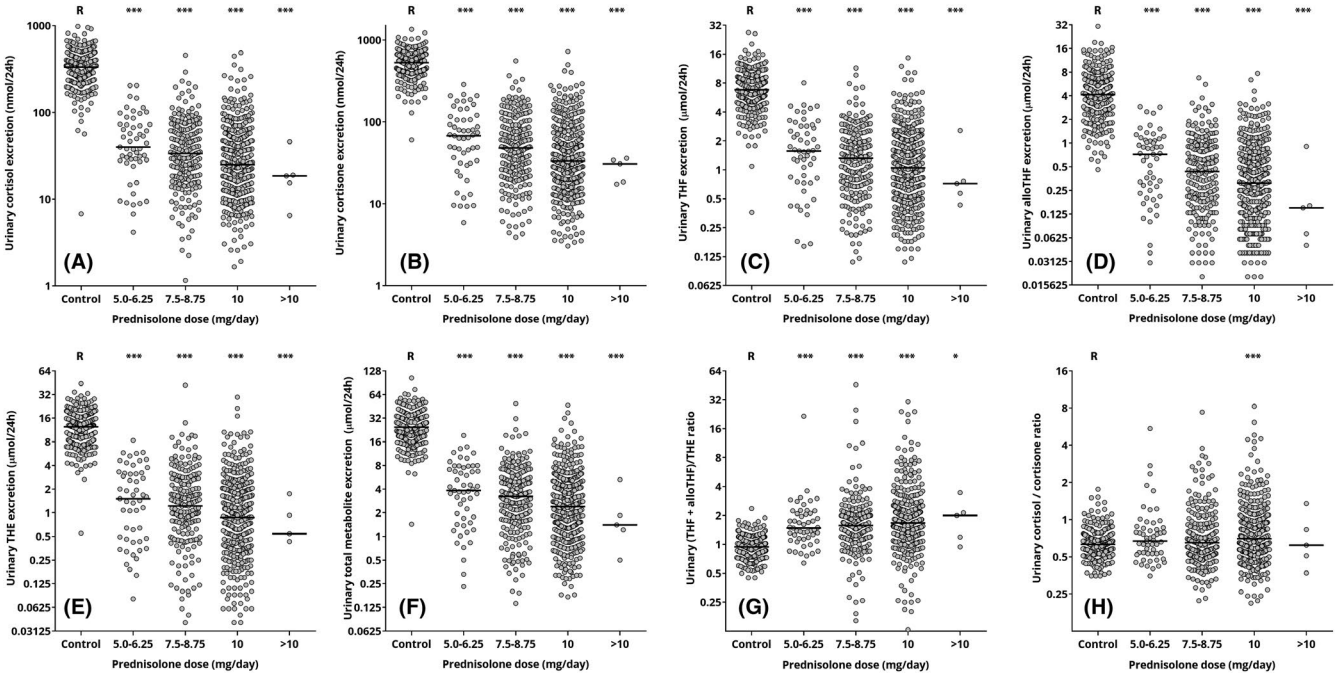

FIGURE 2 Urinary [A] cortisol excretion, [B] cortisone excretion, [C] tetrahydrocortisol (THF) excretion, [D] allotetrahydrocortisol (alloTHF) excretion, [E] tetrahydrocortisone (THE) excretion, [F] total endogenous glucocorticoid (GC) metabolite excretion, [G] (THF + alloTHF)/THE ratio, and [H] cortisol/cortisone ratio in renal transplant recipients according to prednisolone dose compared with healthy controls, which are referred to as R (reference). *P < .05, ***P < .001

BSA, hsCRP, daily prednisolone dose, and eGFR. Because use of carbamazepine is known to be able to affect prednisolone metabolism,[25,26] we also performed analyses in which we adjusted for use of carbamazepine (model 7). To allow for comparison of strengths of associations, log-transformed urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF/THE) ratio, and cortisol/cortisone ratio were standardized to *Z*-scores and used as continuous variables in further analyses. It is known that 24 hour urine collection can be subject to collection errors. Comparison of measured urinary creatinine excretion with estimated urinary creatinine excretion and exclusion of the 2.5% of outliers at both sides of the difference between the two is an accepted method to control for potential collection errors.[27] To account for potential effects of collection errors, we performed sensitivity analyses, in which we repeated analyses for associations of urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF)/THE ratio, and cortisol/cortisone ratio with all-cause mortality, cardiovascular mortality, and mortality from infectious cause with exclusion of the RTR with potential inadequate 24 hours urine collection, defined as the upper and lower 2.5% difference between the estimated and measured creatinine excretion rate.[27]

