

Calcification Propensity and Survival among Renal Transplant Recipients

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ABSTRACT

Calciprotein particle maturation time (T_{50}) in serum is a novel measure of individual blood calcification propensity. To determine the clinical relevance of T_{50} in renal transplantation, baseline serum T_{50} was measured in a longitudinal cohort of 699 stable renal transplant recipients and the associations of T_{50} with mortality and graft failure were analyzed over a median follow-up of 3.1 years. Predictive value of T_{50} was assessed for patient survival with reference to traditional (Framingham) risk factors and the calcium-phosphate product. Serum magnesium, bicarbonate, albumin, and phosphate levels were the main determinants of T_{50} , which was independent of renal function and dialysis vintage before transplant. During follow-up, 81 (12%) patients died, of which 38 (47%) died from cardiovascular causes. Furthermore, 45 (6%) patients developed graft failure. In fully adjusted models, lower T_{50} values were independently associated with increased all-cause mortality (hazard ratio, 1.43; 95% confidence interval, 1.11 to 1.85; $P=0.006$ per SD decrease) and increased cardiovascular mortality (hazard ratio, 1.55; 95% confidence interval, 1.04 to 2.29; $P=0.03$ per SD decrease). In addition to age, sex, and eGFR, T_{50} improved prognostication for all-cause mortality, whereas traditional risk factors or calcium-phosphate product did not. Lower T_{50} was also associated with increased graft failure risk. The associations of T_{50} with mortality and graft failure were confirmed in an independent replication cohort. In conclusion, reduced serum T_{50} was associated with increased risk of all-cause mortality, cardiovascular mortality, and graft failure and, of all tested parameters, displayed the strongest association with all-cause mortality in these transplant recipients.

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Kidney transplantation considerably improves the prognosis of patients with ESRD; yet in renal transplant recipients (RTR) the annual rate of fatal or nonfatal cardiovascular events remains much higher compared with the general population.^{1,2} Kidney transplantation restores renal function but incompletely mitigates cardiovascular risk. This appears to be mainly a result of the markedly accelerated

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vascular calcification in RTR. Vascular calcification progresses substantially even in stable RTR and is an established predictor of morbidity and mortality.^{3–5} The so-called traditional risk factors tobacco use, diabetes, obesity, hypertension, and dyslipidemia (*i.e.*, the Framingham risk factors) have been identified as independent predictors of cardiovascular disease after kidney transplantation.⁶

Biomineralization is a tightly regulated and location-specific process, determined by both inhibitors and promoters of calcification. In serum, precipitation of supersaturated calcium and phosphate is prevented by the formation of primary calciprotein particles (CPPs).^{7,8} Primary CPPs spontaneously undergo topological rearrangement and convert to secondary CPPs.⁷ The formation of CPPs can be regarded as a defense mechanism against ectopic calcifications. Circulating CPPs can be detected in blood in pathologic situations like CKD, and their levels have been associated with aortic stiffness and vascular calcification.^{9,10}

Recently, a novel *in vitro* blood test was developed that monitors the calcification propensity, *i.e.*, the transformation time (T_{50}) of primary to secondary CPPs in serum.¹¹ A long delay of T_{50} indicates a high residual capacity of the patient's serum to prevent the formation of secondary CPPs and is therefore indicative of an intact endogenous defense against calcification.

In this study, we aimed to quantify calcification propensity in stable RTR and to determine its association with all-cause mortality, cardiovascular mortality, and graft failure risk by using the CPP maturation time (T_{50}) in serum. Furthermore, we compared the predictive value of serum T_{50} for the risk of mortality with the established “traditional” cardiovascular risk factors, *i.e.*, the “Framingham risk factors” and the calcium-phosphate product. Finally, we performed mediation analyses to identify potential causal pathways shared among T_{50} and two established nontraditional cardiovascular risk factors in RTR: N-terminal-pro brain natriuretic peptide (NT-proBNP)¹² and fibroblast growth factor 23 (FGF23).¹³

RESULTS

Study Population

All chronically kidney transplanted patients from the University Medical Center Groningen, transplanted for more than 1 year, were recruited for this study ($n=699$, 57% male, age 53 ± 13 years). The median time after kidney transplantation upon inclusion in the cohort was 5.4 (1.9–12.1) years. Serum T_{50} was normally distributed and mean serum T_{50} was 286 ± 62 minutes (Supplemental Figure 1). Baseline patient characteristics according to tertiles of T_{50} are presented in Table 1. Calcification propensity was significantly increased (*i.e.*, lower serum T_{50}) in RTR who had received a kidney from a deceased donor compared with RTR with a living donor (278 ± 62 versus 299 ± 58 minutes, respectively; t test $P<0.001$). Furthermore, there was a borderline significant difference between preemptive and nonpreemptive transplant recipients (296 ± 57

versus 284 ± 62 minutes, respectively; t test $P=0.07$). Multivariable linear regression analyses revealed that serum magnesium, albumin, venous bicarbonate, and parathyroid hormone (PTH) were positively associated, and phosphate, hemoglobin, and the use of calcineurin inhibitors (CNI) or vitamin K antagonists were inversely associated with serum T_{50} (Supplemental Table 1; model $R^2=0.41$). Of note, in multivariate analyses renal function and dialysis vintage were not independently associated with serum T_{50} .

Serum T_{50} and Mortality

During a median follow-up of 3.1 (interquartile range [IQR] 2.7–3.9) years, 81 out of 699 patients died (12%). Kaplan–Meier analysis revealed an increased all-cause mortality with tertiles of decreasing serum T_{50} (Figure 1A; log-rank $P<0.001$); first tertile $n=43$ (19%), second tertile $n=24$ (10%), and third tertile $n=14$ (6%); chi-squared test $P<0.001$.

