Title page:

Vitamin D as a risk factor for patient survival after kidney transplantation; a prospective observational cohort study

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Abstract

Background

Short-term survival after kidney transplantation is excellent, but long-term survival remains low and is equivalent to non-end stage renal disease patients with many invasive malignancies. The aim of the study was to explore vitamin D status in the early phase after transplantation as a prognostic marker for long-term graft- and patient survival.

Methods

All first-time kidney transplant recipients between October 2007 and October 2012 in Norway were included. Vitamin D was measured ten weeks post-transplant. Information on graft failure and death was obtained from the Norwegian Renal Registry.

Results

Seven hundred and sixty-two first-time kidney transplant recipients were included, median age 57 years, with a median follow-up of 82 months. In the follow-up period there were 172 graft failures (23%) and 118 deaths (15%). Eighty-six percent of the transplant recipients with sufficient vitamin D levels were alive with a well-functioning graft after five years using Kaplan-Meier survival estimates, compared with 79% and 76% of the patients with vitamin D deficiency and insufficiency, respectively (p=0.006).

Conclusion

In a nation-wide cohort of 762 first-time kidney transplant recipients, long term graft- and patient survival were better in recipients with vitamin D sufficiency ten weeks post-transplant compared with those with vitamin D deficiency and insufficiency.

Introduction

Chronic Kidney Disease-Mineral and Bone Disorder is a term that encompasses a constellation of abnormalities in the calcium-phosphate homeostasis seen in most patients with chronic kidney disease (CKD). Disturbances in the mineral and bone metabolism develop early in the progression of the disease, leading to calcification of the vasculature and soft tissue, renal bone disease and increased cardiovascular morbidity and mortality[1]. Increased levels of fibroblast growth factor 23 (FGF23) and subsequent increased levels of parathyroid hormone (PTH) increase the urinary excretion of phosphate, reduce the production of active vitamin D, compensating and resulting in near-normal levels of serum phosphate and calcium. As the disease progresses towards end-stage renal disease (ESRD) the compensatory mechanisms are overwhelmed, resulting in hyperphosphatemia, hypocalcemia, abnormal bone turnover and extra-skeletal calcification[2].

Vitamin D3 (cholecalciferol) is important for calcium and bone homeostasis[3]. In humans, vitamin D3 is primarily produced from 7-dehydrocholesterol in the skin by exposure to sunlight, and only a small amount comes from dietary sources or supplements. Some supplements may contain the plant analog, vitamin D2 (ergocalciferol). The formation of the fully active vitamin D metabolite, 1,25-dihydroxyvitamin D (calcitriol), requires a two-step hydroxylation, the first step in the liver and the second step in the kidneys[4]. Vitamin D is essential for intestinal and renal handling of calcium and phosphate, as well as for bone turnover through interplay with PTH and FGF23[5,6]. Serum levels of 25(OH)D reflect vitamin D body stores, and are used as a measure of vitamin D status[7].

A large number of observational studies indicate that vitamin D has a number of effects beyond the regulation of the mineral metabolism. The almost universal

presence of vitamin D receptors on different cell types may explain why 25(OH)D deficiency is associated with diseases such as cardiovascular disease[8], cancer[9], diabetes[10], and overall mortality[11].

A kidney transplantation is the preferred renal replacement therapy for most patients with ESRD[12], improving both quality of life, and long-term survival[13]. Short-term results after kidney transplantation are excellent. Long-term prognosis is better than in chronic dialysis, but annual rates of graft failure beyond the first year post-transplant have remained largely unchanged since the 1990s[14]. Five year survival after transplantation is still comparable to the prognosis associated with many invasive malignancies[15].

The aim of the study was to explore the association between vitamin D status ten weeks post-transplant and long-term graft- and patient survival.

Patients and methods

The study is a prospective, observational cohort study, where all patients receiving a first-time kidney transplant between October 15th 2007 and October 18th 2012 in Norway were included. In Norway there is one solid organ transplantation center, Oslo University Hospital, Rikshospitalet (OUS-RH), serving the entire country, without selection bias. All 762 adult first-time single-kidney transplant recipients in the period were included in the study, Figure 1.

The standard immunosuppressive regimen included the use of basiliximab and methylprednisolone induction. The maintenance immunosuppression consisted of a calcineurin inhibitor (CNI) in combination with mycophenolate (95%) and prednisolone (100%). From 2007 to 2011, tacrolimus (Tac) was the preferred CNI to patients younger than 50 years and ciclosporine A (CsA) to older patients, patients

with high BMI or impaired glucose tolerance. From 2012, tacrolimus was preferred CNI to all patients except those with impaired glucose tolerance. In the total cohort 53% used Tac and 46% CsA. A mammalian target of rapamycin inhibitor (m-TOR) was substituted for a CNI in a small number of patients (n=10) due to side effects, and azathioprine was used in one patient in combination with CsA. National guidelines regulate choice of immunosuppressive regime, changes in regime and follow-up by local nephrologists.