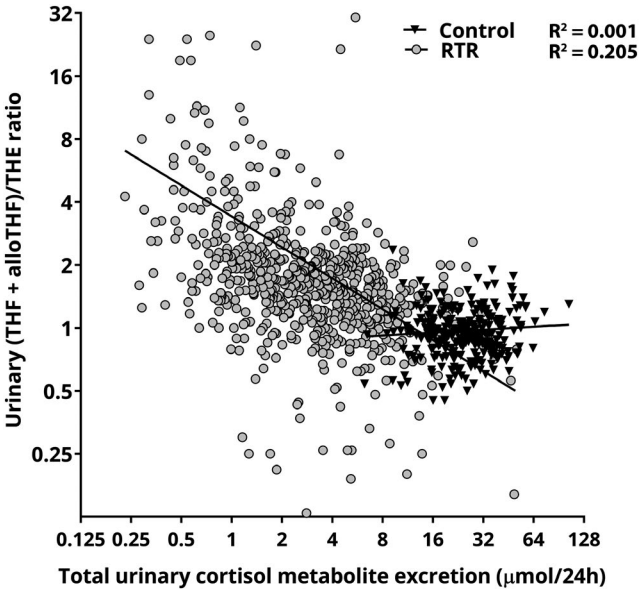

FIGURE 3 Association of total urinary endogenous glucocorticoid (GC) metabolite excretion with urinary (THF + alloTHF)/THE ratio in RTR and healthy controls. Controls: St. β = 0.03, 95%CI −0.09; 0.15, P = .65; RTR: St. β = −0.45, 95%CI −0.51; −0.40, P < .001

3 | RESULTS

3.1 | Study population

We included 693 stable RTR and 275 healthy controls. RTR had a mean age of 53 ± 13 years; 57% of RTR were men. At baseline, they were 5.3 [IQR, 1.8-12.0] years after transplantation, had a mean eGFR of 52 ± 20 mL/min/1.73 m^2, a median urinary protein excretion

of 0.2 [IQR, 0.0-0.4] g/24 h with a range from 0.0 g/24 h to 8.9 g/24 h and a median albumin excretion of 41 [IQR 10.7-182.4] mg/24 h with a range from 0.02 mg/24 h to 7981.6 mg/24 h. Of the RTR, 13 (1.9%) were transplanted before 1980, 43 (6.1%) were transplanted between 1980 and 1990, 159 (23.0%) were transplanted between 1990 and 2000, and 478 (69.0%) were transplanted after 2000. All RTR were treated with prednisolone as maintenance immunosuppressive therapy, with additional treatment with either cyclosporine (39%) or tacrolimus (18%) and/or mycophenolate mofetil (66%) or azathioprine (17%). Median daily prednisolone dose was 10.0 [7.5-10.0] mg/day. Forty-nine RTR (7%) used 5 mg prednisolone per day, 6 RTR (1%) used 6.25 mg/d, 220 RTR (32%) used 7.5 mg/d, 7 RTR (1%) used 8.75 mg/d, 406 RTR (59%) used 10 mg/d, and 5 RTR (1%) used more than 10 mg/d once daily. Of all RTR, 7 (1.0%) used carbamazepine.

As healthy controls, we included 275 healthy kidney donors. They had a mean age of 53 ± 11 years; 48% of healthy controls were men. They had a mean eGFR of 91 ± 14 mL/min/1.73 m^2 and median urinary protein excretion of 0.0 [IQR, 0.0-0.1] g/24 h, with a range from 0.0 g/24 h to 0.35 g/24 h. Median albumin excretion was 5.2 [IQR, 3.2-8.9] mg/24 h, with a range from 0.02 mg/24 h to 128.5 mg/24 h. Compared with healthy controls, RTR had higher BMI and waist circumference, lower creatinine excretion, higher blood pressure, higher triglycerides, lower urinary sodium and potassium excretion, higher hsCRP, and worse kidney function. None of the healthy controls, but 24% of RTR had diabetes mellitus (Table 1).

3.2 | Urinary cortisol parameters in RTR and healthy controls

Urinary excretion of cortisol, cortisone, THF, alloTHF, THE, and total endogenous glucocorticoid metabolites was about a 10-fold lower in RTR than in healthy controls ($P < .001$ for all metabolites; Table 1). In contrast, urinary (THF + alloTHF)/THE ratio and cortisol/cortisone ratio were significantly higher ($P < .001$ and $P = .002$, respectively, Table 1). Urinary excretion of cortisol, cortisone, THF, alloTHF, THE, and total endogenous glucocorticoid metabolites significantly decreased with increasing prednisolone dose (P for trend <.001 for all metabolites; Figure 2A-F), and there was a trend toward a significant increase in urinary (THF + alloTHF)/THE ratio with increasing prednisolone dose (P for trend = .08; Figure 2G). However, there was considerable inter-individual variation in urinary cortisol metabolite excretion and urinary (THF + alloTHF)/THE ratio in RTR treated with the same prednisolone dose (Figure 2A-G). There was no significant difference in urinary cortisol/cortisone ratio for different prednisolone doses (Figure 2H). Interestingly, there was a strong inverse association of urinary (THF + alloTHF)/THE ratio with total urinary endogenous glucocorticoid metabolite excretion in RTR (st.$\beta = -0.45$, $P < .001$; Figure 3), whereas there was no significant positive association in healthy controls (st.$\beta = 0.03$, $P = .65$; Figure 3). The strong inverse association of urinary (THF + alloTHF)/THE ratio with total endogenous glucocorticoid metabolite excretion in RTR remained significant, after adjustment for age, sex, BSA, eGFR, and daily prednisolone dose (st. $\beta = -0.43$, $P < .001$).