Also, in univariable Cox regression analysis of serum T_{50} as a continuous variable, serum T_{50} was significantly associated with all-cause mortality (hazard ratio [HR], 1.69; 95% confidence interval [95% CI], 1.37 to 2.09; $P<0.001$ per SD decrease, Table 2). Hazard ratios were consistent in subgroup analyses (Figure 2). This association remained significant after adjustment for potential confounders, including recipient age and gender, eGFR (Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI]), albuminuria, the traditional risk factors (current smoking, systolic BP, body mass index, current diabetes and LDL cholesterol), high-sensitivity C-reactive protein, CNI use, dialysis vintage and type of kidney transplantation (living versus deceased donor) (final model HR, 1.43; 95% CI, 1.11 to 1.85; $P=0.006$ per SD decrease) (Table 2). When analyzed according to tertiles of T_{50} , patients in the lowest T_{50} tertile (*i.e.*, higher calcification propensity) were at a considerably higher mortality risk (final model HR, 2.86; 95% CI, 1.41 to 5.80) than patients in the middle (final model HR, 1.58; 95% CI, 0.76 to 3.32) or higher T_{50} tertile (reference, $P[\text{trend}]=0.002$).

During follow-up, 38 (47%) of the 81 patients who died, died due to cardiovascular causes. Other causes of death were infection (24%), malignancy (16%), and miscellaneous and other causes (14%). Kaplan–Meier analysis revealed an increased cardiovascular mortality with tertiles of decreasing serum T_{50} (Figure 1B; log-rank $P<0.001$). In Cox regression analysis, serum T_{50} was significantly associated with cardiovascular mortality independent of potential confounders (final model HR, 1.55; 95% CI, 1.04 to 2.29; $P=0.03$ per SD decrease) (Table 2).

To compare the performance of serum T_{50} with other biochemical measures of calcification as individual predictors of all-cause mortality, separate Cox regression analyses were performed for each variable. As indicated in Figure 3, serum T_{50} displayed the strongest association with all-cause mortality of all tested parameters, both in unadjusted and adjusted models. To identify possible pathophysiological pathways of T_{50} , the associations between T_{50} and FGF23, a phosphaturic hormone or NT-proBNP, a marker of cardiac dysfunction, and all-cause

Table 1. Baseline patient characteristics of the study population presented as tertiles of serum T_{50} in stable renal transplants

Characteristics	Renal transplant recipients Tertiles of T_{50}				P Value
	Overall n=699	Tertile 1 n=231	Tertile 2 n=233	Tertile 3 n=235	
T_{50} (minutes)	286±62	<262	262–311	>311	<0.001 ^a
Demographics					
Age, years	53.0±12.8	53.6±12.7	53.6±13.0	51.9±12.6	0.26
Male gender, n (%)	398 (57)	135 (58)	128 (55)	135 (57)	0.73
Current smoker, n (%)	84 (13)	31 (15)	25 (11)	28 (13)	0.62
Current diabetes, n (%)	170 (24)	62 (27)	63 (27)	45 (19)	0.08
BMI, kg/m ²	26.7±4.8	26.9±5.1	26.8±4.6	26.2±4.6	0.27
Systolic BP, mmHg	135.9±17.4	136.6±17.9	136.0±16.6	135.1±17.6	0.66
Diastolic BP, mmHg	82.5±10.9	82.7±10.9	82.5±10.2	82.4±11.7	0.95
Heart rate, bpm	68.6±11.9	69.2±13.0	68.8±11.6	67.9±11.1	0.49
Renal Transplantation					
Transplant vintage, years	5.4 [1.9–12.1]	6.1 [1.7–12.8]	5.3 [2.0–12.3]	5.2 [1.8–10.1]	0.54
Living donor, n (%)	236 (34)	61 (27)	77 (34)	98 (42)	0.002 ^a
Pre-emptive transplant, n (%)	105 (15)	28 (12)	35 (15)	42 (18)	0.22
Dialysis vintage, months	27 [9–52]	29 [10–55]	25 [10–48]	25 [8–51]	0.22
HLA mismatches, n	2 [1–3]	2 [1–3]	2 [1–3]	2 [1–3]	0.50
Age donor, years	42.8±15.6	41.2±16.2	43.2±15.3	43.8±15.2	0.19
Acute rejection, n (%)	186 (27)	69 (30)	67 (29)	50 (21)	0.07
Laboratory measurements					
Hemoglobin, mmol/L	8.2±1.1	8.0±1.1	8.3±1.1	8.4±1.0	<0.001 ^a
HbA1c, %	6.00±0.83	6.02±0.88	6.08±0.89	5.85±0.69	0.01 ^a
eGFR, CKD-EPI (mL/min per 1.73 m ²)	52.4±20.2	46.6±20.3	53.3±19.3	56.7±19.5	<0.001 ^a
Corrected calcium, mmol/L	2.41±0.15	2.37±0.14	2.40±0.14	2.45±0.15	<0.001 ^a
Phosphate, mmol/L	0.96±0.21	1.07±0.24	0.95±0.17	0.87±0.18	<0.001 ^a
Magnesium, mmol/L	0.95±0.12	0.93±0.13	0.95±0.11	0.98±0.11	<0.001 ^a
PTH, pmol/L	9.0 [5.9–14.7]	8.7 [5.2–15.9]	9.1 [6.2–14.4]	9.1 [6.3–14.1]	0.48
Venous pH	7.37±0.04	7.36±0.05	7.37±0.04	7.37±0.04	<0.001 ^a
Venous HCO ₃ ⁻ , mmol/L	24.6±3.1	23.6±3.6	24.5±2.6	25.8±2.6	<0.001 ^a
hsCRP, mg/L	1.6 [0.7–4.6]	1.9 [0.8–6.2]	1.5 [0.8–4.4]	1.4 [0.6–3.5]	0.01 ^a
Albumin, g/L	43.0±3.0	42.1±3.1	42.8±2.7	44.0±2.8	<0.001 ^a
Alkaline phosphatase, U/L	67 [54–83]	67 [53–84]	69 [55–83]	66 [51–83]	0.21
Total cholesterol, mmol/L	5.0 [4.4–5.8]	4.9 [4.2–5.9]	4.9 [4.3–5.6]	5.2 [4.5–5.8]	0.03 ^a
HDL cholesterol, mmol/L	1.3 [1.1–1.6]	1.3 [1.1–1.6]	1.3 [1.0–1.6]	1.3 [1.1–1.7]	0.09
LDL cholesterol, mmol/L	2.9 [2.3–3.5]	2.8 [2.2–3.6]	2.9 [2.3–3.4]	2.9 [2.5–3.6]	0.08
Triglycerides, mmol/L	1.68 [1.25–2.30]	1.71 [1.24–2.47]	1.67 [1.25–2.23]	1.66 [1.24–2.24]	0.65
Albuminuria, mg/24 h	40 [11–182]	82 [16–300]	36 [9–133]	25 [9–112]	<0.001 ^a
Medication					
Antihypertensives, n (%)	616 (88)	211 (91)	202 (87)	203 (86)	0.18
Statins, n (%)	371 (53)	135 (58)	125 (54)	111 (47)	0.05 ^a
Calcium supplements, n (%)	150 (21)	72 (31)	47 (20)	31 (13)	<0.001 ^a
Vitamin D supplements, n (%)	171 (24)	73 (32)	45 (19)	53 (23)	0.006 ^a
Vitamin K antagonists, n (%)	78 (11)	37 (16)	20 (9)	21 (9)	0.02 ^a
Prednisone, mg/d	10 [7.5–10]	10 [7.5–10]	10 [7.5–10]	10 [7.5–10]	0.17
Calcineurin inhibitors, n (%)	400 (57)	153 (66)	133 (57)	114 (49)	0.001 ^a
Proliferation inhibitors, n (%)	584 (84)	182 (79)	202 (87)	200 (85)	0.05
Sirolimus, n (%)	13 (2)	3 (1)	4 (2)	6 (3)	0.69