Approximately ten weeks post-transplant all patients were examined at the laboratory for Renal Physiology at OUS-RH. At this investigation, vital signs, blood pressure and clinical chemistry data were captured. In addition, patients were subjected to an aortic (carotid-femoral) pulse wave velocity (aPWV) investigation. Plasma and serum samples for analysis of 25(OH)D, neutrophil gelatinase-associated lipocalin (NGAL) and calcium propensity score (T_{50}) were obtained and stored at -70º C in the Diagnostic and Treatment biobank “Nyrefysiologisk laboratorium” (Biobank nr. 266-2005-142234). 25(OH)D was measured en bloc from the biobanked serum on the complete cohort in 2015, the results were not reported to the treating physicians. Patients with a functioning kidney-graft one-year post-transplant were invited to OUS-RH for clinical examination and a laboratory follow-up. From 2009 a routine protocol kidney graft biopsy was included in the examination. Long-term outcomes were obtained from the Norwegian Renal Registry (NRR) where annual data is collected on the entire Norwegian transplant population. The reporting to NRR is closely monitored, and the reply-rate is close to 100%. The study was approved by the Regional Medical and Health Research Commities (REC) South-East Norway (2014/455). Written informed consent was obtained from all patients before any data and biological material was included in the NRR and the biobank at OUS-RH.

Patients were followed from time of transplantation until graft failure, death or end of study at October 18th 2017.

Blood samples were collected from fasting patients, and routine lab samples were analyzed at the biochemical department at OUS-RH. 25(OH)D, T_{50} and NGAL were analyzed in biobanked serum at Stavanger University Hospital, Calciscon AG and OUS-RH, respectively.

Serum creatinine values were calibrated to the isotope dilution mass spectrometry method (reference range: females 45-90 µmol/L; males 60-105 µmol/L), and eGFR was estimated using the CKD Epidemiology Collaboration equation[16]. Serum 25(OH)D was quantified by liquid-liquid extraction, derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione reagent (PTAD, Sigma-Aldrich, St. Louis, MO, USA), and analyzed by liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) at the laboratory of medical biochemistry at Stavanger University Hospital. The previously reported method[17] has been incorporated in the routine laboratory workflow with some modifications. Briefly, 50 µL serum is mixed with 350 µL of 0.2M magnesium sulfate in a 96-well deepwell plate followed by 900 µL of acetone + heptane (1+1) containing 4 ng/mL of D6-labelled 25(OH)D3 and D3-labelled 25(OH)D2. After one min shaking and centrifuging with 4000xG at 4°C for 11 min, a 300 µL volume of the top heptane layer was transferred to a new 96-well microplate and evaporated to dryness in a vacuum centrifuge at 60°C for 30 min. Finally, 100 µL of 0.5 mg/mL PTAD in dry acetonitrile was added to the microplate, and 1 µL of sample volume was analyzed by an Acquity UPLC coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). The analytes were separated on a 2.1 x 50 mm C18 BEH reversed phase column (Waters) and eluted in a mobile phase consisting of 61 % of acetonitrile mixed with 0.1 % ammonium hydroxide.

Measurement ranges were 1-350 nmol/L for 25(OH)D3 and 4-350 nmol/L for 25(OH)D2. Method quality was monitored by four different quality control samples, resulting in a method CV of less than 6 %. The laboratory participates in the Vitamin D External Quality Assessment Scheme (DEQAS, London, UK) and has received the proficiency certificate for the analysis of 25(OH)D. We used the National Institute of Health definition for vitamin D levels, with vitamin D deficiency defined as serum 25(OH)D concentrations <30 nmol/L (<12 ng/mL), insufficiency 30-50 nmol/L (12-20 ng/mL), sufficiency >50 nmol/L (>20 ng/mL), and levels >125 nmol/L (>50 ng/mL) as potentially toxic levels[18,19].

NGAL is a small protein belonging to the lipocalin superfamily, which increases during cellular stress and in acute kidney injury[20]. NGAL was analyzed by enzyme immunoassay using antibodies from R&D systems (Stillwater, Minnesota, USA). Inter- and intraassay coefficients of variation of 4.8% and 2.3%, respectively.

Calcification propensity score (T_{50}) is a blood test evaluating the propensity of blood to resist calcification by measuring the calcification inhibitory forces present in individual blood samples. Short T_{50} is consistent with accelerated transformation and low calcification resistance, whereas delayed transformation reflects intact calcification resistance[21]. T_{50} was determined using a method that measures the time of in vitro transformation from primary to secondary calciprotein particles, and has been described previously[21]. 40µl serum was exposed to high and supersaturated concentrations of calcium (35µl) and phosphate (25µl) solutions in triplicate in 384-well plates. The transformation step was monitored at 37°C using time-resolved nephelometry (bmg labtech, Ortenberg, Germany). Nonlinear regression curves were calculated to determine 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[21,22] .