3.3 | Cross-sectional associations of clinical and biochemical parameters with urinary cortisol parameters in RTR

In univariate linear regression analysis, male sex was positively associated with urinary cortisol and total endogenous glucocorticoid metabolite excretion (st.$\beta = 0.13$, $P = .001$ and st.$\beta = 0.19$, $P < .001$, respectively). Associations for age were borderline positive (st.$\beta = 0.07$, $P = .05$ and st.$\beta = 0.07$, $P = .07$, respectively). Age and sex were neither associated with urinary (THF + alloTHF)/THE ratio nor with cortisol/cortisone ratio. In age- and sex-adjusted linear regression analyses, daily prednisolone dose was inversely associated with urinary cortisol and total endogenous glucocorticoid metabolite excretion (st.$\beta = -0.18$, $P < .001$ and st.$\beta = -0.18$, $P < .001$, respectively), whereas it was positively associated with urinary (THF + alloTHF)/THE ratio (st.$\beta = 0.09$, $P = .03$) (Table 2). Other age- and sex-adjusted associations with urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, urinary (THF + alloTHF)/THE ratio, and cortisol/cortisone ratio are presented in Table 2. In multivariable regression analyses with backward elimination, we found age, eGFR, daily prednisolone dose, hsCRP, and creatinine excretion to be independently associated with urinary cortisol excretion; age, male sex, BSA, eGFR, daily prednisolone dose, hsCRP, and creatinine excretion to be independently associated with total urinary endogenous glucocorticoid metabolite excretion; daily prednisolone dose, eGFR, and hsCRP to be independently associated with urinary (THF + alloTHF)/THE ratio; and age, male sex, hsCRP, and creatinine excretion to be independently associated with urinary cortisol/cortisone ratio (Table 3).

3.4 | Endogenous glucocorticoid metabolites and mortality

During a median follow-up of 5.3 [4.7-6.1] years, 147 of 693 (21%) RTR died, of whom 58 (8%) from cardiovascular causes and 42 (6%) from infectious causes. In age- and sex-adjusted Cox regression survival analyses, total urinary endogenous glucocorticoid metabolite excretion was inversely associated with all-cause mortality (HR 0.67 [95% CI, 0.53-0.83]; $P < .001$ per SD increase), whereas urinary (THF + alloTHF)/THE ratio (HR 1.37 [95% CI, 1.19-1.57]; $P < .001$) and urinary cortisol/cortisone ratio (HR 1.22 [95% CI, 1.06-1.41]; $P = .006$) were positively associated with mortality (Table 4). The associations of total urinary endogenous glucocorticoid metabolite excretion and urinary (THF + alloTHF)/THE ratio with all-cause mortality remained independent of adjustment for

TABLE 2 Age- and sex-adjusted associations of clinical and biochemical parameters with urinary glucocorticoid excretion in renal transplant recipients