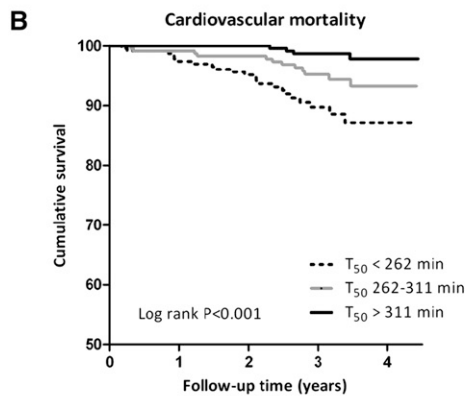
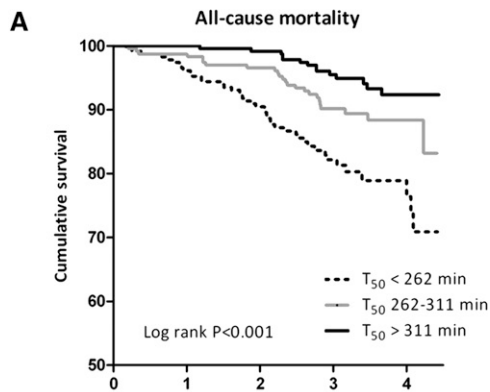
^aDifferences were tested by ANOVA or Kruskal–Wallis test for continuous variables and with chi-squared test for categorical variables.

Data are represented as mean±SD or median [IQR]. BMI, body mass index; HbA1c, glycated hemoglobin; HCO₃⁻, bicarbonate; hsCRP, high-sensitivity C-reactive protein.

mortality, was tested using mediation analyses (Supplemental Figure 2). The indirect effect of T_{50} on mortality via FGF23 was not significant. In contrast, the indirect effect of T_{50} on mortality via NT-proBNP was significant ($P<0.05$) with a magnitude of 10.7%.

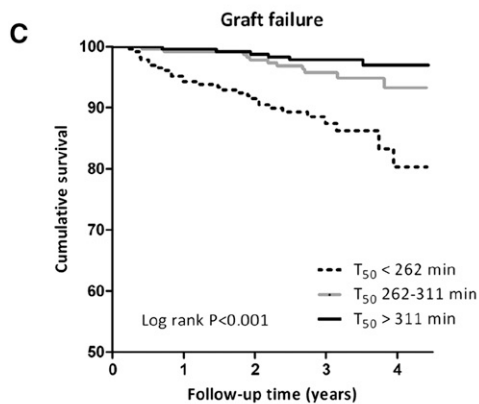
Predictive Value of Serum T_{50} for All-Cause Mortality

The discrimination performance of different models was tested with Harrell's concordance statistic (c-statistic). Serum T_{50} alone had a c-statistic (95% CI) of 0.67 (0.61 to 0.73). When added to a basic model of recipient age, gender, and eGFR



No. at risk for Figures 1A and 1B

	0	1	2	3	4
Tertile 1	231	222	202	97	31
Tertile 2	233	230	223	131	43
Tertile 3	235	235	232	165	45



No. at risk

	0	1	2	3	4
Tertile 1	231	209	184	83	25
Tertile 2	233	228	219	126	39
Tertile 3	235	234	229	160	44

Figure 1. Kaplan–Meier curve for mortality and graft failure. (A) All-cause mortality, (B) cardiovascular mortality, and (C) death-censored graft failure among renal transplant recipients according to tertiles of baseline T_{50} .