Aortic (carotid-femoral) pulse wave velocity (aPWV) is the gold standard for non-invasive measurement of aortic stiffness[23] and has previously been described in detail[24]. Shortly summarized, aPWV measurements were done in the morning with patients in a quiet room. aPWV was measured with the SphygmoCor apparatus version 8.0 (AtCor Medical, New South Wales, Australia), and a validated tonometer (SPT-304, Millar Instruments, Houston, Texas). Pulse waves in the carotid and contralateral femoral artery were measured sequentially, with femoral measurements done in the non-transplanted side. Integrated software was used to calculate carotid-femoral pulse wave transit time with a simultaneously recorded electrocardiogram as reference. Pulse wave travel distance was measured as the distance from the suprasternal notch to the umbilicus plus ten cm based on recommendations by the manufacturer[24].

From 2009 a protocol graft biopsy was obtained 1 year±2 months post-transplant. Two cores were obtained with ultrasound guidance using an 18 gauge spring-loaded biopsy gun, one for histology (hematoxylin-eosin and saffron, periodic acid-Schiff and Masson trichrome) and one for C4d. Both biopsies contained at least one glomerular and one arterial profile and sufficient tubulointerstitial tissue to grade inflammation (i), tubulitis (t) interstitial fibrosis (ci), and tubular atrophy (ct). One year biopsy data was available in 510 of the 584 kidney transplantations done after 2009 included in this study. Some recipients did not have a functioning graft one year post-transplant, some refused the procedure, or were already biopsied on indication. Protocol biopsies were assessed by one of four dedicated renal pathologists and graded according to revisited Banff 2007 classification[25]. Interstitial fibrosis was scored from

zero to three and categorized into two groups with score zero and one vs. two and three.

Statistical analysis

Normally distributed data is presented as mean±SD, not normally distributed data as median and range.

Survival analysis was performed using Kaplan Meier estimate and Cox proportional hazard analysis including 25(OH)D and other relevant parameters as explanatory variables. The selection of variables to be entered into the Cox regression model was based on clinical knowledge and previous publications. The proportionality assumption was checked using a log-minus-log plot.

To compare the different variable-distribution between vitamin D groups, ANOVA was used in numeric variables, and chi-square in categorical variables.

A linear regression model was performed with vitamin D as dependent variable and sex, age, BMI, ten-week eGFR, iPTH, calcium, phosphate, albumine, preTx diabetes, combined vascular morbidity (a history of coronary and/or cerebral and/or peripheral vascular disease), time on dialysis, rejection episodes and donor status (living vs deceased donor) as independent variables measured at ten weeks post-transplant.

Three different Cox regression models were performed using death, graft failure and death-censored graft loss as dependent variables. Sex, age, BMI, ten-week eGFR, iPTH, calcium, vitamin D, phosphate, albumine, preTx diabetes, vascular comorbidity, time on dialysis, rejection episodes and donor status were used as independent variables in all models. All adjustment variables were set to their empirical means when computing the adjusted survival curves. Patients without events were censored at October 18th 2017.

ANOVA and paired samples t-tests were used to compare creatinine levels at ten weeks post-transplant and at one-year.

Statistical analysis was performed using IBM SPSS Statistics 24. P-value ≤ 0.05 was considered statistically significant.

Results

Vitamin D status and renal diagnosis in first-time kidney transplant recipients

From a total of 762 first-time transplant recipients, 148 (19%) had 25(OH)D levels <30 nmol/L (vitamin D deficiency), 351 (46%) had 25(OH)D levels from 30 to 50 nmol/L (vitamin D insufficiency), and 263 (35%) had 25(OH)D levels >50nmol/L (vitamin D sufficiency) ten weeks post-transplant. None had 25(OH)D levels >125 nmol/L. The baseline characteristics are given in Table 1. The most common causes for ESRD in this population are hypertensive nephropathy, glomerulonephritis, polycystic kidney disease, IgA nephropathy and diabetic nephropathy. The renal diagnoses for ESRD were not different between the groups of patients in the different vitamin D categories, Table 2.

Predictors of vitamin D

Patients with vitamin D sufficiency ten weeks post-transplant had significantly lower eGFR and higher cholesterol compared with patients with vitamin D deficiency and insufficiency, but a larger proportion had a pre-emptive transplant or a living donor graft. The prevalence of a history of cerebrovascular disease (transitory ischemic attack (TIA), cerebrovascular infarction or - bleeding) at time of transplantation was significantly higher in the group of patients with vitamin D insufficiency compared with

patients with vitamin D sufficiency, but creatinine levels were not different between the three groups.