Variable (n = 693)	Cortisol	Total endogenous GC metabolites	(THF + alloTHF)/THE ratio	Cortisol/cortisone ratio
Recipient demographics				
Weight (kg)	0.02	0.14***	0.05	−0.04
Waist (cm)	0.00	0.11**	0.04	0.02
BMI (kg/m^2)	−0.01	0.10**	0.02	−0.02
BSA (m^2)	0.04	0.15***	0.05	−0.06
Muscle mass				
Creatinine excretion (mmol/24 h)	0.17***	0.20***	−0.03	−0.14**
Blood pressure				
SBP (mm Hg)	−0.02	0.01	0.02	0.07
DBP (mm Hg)	0.00	0.05	0.00	−0.00
No. of antihypertensive drugs (n)	−0.08*	−0.06	0.11**	0.05
Sodium & potassium homeostasis				
Serum Na (mmol/l)	0.06	0.13***	−0.10**	0.02
Serum K (mmol/l)	−0.10**	−0.08*	0.03	0.10**
Urinary Na excretion (mmol/24 h)	0.12**	0.13***	0.01	−0.03
Urinary K excretion (mmol/24 h)	0.18***	0.16***	−0.07	−0.14***
Fractional Na excretion (%)	−0.13***	−0.12**	0.18***	0.18***
Fractional K excretion (%)	−0.09*	−0.10**	0.15***	0.10**
Urinary Na/K ratio (mmol/mmol)	−0.01	−0.01	0.05	0.10**
Lipids				
Total cholesterol (mmol/l)	−0.09*	−0.05	−0.00	−0.04
HDL cholesterol (mmol/l)	0.02	−0.06	0.02	−0.17***
LDL cholesterol (mmol/l)	−0.05	−0.01	−0.06	0.01
Triglycerides (mmol/l)	−0.08*	0.02	0.07	0.11**
Statin use, n (%)	−0.08*	−0.06	0.11**	0.02
Diabetes				
Glucose (mmol/l)	−0.11**	−0.10**	−0.03	0.04
HbA_{1c} (%)	0.02	0.04	−0.02	−0.04
Diabetes mellitus, n (%)	0.01	0.02	0.02	0.02
Antidiabetic drug use, n (%)	0.03	0.02	−0.02	0.07
Inflammation				
hsCRP (mg/l)	0.15***	0.10**	−0.07	−0.15***
Serum total protein (g/l)				
Serum albumin (g/l)				
Kidney function				
Serum creatinine (µmol/l)	−0.21***	−0.18***	0.24***	0.23***
eGFR (ml/min*1.73)	0.20***	0.18***	−0.24***	−0.23***
Creatinine clearance (ml/min)	0.25***	0.25***	−0.19***	−0.27***
Urinary protein excretion (g/24 h)	−0.07	−0.07	0.16***	0.06
Urinary albumin excretion (mg/24 h)	−0.03	−0.02	0.11**	0.07
Transplantation				
Transplant vintage (y)	0.02	0.02	−0.03	−0.05
Previous dialysis duration (y)	0.02	0.04	0.05	0.08

(Continues)

TABLE 2 (Continued)

Variable (n = 693)	Cortisol	Total endogenous GC metabolites	(THF + alloTHF)/THE ratio	Cortisol/cortisone ratio
Warm ischemia times (min)	0.01	0.03	−0.08	0.03
Cold ischemia times (h)	−0.06	−0.05	−0.02	0.02
Acute rejection, n (%)	−0.02	0.02	0.05	−0.01
Medication				
Prednisolone dose (mg/24 h)	−0.18***	−0.18***	0.09*	0.03
Cyclosporine use, n (%)	−0.04	−0.05	0.00	0.09*
Tacrolimus use, n (%)	−0.01	0.02	0.00	0.02
Mycophenolate mofetil use, n (%)	0.02	0.03	−0.04	0.00
Azathioprine use, n (%)	0.02	0.02	0.06	−0.03
Carmabazepine use, n (%)	0.08*	0.06	0.02	−0.01

Note: Non-normally distributed variables were log-transformed before entering regression analysis. For dichotomous variables, 0 = no and 1 = yes. Results of linear regression analyses with urinary cortisol parameters as dependent variable. Standardized beta-regression coefficients of age- and sex-adjusted associations are shown. Asterisks represent level of significance.

Abbreviations: alloTHF, allotetrahydrocortisol; BMI, body mass index; BSA, body surface area; DBP, diastolic blood pressure, eGFR, estimated glomerular filtration rate; GC, glucocorticoid; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; n/a, not applicable; n.m., not measured; SBP, systolic blood pressure; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

*P < .05, **P < .01, ***P < .001.

potential confounders (including BSA, hsCRP, daily prednisolone dose, and eGFR), whereas the association of urinary cortisol/cortisone ratio with mortality lost significance after adjustment for eGFR (Table 4). Total urinary endogenous glucocorticoid metabolite excretion was inversely associated with mortality from cardiovascular causes (HR 0.67 [95% CI, 0.47-0.95]; P = .02). This association remained independent of adjustment for potential confounders. Finally, urinary (THF + alloTHF)/THE ratio (HR 1.42 [95% CI, 1.23-1.80]; P < .001) and urinary cortisol/cortisone ratio (HR 1.68 [95% CI, 1.33-2.15]; P < .001) were positively associated with mortality from infectious causes. These associations also remained independent of adjustment for potential confounders (Table 4). As carbamazepine is known to be able to affect prednisolone metabolism,[25,26] we also performed analyses in which we adjusted for use of this drug. Adjustment for this use did not materially affect associations of urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF)/THE ratio, and cortisol/cortisone ratio with all-cause mortality, cardiovascular mortality, and mortality from infectious cause. In sensitivity analyses in which we controlled for potential collection errors by exclusion of outliers for the difference between estimated 24 hours urinary creatinine excretion and measured 24 hours urinary creatinine excretion, we found no material differences in associations of urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF)/THE ratio, and cortisol/cortisone ratio with all-cause mortality, cardiovascular mortality, and mortality from infectious cause between these sensitivity analyses and the primary analyses (see Table S1 for results of the sensitivity analyses).