(c-statistic, 0.72; 95% CI, 0.66 to 0.78), the serum T_{50} yielded higher Harrell’s c values than Framingham risk factors or calcium-phosphate product: c-statistics 0.75 (95% CI, 0.70 to

0.80); 0.73 (95% CI, 0.67 to 0.79); 0.72 (95% CI, 0.66 to 0.78), respectively; see Supplemental Table 2. The model containing serum T_{50} , but not the model containing Framingham risk factors, improved significantly according to the integrated discrimination improvement index (IDI, 1.6%; $P=0.03$ versus 0.8%, $P=0.3$; see Supplemental Table 2). Net reclassification improvement (NRI) analysis showed that addition of serum T_{50} to a basic model of recipient age, gender, and eGFR improved classification in both higher and lower risk categories (NRI 14%, $P=0.002$; see Table 3), whereas addition of traditional risk factors or calcium-phosphate product did not improve classification (data not shown, NRI -2% , $P=0.70$; NRI 0.7%, $P=0.80$, respectively).

Serum T_{50} and Graft Failure

After a median follow-up of 3.1 (IQR, 2.6–3.8) years, 45 of 699 patients (6%) developed graft failure. The main reason for the allograft loss was chronic transplant dysfunction (71%). Other causes for graft failure were relapse of the original kidney disease (9%), infection (9%), vascular disease (4%), acute rejection (4%), and unknown cause (2%). The patients who developed graft failure had lower serum T_{50} compared with the patients who did not develop graft failure (235 ± 68 versus 289 ± 60 , respectively, $P<0.001$). Competing risks analyses using a subdistribution proportional hazards model indicated that all-cause mortality was not a competing risk for graft failure. Kaplan–Meier analysis revealed a graded increase in risk of death-censored graft failure according to descending tertiles of serum T_{50} (Figure 1C; log-rank $P<0.001$). In univariable analyses of serum T_{50} as a continuous variable, serum T_{50} was significantly associated with death-censored graft failure (HR, 2.61; 95% CI, 1.94 to 3.50; $P<0.001$ per SD decrease). This association remained significant after adjustment for potential confounders, including recipient age and gender, eGFR (CKD-EPI), and albuminuria or CNI use, dialysis vintage, and the type of donor (Supplemental Table 3). Furthermore, patients in the lowest T_{50} tertile were at a higher graft failure risk compared with patients in the higher T_{50} tertile (HR, 6.38; 95% CI, 2.63 to 15.48; $P<0.001$) (Supplemental Table 3). Serum T_{50} displayed significant associations with graft failure in adjusted models in separate Cox regression analyses (Supplemental Figure 3).

Independent Replication Cohort

Renal transplant recipients ($n=198$, 68% male, age 52.9 ± 13.0 years) were included at 5.2 (IQR, 1.8–10.2) years after kidney transplantation. Diabetes mellitus was present in 24%, mean body mass index was 26.1 ± 4.4 kg/m², mean systolic BP was 137 ± 19 mmHg and mean diastolic BP was 85 ± 10 mmHg, 9% of the patients were transplanted preemptively, median dialysis vintage was 17 (7–36) months, mean eGFR was 49.2 ± 18.9 ml/min per 1.73 m², mean phosphate was 1.05 ± 0.26 mmol/L, mean (corrected) calcium was 2.45 ± 0.14 mmol/L and median PTH was 6.3 (4.3–9.7) pmol/L. At study inclusion, immunosuppressive treatment was mainly based on a calcineurin-inhibitor

Table 2. Associations of calcification propensity, serum T_{50} , with all-cause mortality and cardiovascular mortality in stable renal transplant recipients

	High	Intermediate HR (95% CI)	Low HR (95% CI)	P (trend) Value	Serum T_{50} continuous	
					HR (95% CI) per SD	P Value
All-cause mortality ($n_{\text{events}}/n_{\text{total}}=81/699$)						
Model 1 ^a	1.0 (Ref)	1.91 (0.99–3.70)	4.09 (2.23 to 7.49)	<0.001	1.69 (1.37 to 2.09)	<0.001
Model 2 ^b	1.0 (Ref)	1.77 (0.91–3.42)	3.77 (2.06 to 6.91)	<0.001	1.69 (1.36 to 2.09)	<0.001
Model 3 ^c	1.0 (Ref)	1.77 (0.90–3.49)	3.54 (1.88 to 6.65)	<0.001	1.57 (1.26 to 1.97)	<0.001
Model 4 ^d	1.0 (Ref)	2.03 (0.98–4.19)	3.50 (1.74 to 7.01)	<0.001	1.56 (1.23 to 2.00)	<0.001
Model 5 ^e	1.0 (Ref)	1.58 (0.76–3.32)	2.86 (1.41 to 5.80)	0.002	1.43 (1.11 to 1.85)	0.006
Cardiovascular mortality ($n_{\text{events}}/n_{\text{total}}=38/699$)						
Model 1 ^a	1.0 (Ref)	3.25 (0.88–12.02)	7.18 (2.09 to 24.72)	0.001	1.70 (1.19 to 2.43)	0.003
Model 2 ^b	1.0 (Ref)	3.12 (0.84–11.55)	6.82 (1.98 to 23.50)	0.001	1.71 (1.18 to 2.46)	0.004
Model 3 ^c	1.0 (Ref)	3.02 (0.82–11.19)	6.12 (1.75 to 21.44)	0.002	1.61 (1.10 to 2.36)	0.01
Model 4 ^d	1.0 (Ref)	2.75 (0.74–10.30)	6.07 (1.72 to 21.44)	0.002	1.59 (1.08 to 2.35)	0.02
Model 5 ^e	1.0 (Ref)	2.63 (0.70–9.92)	5.58 (1.58 to 19.72)	0.003	1.55 (1.04 to 2.29)	0.03

Data are presented as hazard ratio (HR) plus 95% CI according to tertiles of serum T_{50} and per SD serum T_{50} decrease. Mean \pm SD, T_{50} : 286 \pm 62 minutes.