4 | DISCUSSION

To our knowledge, this study is the first to investigate endogenous glucocorticoid production and 11β-HSD activities in a large cohort of stable outpatient RTR, who are chronically treated with prednisolone. We found that urinary excretion of cortisol and its metabolites was decreased in RTR compared with healthy controls, whereas urinary (THF + alloTHF)/THE and cortisol/cortisone ratios were increased. In addition, there was considerable inter-individual variation in these parameters. Both decreased total urinary endogenous glucocorticoid metabolite excretion and increased urinary (THF + alloTHF)/THE ratios were associated with increased risk of mortality long-term after kidney transplantation, independent of potential confounders, including daily prednisolone dose, body composition, and kidney function.

Endogenous glucocorticoid production, as measured by 24-hour urinary cortisol and total endogenous glucocorticoid metabolite excretion, was decreased in prednisolone-treated RTR compared with healthy controls. In addition, there seemed to be a dose-dependent effect of prednisolone, with lower endogenous glucocorticoid production in RTR treated with a higher daily prednisolone dose. This is in line with previous studies showing that chronic prednisolone treatment suppresses endogenous glucocorticoid production after kidney transplantation.[8-10] Interestingly, we found that there was considerable variation in endogenous glucocorticoid production within groups of RTR who were treated with the same daily prednisolone dose. This finding could be a first step toward individualized dosing of glucocorticoids in this patient group. Possibly, this could decrease over- or

TABLE 3 Independent associates of urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF)/THE ratio, and cortisol/cortisone ratio

	Cortisol		Total endogenous GC metabolites		(THF + alloTHF)/THE ratio		Cortisol/cortisone ratio	
Variable (n = 693)	**St. β**	***P*-value**	**St. β**	***P*-value**	**St. β**	***P*-value**	**St. β**	***P*-value**
Age (y)	0.12	.001	0.11	.007	-	-	−0.16	<.001
Male sex (yes)	-	-	−0.10	.02	-	-	−0.13	.003
Body surface area (m^2)	-	-	0.09	.04	-	-	-	-
eGFR (mL/min*1.73)	0.23	<.001	0.20	<.001	−0.24	<.001	−0.21	<.001
Daily prednisolone dose (mg/d)	−0.17	<.001	−0.19	<.001	0.09	.03	-	-
hsCRP (mg/L)	0.18	<.001	0.13	<.001	−0.08	.04	−0.10	.01
Creatinine excretion (mmol/24 h)	0.22	<.001	0.17	<.001	-	-	−0.13	.003

Note: St. β: standardized beta coefficients. Non-normally distributed variables were log-transformed before entering regression analysis. For dichotomous variables, 0 = no and 1 = yes. Results of linear regression analyses with backward elimination, with urinary cortisol parameters as dependent variable. Only standardized beta-regression coefficients of significant covariates in the final model for each parameter are shown.
Abbreviations: alloTHF, allotetrahydrocortisol; eGFR, estimated glomerular filtration rate; GC, glucocorticoid; hsCRP, high sensitivity C-reactive protein; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

underimmunosuppression and therefore minimize the undesirable side effects of corticosteroids.

Using stable isotope tracers the daily cortisol production rate in healthy subjects has been estimated at approximately 6-10 mg/m^2/d,[28,29] which is equivalent to 3-5 mg of prednisolone per day. Thus, theoretically, when a patient is chronically treated with a daily dose of prednisolone greater than 5 mg/d, and certainly if the dose is higher than 7.5 mg/d, one might surmise that HPA-axis activity is entirely suppressed, with no residual endogenous cortisol synthesis present. However, our data, showing endogenous cortisol synthesis in RTR with daily prednisolone doses of 7.5 mg and higher, indicate that this is not what actually happens. Indeed, biological half-life of prednisolone is only 2-4 hours in stable outpatient RTR.[30-34] Thus, a one time a day daily dose, like we and other centers usually prescribe, may not fully cover the normal diurnal pattern of endogenous cortisol requirement and synthesis and leave room for endogenous production. Furthermore, individual differences in prednisolone pharmacokinetics and variation in glucocorticoid sensitivity could potentially lead to considerable variation in HPA-axis activity in patients treated with the same prednisolone dose.[7] Therefore, it quite conceivable that under a standard once daily dosing regimen with relatively low dosages, there can be incomplete suppression of the HPA axis and that there is considerable variation in the extent to which there is suppression.