^aModel 1: crude.

^bModel 2: adjusted for age and gender.

^cModel 3: adjusted for model 2 plus eGFR (CKD-EPI) and albuminuria.

^dModel 4: adjusted for model 3 plus current smoking, body mass index, diabetes mellitus, systolic BP and LDL cholesterol.

^eModel 5: adjusted for model 4 plus high-sensitivity C-reactive protein, calcineurin inhibitor use, dialysis vintage and type of kidney transplant (living versus deceased donor).

(77%) combined with prednisone (93%). All but eight patients received at least one antihypertensive drug (96%).

During 5.1 (IQR, 4.9–5.3) years of follow-up, 28 (14%) patients died and 14 (7%) patients developed graft failure. In univariable Cox regression analyses, serum T_{50} was significantly associated with all-cause mortality (HR, 1.68; 95% CI, 1.03 to 2.74; $P=0.04$ per SD decrease) and death-censored graft failure (HR, 3.80; 95% CI, 1.53 to 9.45; $P=0.004$ per SD decrease).

DISCUSSION

The measurement of the CPP maturation time (T_{50}), an integrated functional measure of calcification propensity, is a novel approach in medicine. T_{50} quantifies the effects of calcification-promoting and -inhibiting forces in serum by monitoring the spontaneous transformation of spherical primary CPPs, which contain amorphous calcium phosphate, to spindle-shaped secondary CPPs, which contain crystalline calcium phosphate.¹¹ The major findings of this study are that serum T_{50} is associated with all-cause and cardiovascular mortality and graft failure in stable RTR. In both unadjusted and adjusted models, these associations were already observed after 3 years of follow-up and were independent of established risk factors. The associations of T_{50} with all-cause mortality and graft failure were confirmed in an independent cohort. Corroborating and extending these findings, calcification propensity had additive value for patient survival prediction in addition to recipient age, gender, and eGFR.

The *in vitro* T_{50} measurement is very stable intraindividually.¹⁴ Within-subject variance was 19% and between-subject variance was 81%, *i.e.*, compared with serum phosphate,

serum T_{50} measurement yielded a higher intraclass correlation coefficients (0.63; 95% CI, 0.34 to 0.71; and 0.81, 95% CI, 0.68 to 0.91, respectively), indicating a higher stability of T_{50} than serum phosphate in individuals over time.¹⁴

Serum phosphate, magnesium, venous bicarbonate and albumin were the strongest determinants of T_{50} in our multivariable linear regression model. This model explained about 40% of the variation of T_{50} . Amorphous calcium phosphate is formed as the first mineral phase and consists of so-called Posner clusters ($\text{Ca}_9[\text{PO}_4]_6$),¹⁵ when calcium and phosphate concentrations are raised in aqueous solutions. This amorphous mineral phase is then spontaneously transformed via octacalcium phosphate ($\text{Ca}_8\text{H}_2[\text{PO}_4]_6$) to hydroxyapatite ($\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$).¹⁶ *In vitro* data demonstrate that the molecules found in our multivariate analysis have direct accelerating (phosphate) or delaying (magnesium, bicarbonate, and albumin) effects on this transformation process.^{11,17–19} Although PTH was also an independent determinant of serum T_{50} , PTH did not differ among the tertiles of serum T_{50} . The explanation for this seeming discrepancy is probably that continuous analysis has more statistical power to detect associations than categorical analysis. Interestingly, total as well as albumin-corrected calcium did not have a strong impact on T_{50} , a result also in line with previous *in vitro* and clinical data.^{11,14} After multivariable modeling, serum T_{50} lost its significant association with renal function in stable RTR, corroborating data previously obtained in CKD patients.¹⁴ Of note, the multivariable model did not include dialysis vintage, indicating that T_{50} is independent of time of previous uremic exposure in ESRD before transplantation.

Our results extend recently published findings in predialysis CKD patients, where T_{50} was associated with all-cause mortality.¹⁴ In the renal transplant population, several parameters

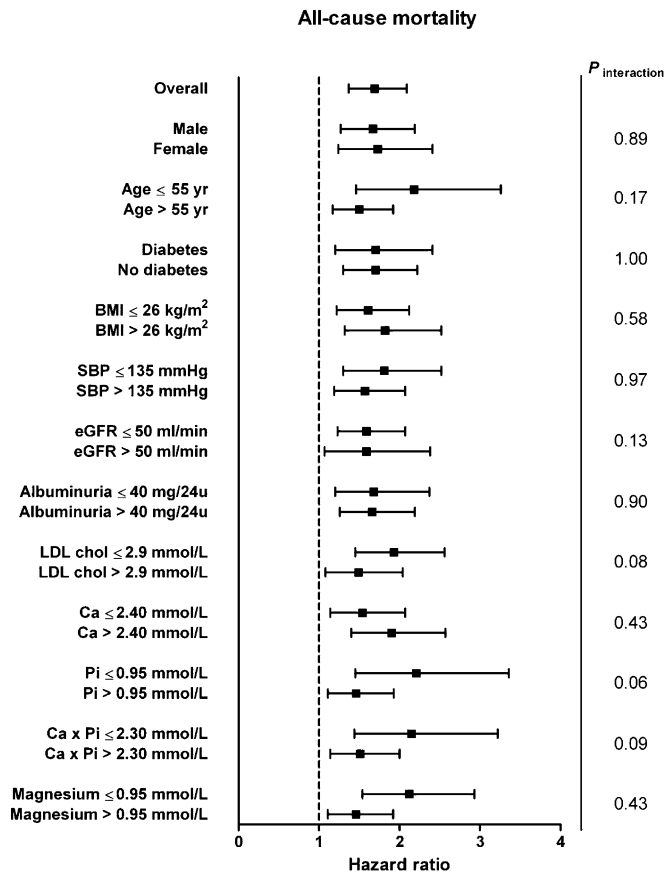


Figure 2. Forest plot of sub-analyses of serum T_{50} demonstrating that hazard ratios for all-cause mortality were consistent in different subgroups.

of calcium-phosphate metabolism have been associated with adverse outcome.²⁰ In addition, traditional cardiovascular risk factors in the general population (*i.e.*, the Framingham risk factors) are predictive of cardiovascular events and therefore should also be considered in RTR according to current guidelines.⁶ Interestingly, these risk factors had no predictive value in our study. This indicates that calcification propensity T_{50} as an integrated value representing non-traditional risk factors may be of clinical relevance as a prognostic novel biomarker in RTR.

Pathophysiologically, the close association of T_{50} with all-cause and cardiovascular mortality is potentially related to its association with naturally occurring CPPs in renal patients. CPPs have been found in different stages of preclinical and clinical CKD and it is prudent to assume that these particles can also be encountered in RTR.^{9,21,22} Calcification propensity measured as T_{50} , had a higher predictive value for future events and survival than the direct determination of circulating CPPs.¹⁴

T_{50} might share common pathophysiologic pathways with established markers of mineral metabolism or impairment of cardiac function. We, therefore, tested whether the association between T_{50} and mortality was modulated by FGF23, an important regulator of mineral metabolism, or

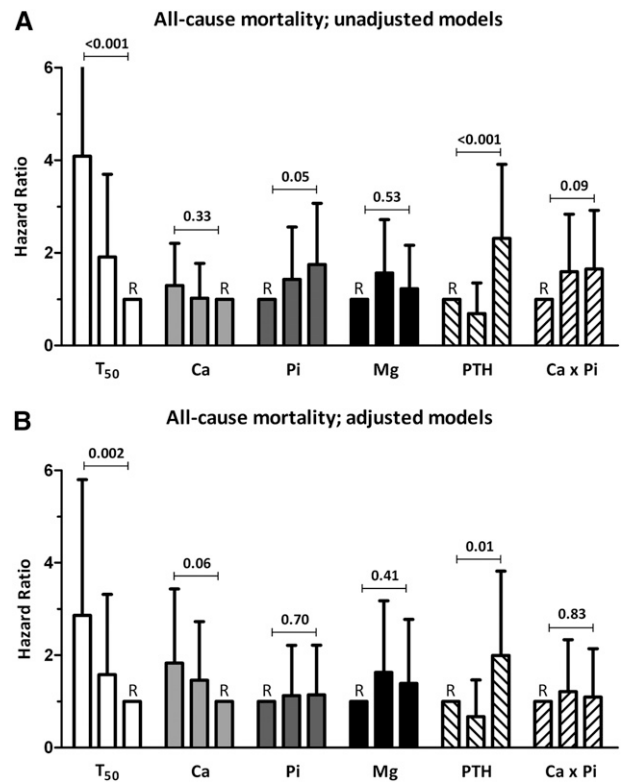


Figure 3. Comparative analysis of serum T_{50} with serum corrected calcium (Ca), phosphate (Pi), magnesium (Mg), parathyroid hormone (PTH) and calcium-phosphate (Ca x Pi) product, displayed per tertile as independent risk factors for all-cause mortality. Bars represent hazard ratio with 95% CI. (A) Crude analyses. (B) Adjustment for age, gender, eGFR, albuminuria, Framingham risk factors (systolic BP, body mass index, smoking, diabetes mellitus and LDL cholesterol), high-sensitivity C-reactive protein (hsCRP), calcineurin inhibitor use, dialysis vintage and type of donor (living versus deceased). Tertile with the lowest risk on mortality served as reference group (R).

NT-proBNP, a strong predictor of mortality in RTR.¹² Although no effect of FGF23 was found, NT-proBNP had a small but significant modulating effect on the association between T_{50} and mortality. This indicates that the mechanism by which T_{50} may exert its effects includes a pathophysiologic pathway and mechanism partially shared with NT-proBNP. We propose that a higher calcification propensity, by promoting vascular calcification over time, may predispose to an adverse hemodynamic state (volume overload, cardiac dysfunction), as reflected by higher NT-proBNP levels.^{23,24} Our data suggest that this pathway underlies at least in part the association between a lower T_{50} and a higher mortality risk.

Interestingly, T_{50} was also associated with graft failure in two independent cohorts, indicating that a tight control of calcification propensity may also be of high relevance for single organs or organ systems. A hypothetical causal relation may exist through the stiffness of the renal vasculature and intrarenal resistance, a marker currently used in renal ultrasound imaging

Table 3. Net reclassification improvement based on serum T_{50}

Without serum T_{50} ^a	With serum T_{50}			Total (n)
	<5%	5%–10%	>10%	
Patients with all-cause mortality ^{b,c}				
<5%	4	4 ^d		8
5%–10%		9	4 ^d	13
>10%		3 ^e	57	60
Total	4	16	61	81
Patients without all-cause mortality ^{b,c}				
<5%	176	17 ^e		193
5%–10%	43 ^d	90	22 ^e	155
>10%	3 ^d	40 ^d	225	268
Total	222	147	247	616

^aMultivariable model including recipient age and gender and eGFR (CKD-EPI).