In previous studies, Bergmann et al[7] showed that time after transplantation, sex, bodyweight, and kidney function are important determinants of variability in prednisolone pharmacokinetics. They also showed that in elderly transplant recipients, despite decreased prednisolone catabolism, endogenous cortisol was also higher, suggesting less suppression of endogenous cortisol production by prednisolone.[7,35] In line with this, we found lower daily prednisolone dose, higher age, male sex, and better kidney function to be independently associated with higher total urinary endogenous glucocorticoid metabolite excretion.

Urinary (THF + alloTHF)/THE ratio, and to a lesser extend urinary cortisol/cortisone ratio, was increased in RTR compared with healthy controls, indicating altered 11β-HSD enzyme activities. There seemed to be a weak dose-dependent effect of prednisolone on urinary (THF-alloTHF)/THE ratio, with higher (THF-alloTHF)/THE ratios in RTR treated with a higher daily prednisolone dose. There was no dose-dependent effect on urinary cortisol/cortisone ratio. To our knowledge, there are no previous clinical studies in RTR measuring urinary (THF-alloTHF)/THE ratio or cortisol/cortisone ratio or studying the effect of prednisolone on these parameters. Nevertheless, our findings are in line with findings in one experimental study in rats showing increased urinary (THF-alloTHF)/THE ratio after syngeneic kidney transplantation,[36] and with one case report, showing that urinary (THF-alloTHF)/THE ratio, but not cortisol/cortisone ratio, remained high after kidney transplantation in a patient with the syndrome of apparent mineralocorticoid excess (AME), who was chronically treated with methylprednisolone after transplantation.[37] In addition, our results of a dose-dependent effect of prednisolone on urinary (THF-alloTHF)/THE ratio are in line with two studies in patients with adrenal insufficiency treated with exogenous hydrocortisone, which showed increased urinary (THF + alloTHF)/THE ratio in patients treated with higher hydrocortisone doses.[13,14]

Higher urinary (THF + alloTHF)/THE and cortisol/cortisone ratios in RTR suggest that peripheral cortisol balance as maintained by 11β-HSD enzymes has shifted toward cortisol production rather than inactivation. Since 11β-HSD2 is known to metabolize cortisol to cortisone and the counter-enzyme 11β-HSD1 is known to regenerate cortisol back from cortisone, a shift toward cortisol production could either be attributable to a relative decrease in enzymatic activity of the former or a relative increase in activity of the latter. The 11β-HSD2 enzyme is mainly localized in classic mineralocorticoid tissue such as the kidney, where it protects the mineralocorticoid receptor from stimulation by cortisol. From previous studies, it is known

TABLE 4 Associations of urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF)/THE ratio, and cortisol/cortisone ratio with all-cause mortality, cardiovascular mortality, and mortality from infectious cause

	All-cause mortality (n^{events} = 147/693)		Cardiovascular mortality (n^{events} = 58/693)		Mortality from infectious cause (n^{events} = 42/693)	
	HR [95% CI]	*P*-value	HR [95% CI]	*P*-value	HR [95% CI]	*P*-value
Cortisol						
Model 1	0.80 [0.63-1.01]	.06	0.88 [0.61-1.28]	.5	0.90 [0.58-1.39]	.6
Model 2	0.81 [0.64-1.02]	.07	0.88 [0.61-1.29]	.5	0.91 [0.59-1.42]	.7
Model 3	0.75 [0.59-0.96]	.02	0.79 [0.54-1.16]	.2	0.88 [0.56-1.37]	.6
Model 4	0.76 [0.60-0.97]	.02	0.78 [0.53-1.15]	.2	0.90 [0.57-1.41]	.6
Model 5	0.86 [0.67-1.10]	.2	0.91 [0.61-1.35]	.6	1.01 [0.64-1.62]	.9
Model 6	0.90 [0.75-1.07]	.3	0.92 [0.70-1.22]	.5	1.01 [0.73-1.40]	.9
Model 7	0.91 [0.76-1.08]	.3	0.94 [0.71-1.24]	.6	1.02 [0.74-1.42]	.9
Total GC metabolites						
Model 1	0.67 [0.53-0.83]	<.001	0.67 [0.47-0.95]	.02	0.73 [0.48-1.12]	.15
Model 2	0.67 [0.54-0.85]	.001	0.66 [0.46-0.95]	.02	0.77 [0.50-1.18]	.2
Model 3	0.64 [0.51-0.81]	<.001	0.61 [0.43-0.88]	.007	0.74 [0.48-1.15]	.18
Model 4	0.64 [0.51-0.81]	<.001	0.60 [0.42-0.87]	.006	0.77 [0.49-1.19]	.2
Model 5	0.70 [0.55-0.88]	.003	0.65 [0.45-0.95]	.02	0.83 [0.53-1.31]	.4
Model 6	0.76 [0.64-0.91]	.003	0.72 [0.55 0.95]	.02	0.87 [0.62-1.23]	.4
Model 7	0.77 [0.64-0.92]	.004	0.73 [0.55-0.96]	.03	0.88 [0.63-1.24]	.5
(THF + alloTHF)/ THE ratio						
Model 1	1.37 [1.19-1.57]	<.001	1.24 [0.98-1.58]	.07	1.42 [1.23-1.80]	.003
Model 2	1.37 [1.20-1.58]	<.001	1.24 [0.98-1.57]	.07	1.44 [1.14-1.82]	.002
Model 3	1.41 [1.23-1.62]	<.001	1.31 [1.03-1.67]	.03	1.47 [1.16-1.86]	.002
Model 4	1.42 [1.23-1.63]	<.001	1.30 [1.02-1.66]	.03	1.48 [1.16-1.88]	.002
Model 5	1.34 [1.16-1.55]	<.001	1.21 [0.94-1.55]	.14	1.42 [1.10-1.82]	.006
Model 6	1.38 [1.18-1.16]	<.001	1.23 [0.93-1.62]	.15	1.43 [1.11-1.90]	.006
Model 7	1.38 [1.18-1.61]	<.001	1.23 [0.93-1.62]	.15	1.45 [1.11-1.90]	.006
Cortisol/cortisone ratio						
Model 1	1.22 [1.06-1.41]	.006	1.12 [0.89-1.42]	.3	1.68 [1.33-2.15]	<.001
Model 2	1.22 [1.06-1.41]	.007	1.12 [0.89-1.42]	.3	1.66 [1.31-2.12]	<.001
Model 3	1.20 [1.03-1.39]	.02	1.08 [0.84-1.37]	.6	1.65 [1.30-2.12]	<.001
Model 4	1.20 [1.03-1.39]	.02	1.08 [0.84-1.38]	.5	1.65 [1.29-2.11]	<.001
Model 5	1.14 [0.98-1.33]	.09	1.00 [0.79-1.28]	1.0	1.63 [1.27-2.11]	<.001
Model 6	1.17 [0.98-1.38]	.08	1.00 [0.77-1.31]	1.0	1.74 [1.31-2.32]	<.001
Model 7	1.16 [0.98-1.38]	.09	1.00 [0.77-1.31]	1.0	1.73 [1.30-2.31]	<.001

Note: Data are presented as hazard ratio (HR) per standard deviation increase in log-transformed urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, urinary cortisol/cortisone ratio, and urinary (THF + alloTHF)/THE ratio, plus 95% confidence interval (CI). Model 1 = age- and sex-adjusted associations; Model 2 = as model 1 + additional adjustment for body surface area; Model 3 = as model 2 + additional adjustment for hsCRP; Model 4 = as model 3 + additional adjustment for daily prednisolone dose; Model 5 = as model 4 + additional adjustment for eGFR CKD-EPI; Model 6 = as model 5 + additional adjustment for cardiovascular risk factors; Model 7 = as model 6 + additional adjustment for use of carbamazepine.

Abbreviations: alloTHF, allotetrahydrocortisol; GC, glucocorticoid; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

The gray shades present significant values for (almost) every model.

that expression of 11β-HSD2 is reduced in kidney failure.[38] Since we found a strong inverse association of (THF + alloTHF)/THE and cortisol/cortisone ratios with kidney function, this could also be true for our study. However, it has recently been suggested that exogenous steroids, such as prednisolone, could also influence peripheral cortisol balance by induction of 11β-HSD1, thereby creating a feed-forward mechanism enhancing systemic cortisol exposure.[13,14,22] Taking this into consideration, both decreased urinary cortisol excretion and increased (THF + alloTHF)/THE and cortisol/cortisone ratios in our study could be a reflection of the pharmacological

effects of prednisolone. This is supported by the very strong inverse association of total urinary endogenous glucocorticoid excretion with (THF + alloTHF)/THE ratio we found in our study, which was independent of kidney function and body composition.

Intriguingly, we found that both decreased urinary cortisol excretion and increased (THF + alloTHF)/THE and cortisol/cortisone ratios were associated with increased risk of mortality after kidney transplantation, which was independent of confounders such daily prednisolone dose and kidney function. One could postulate that the decreased urinary cortisol excretion and the apparent dysfunction of 11β-HSD activity independent of prednisolone dose may be linked to variation in inter-subject sensitivity to prednisolone.

In addition, in secondary analyses decreased urinary cortisol excretion was associated with increased risk of mortality from cardiovascular causes and increased (THF + alloTHF)/THE and cortisol/cortisone ratios with mortality from infectious cause. To our knowledge, there are no studies in RTR which investigated the association of urinary cortisol metabolism and mortality long-term after kidney transplantation. However, there are studies in other populations, such as patients with rheumatoid arthritis,[39-41] pituitary adenoma,[42] acromegaly,[20] and alcoholic hepatitis,[43] showing that treatment with higher doses of corticosteroids is associated with an increased risk of mortality.