^bNet reclassification improvement was 14%, $P=0.002$.

^c697 RTR were included in this analysis.

^dNumbers of patients who were correctly reclassified by the model with serum T_{50} .

^eNumbers of patients who were incorrectly reclassified by the model with serum T_{50} .

for the prediction of renal aging and rejection.^{25,26} Alternatively, but not mutually exclusively, the mineralization propensity within the tubular system might be related to T_{50} and to the progression of renal failure. This hypothesis was initially proposed as the *precipitation-calcification hypothesis*, and is currently being reconsidered in the field of renal failure progression and premature aging.^{27–29}

T_{50} might be of clinical use for the early detection of patients at high mortality risk. Furthermore, serum T_{50} may be of use as a guide for the treatment of renal patients. Therapeutic strategies to improve serum calcification propensity should be identified in future clinical trials. Based on our analysis of T_{50} determinants and previous *in vitro* data, interventions lowering serum phosphate (*i.e.*, dietary restriction and/or phosphate binders) or supplementation of magnesium or bicarbonate may be suitable to improve serum T_{50} as an intermediate end point.

Our study has several limitations and strengths. Although prospective, it is of observational nature and therefore a causality of the associations found, although likely, cannot be proven. The prespecified all-cause mortality risk categories (<5%/ 5%–10%/ >10%) used in the NRI analysis have not been validated. The absolute meaning of these risk categories should be interpreted with caution. Furthermore, no clinical data on vascular calcification as an intermediate end point were available. On the other hand, strengths include the complete follow-up and uniform single-center handling of samples along with the hard and clinically relevant end points (all-cause and cardiovascular mortality, and graft failure) and the confirmation of the major findings in a replication cohort.

In conclusion, increased serum calcification propensity (*i.e.*, lower serum T_{50}) is a novel *in vitro* diagnostic tool and potent predictor and functional biomarker of all-cause and

cardiovascular mortality and of graft failure in long-term RTR, which substantially improves mortality prognostication. Intervention studies based on T_{50} measurements are needed to clarify whether therapeutic targeting of serum T_{50} improves patient and graft survival after kidney transplantation.

CONCISE METHODS

Research Design and Subjects

For this longitudinal cohort study, RTR with a functioning graft for over 1 year were recruited. A total of 707 out of 817 eligible RTR (87%) who visited the outpatient clinic of the University Medical Center Groningen, The Netherlands between November 2008 and June 2011 were included.^{30,31} This cohort design yielded a cohort representative of our outpatient clinic of prevalent RTR with a median time after transplantation of 5.4 (IQR, 1.9–12.1) years. T_{50} was finally measured in 699 RTR with available serum samples (99%). The Institutional Review Board approved the study protocol (METC 2008/186), which adhered to the Declaration of Helsinki.

Clinical End Points

The primary end point of this study was all-cause mortality and the secondary end points were cardiovascular mortality (defined as death due to cerebrovascular disease, ischemic heart disease, heart failure, or sudden cardiac death) and death-censored graft failure (defined as restart of dialysis or retransplantation). End points were recorded until the end of May 2013; median follow-up was 3.1 (IQR, 2.7–3.9) years. There was no loss to follow-up.

Clinical and Laboratory Measurements

Relevant transplant characteristics such as date of transplantation, donor characteristics, HLA mismatches, dialysis vintage and acute rejection were extracted from the local University Medical Center Groningen renal transplantation database. Cause of death or graft failure was obtained from patient records. Further details of the cohort have been published previously.^{30,31} In short, upon entry into the cohort, blood was drawn in the morning after completion of a 24-hour urine collection. Routine plasma and urine analyses, including electrolytes, creatinine, and albumin, were performed using standard laboratory procedures. Serum cholesterol, triglycerides, alkaline phosphatase, hemoglobin A1c, high-sensitivity C-reactive protein, and NT-proBNP were also directly analyzed using standard laboratory procedures. Serum samples were stored at -80°C . The eGFR was calculated using the CKD-EPI equation.³² Serum calcium was adjusted for hypoalbuminemia (<40 g/L); corrected calcium = serum calcium (mmol/L) + $0.02 \times (40 - \text{serum albumin [g/L]})$. Serum intact FGF23 levels were determined using a commercially available ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan). Intra-assay and interassay coefficients of variation are $<10\%$ and $<14\%$, respectively.³³ Serum magnesium was measured using the xylydyl blue method.³⁴

Serum T_{50} Measurement

The T_{50} calcification propensity test has been described previously.¹¹ For the present study, the test was performed with minor changes that

did not materially change the test when compared with the original description. Here, serum samples were measured in triplicate in 384-well plates at 37°C over 600 minutes in a Nephelostar nephelometer (BMG Labtech, Ortenberg, Germany). All serum samples were measured in a blinded manner. Stock solutions were a calcium and a phosphate solution. The pH was adjusted to 7.40 at 37°C in both solutions. For measurement, 35 μ l of calcium solution was mixed with 40 μ l serum, and then 25 μ l of the phosphate solution was added. Data analyses of nonlinear regression curves were performed using Microsoft Excel software to determine the half-maximal precipitation time (T_{50}). The analytical coefficients of variation of standards precipitating at 120, 260, and 390 minutes were 7.8%, 5.1%, and 5.9%, respectively.