Several limitations of our study warrant consideration. First, our study was observational in nature. Although we adjusted for several potential confounding variables, including parameters of kidney function, the possibility of residual confounding cannot be excluded. The healthy controls were not sex- and BMI-matched. We corrected for this statistically. However, this can introduce a bias regardless. Of the RTR in our cohort, 18% received tacrolimus, which is low compared to contemporary practice. This can be explained by changes in immunosuppressive regimes over time. In our institution, standard use of cyclosporine was replaced by tacrolimus in 2012.[17] Also, the prednisolone dosage in our cohort was higher than average in RTR patients due to changes in immunosuppressive protocols over time.[17]

Our study design did not allow us to investigate the mechanisms through which altered cortisol metabolism led to increased mortality risk. Therefore, we could only speculate on the causative mechanisms. Second, urinary cortisol metabolism was measured at a single time point only, and therefore, we could not take potential changes over time into account. Furthermore, it cannot be assured that urine collection was complete. However, we performed sensitivity analyses in which we excluded subjects with a relatively large difference between estimated and measured urinary creatinine excretion. These sensitivity analyses did not materially alter the associations of urinary cortisol excretion, total urinary endogenous glucocorticoid metabolite excretion, (THF + alloTHF)/THE ratio, and cortisol/cortisone ratio with all-cause mortality, cardiovascular mortality, and mortality from infectious cause found in the primary analyses. Third, although cortisol/cortisone and (THF + alloTHF)/THE ratios are widely accepted measures of 11β-HSD enzyme activities, it did not allow us to assess separate enzymatic activity of 11β-HSD1 and 11β-HSD2 iso-enzymes. More sophisticated study methodology, for example using radio-labeled cortisol and cortisone tracer infusions, may allow for more accurate estimation of 11β-HSD enzyme activities.[11] Furthermore, we did not diagnose HPA-axis suppression by a synacthen stimulation test, nor did we measure ACTH. However, measurement of urinary excretion of cortisol and its metabolites has the advantage of being relatively easy and much more feasible for large cohort studies.[44] In addition, urinary cortisol excretion is relatively unaffected by the circadian rhythm of cortisol or by varying plasma protein binding capacities compared to serum cortisol.[44,45]

Because prednisolone levels were not measured in the circulation, there is no measure of to what extent patients were actually exposed to exogenous glucocorticoids at a systemic and tissue-specific level, which can be highly variable between subjects. [46]

We used LC-MS/MS to determine the main metabolites of cortisol and cortisone where the gold standard to measure these metabolites is GC-MS.[47] However, our assay is very well developed and validated.[24] Therefore, we do not think that this will influence our results.

Finally, THF, 5a-THF, and THE are not the only metabolites of cortisol and cortisone. Other metabolites include α/β-cortol, α/β-cortolone, and 1β-hydroxyetiocholanolone. However, THF, 5a-THF, and THE account for almost 80% of the metabolites of cortisol and cortisone.[24]

The main strength of this study is that it is, to our knowledge, the first study to measure urinary cortisol in prednisolone-treated RTR. Moreover, it is the first study that specifically addresses both endogenous urinary glucocorticoid excretion and their ratios, representing 11β-HSD activity and mortality in RTR. Other strengths of our study include the well-characterized patient population, the relatively large sample size, and the long-term and complete follow-up. Also, the availability of appropriate healthy controls positively contributed to the reliability of our data.

In conclusion, we show for the first time that urinary cortisol metabolism is altered in prednisolone-treated RTR. Compared with healthy controls, RTR have decreased urinary excretion of cortisol and total endogenous glucocorticoid metabolites and increased urinary (THF + alloTHF)/THE and cortisol/cortisone ratios. Altered cortisol metabolism is associated with increased risk of mortality after kidney transplantation and might reflect the pharmacological effects of prednisolone. Measuring urinary glucocorticoid excretion might prove a future tool to personalize corticosteroid therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Stephan J. L. Bakker and Gerjan Navis: Participated in research design; Stephan J. L. Bakker, Annet Vulto, Laura V. de Vries, Arwin C. Timmermans, Antonio W. Gomes Neto, Daan J. Touw, Margriet F. C. de Jong, André P. van Beek, Robin P. F. Dullaart, and Gerjan Navis: Participated in the writing of the paper; Stephan J. L. Bakker and Laura V. de Vries: Participated in the performance of the research;

Isidor Minović, Martijn van Faassen, and Ido P. Kema: Contributed new reagents or analytic tools; Stephan J. L. Bakker, Annet Vulto, Laura V. de Vries, Arwin C. Timmermans, Daan J. Touw, Margriet F. C. de Jong, André P. van Beek, Robin P. F. Dullaart, and Gerjan Navis: Participated in data analysis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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