Independent Replication Cohort

Between August 2006 and February 2007, all RTR from the outpatient clinic of the Department of Nephrology at the University Hospital Bern were screened for inclusion in this cohort study. Patients were included if they had been transplanted at least 6 months before inclusion and revealed a stable renal graft function (serum creatinine stable within a range of $\pm 20\%$ in the last 3 months) with no signs of acute rejection. Patients with cardiac arrhythmia, hemodynamic significant transplant renal artery stenosis, hydronephrosis $>$ grade 2 or compression of the transplant by adjacent masses, were excluded from the study. A total of 222 RTR were screened and met the general inclusion criteria. Of these, 15 refused consent and seven were excluded because of screening failure. The follow-up was 52 months. Two patients were lost during follow-up. The study was approved by the local ethics committee and registered with the Cochrane renal group database (<http://www.cochrane-renal.org>, CRG110600098).

Routine laboratory values were obtained using standard techniques. The GFR was estimated according to the CKD-EPI formula.³² Serum calcification propensity, T_{50} , was measured as described above.

Statistical Analyses

The data are presented as mean \pm SD, median (IQR), and number (percentage) as indicated. A P value < 0.05 (two-tailed) was considered statistically significant. Statistical analyses were performed using SPSS 20.0 for Windows (IBM SPSS, Chicago, IL), STATA Statistical Software: Release 11 (StataCorp., College Station, TX) and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Variable distribution was tested with histograms and probability plots. For illustrative purposes, the study population was subdivided into tertiles of T_{50} to visualize associations with serum T_{50} . P values for differences in T_{50} tertiles were assessed with ANOVA for normally distributed continuous data, the Kruskal–Wallis test for non-normally distributed data and the chi-squared test for nominal data. Univariable and subsequent multivariable linear regression analyses were used to identify independent determinants of T_{50} . Non-normally distributed variables were transformed to the natural log to fulfill criteria for linear regression analyses. Multivariable linear regression models were constructed using backward selection ($P_{\text{out}} > 0.05$) including variables that were significantly associated with T_{50} in univariable analysis.

Tertiles of serum T_{50} were tested for associations with all-cause mortality and death-censored graft failure by Kaplan–Meier analysis with log-rank testing. Associations of T_{50} with all-cause mortality, cardiovascular mortality or graft failure were further tested by Cox proportional hazards regression analysis with stepwise adjustments for relevant covariates. Non-normally distributed variables were transformed to the natural log before entering the Cox proportional hazards regression analysis models. The full model for all-cause or cardiovascular mortality included adjustment for age, gender, renal function, albuminuria, the Framingham risk factors, high-sensitivity C-reactive protein, CNI use, dialysis vintage, and type of kidney transplantation (living or deceased). The models for graft failure included adjustment for (recipient) age and gender (model 2) plus renal function, albuminuria (model 3), or CNI use, dialysis vintage, and the type of kidney transplantation (living or deceased) (model 4). Cox regression models were built stepwise to keep the number of covariates accurate in relation to the number of events and to avoid over fitting.³⁵

In additional sensitivity analyses, associations of T_{50} with all-cause mortality were tested by Cox proportional hazards regression analysis in subgroups. For continuous variables the subgroups were based on below or above mean or median. To compare the performance of serum T_{50} with serum corrected calcium, serum phosphate, serum magnesium, serum PTH and calcium-phosphate product as individual risk factors for all-cause mortality or graft failure, separate Cox regression analyses were performed for each variable and adjusted for known risk factors of mortality or graft failure, respectively. For each exposure, the first or last tertile served as the reference group (hazard ratio set at 1) depending on which tertile had the lowest risk of an event.

We assessed model discrimination using Harrell's concordance statistic (c -statistic), the NRI and the integrated discrimination improvement index.^{36,37}

Harrell's c -statistic corresponds to the area under the receiver-operating curve for proportional hazards models. Harrell's c -coefficient is the proportion of all usable subject pairs in which the predictions and outcomes are concordant. The value "1" implies a perfect discrimination, whereas the value "0.5" implies a performance comparable to chance. The NRI provides reclassification tables constructed separately for patients with and without events and quantifies the correct movement between categories: upwards for events and downward for non-events. For the NRI we used the following classifications: low ($< 5\%$), intermediate (5%–10%), or high ($> 10\%$) all-cause mortality risk. Addition of serum T_{50} , traditional risk factors or calcium-phosphate product to the basic model including recipient age and gender and eGFR (CKD-EPI) was tested. Improved classification was defined as upward movement in the risk categories for participants who have died and downward movement for participants who survived. The integrated discrimination improvement index represents a continuous measure without *a priori*-defined risk categories. Competing risks analyses were performed using a subdistribution proportional hazards model.³⁸

Possible mediation by FGF23 or NT-proBNP on the association between T_{50} and all-cause mortality was examined (Supplemental Figure 2). The Preacher and Hayes method was used to test magnitude and significance of mediation.^{39,40} First, the total effect of T_{50} on mortality was estimated using logistic regression analysis. Second, the indirect

effect of T_{50} on mortality via FGF23 or NT-proBNP was calculated. Third, the significance of the indirect effect was assessed with bias-corrected bootstrap confidence intervals with 2000 repetitions. Finally, the magnitude of mediation was calculated by dividing the coefficient of the indirect effect by the total effect. Significant mediation ($P < 0.05$) was proven if zero was not between the lower and upper boundary of the 95% confidence interval of the indirect effect.

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DISCLOSURES

A.P. and W.J.-D. are co-founders of Calcisco AG, Bern, Switzerland.

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