Seventy-one percent were treated with active vitamin D or vitamin D analogs prior to transplantation, and eight percent were treated with calcimimetics (six percent used a combination of the two), the distribution was equal in the three vitamin D groups, supplementary table 1. The use of vitamin D analogs and calcimimetics were stopped at the time of transplantation. Vitamin D measurement was not a part of standardized care between 2007 and 2012, anti PTH medication and active vitamin D was prescribed based on PTH and calcium levels post-transplant by the treating nephrologists. We have no data on native vitamin D treatment in the registry pre- or post-transplant. The vitamin D levels were not significantly different between patients transplanted in different seasons of the year.

The linear regression model with vitamin D as dependent variable and eGFR, sex, age, BMI, iPTH, calcium, phosphate, albumine, diabetes, the combined vascular morbidity, time on dialysis, rejection and donor status (living vs deceased donor) as independent variables, left eGFR ($p<0.001$, β -0.132), iPTH ($p<0.001$, β -0.232) the combined vascular morbidity ($p=0.003$, β -4.669), albumine ($p<0.001$, β 0.870), donor status ($p=0.011$, β -3.655), months in dialysis ($p=0.019$, β -0.109) and age ($p<0.001$, β 0.203) as significant influencers on vitamin D, whereas sex ($p=0.887$, β -0.187), BMI ($p=0.843$, β -0.032), calcium ($p=0.909$, β -0.505), phosphate ($p=0.538$, β 0.979), rejection ($p=0.648$, β 1.664) and diabetes ($p=0.058$, β -3.312) did not significantly influence the model.

Vitamin D status and long-term survival, univariate analysis

During a median (range) follow-up of 82 months (1-117) after the ten-week post-transplant control, 118 (15%) recipients died, and 172 (23%) patients lost their grafts. The main causes of death were infection (n=37), malignancy (n=29) and cardiac arrest /myocardial infarction (n=27), table 3. 54 patients lost their grafts, of which 39 were rejections, 5 recurrence of the primary kidney disease in the graft, 2 systemic infections and 8 “other”. The frequency of rejections was highest in the group with low vitamin D.

Patient and graft survival were significantly better in patients with vitamin D sufficiency at ten weeks post-transplant compared with patients with vitamin D insufficiency or deficiency. Crude Kaplan-Meier estimated 5 year survival was 86±2% in recipients with vitamin D sufficiency, compared with 79±2% and 76±3% in recipients with vitamin D deficiency and insufficiency, respectively (p=0.006), Table 4. Using death as the main end-point, the risk of death was 2.3 (1.5-3.6) times higher in patients with vitamin D insufficiency and 1.8 (1.1-3.1) times higher in patients with vitamin D deficiency compared with patients with vitamin D sufficiency, Table 5, Figure 2.

Vitamin D status and long-term survival, multivariable analysis

In the adjusted model the patients with 25(OH)D levels <30 nmol/L, and patients with 25(OH)D levels 30 - 50 nmol/L, had a risk of graft failure or death of 1.6 (1.1-2.4) and 1.6 (1.0-2.7) times higher, respectively, as compared with patients with 25(OH)D levels >50 nmol/L, Table 5, Figure 3. Using graft failure as the main end-point, censoring for death with functioning graft, the results show the same tendency with 1.5 (0.6-3.3) times increased risk of graft failure with vitamin D insufficiency and 1.3

(0.4-3.8) times increased risk of graft failure with vitamin D deficiency, Table 5, Figure 4.

In the Cox regression model with death as dependent variable, age, BMI, vitamin D level, iPTH, diabetes, donorstatus and months in dialysis were significantly associated with death, supporting that high age, low BMI, low vitamin D, long time in hemodialysis, deceased donor and the presence of diabetes are associated with death after kidney transplantation. Sex, eGFR at 10 weeks, calcium, phosphate, albumine, rejection episodes and vascular morbidity were not significantly associated with death, table 6. We found higher risk of death in the group with vitamin D insufficiency (p=0.04), than in the group with vitamin D deficiency (p=0.004) when comparing with vitamin D sufficiency, and overall vitamin D sufficiency shows superior survival (p=0.009) compared with the combination of the other two groups of patients, figure 2.

When using graft failure as the dependent variable, 10 week eGFR and rejection episodes were significantly associated with graft loss, showing that low eGFR and acute rejections are all independent risk factors for graft loss. The vitamin D level 10 weeks post-transplant trends towards a significant association with graft loss. Age, sex, BMI, iPTH, calcium, phosphate, albumine, diabetes, months in dialysis, donor status and vascular morbidity was not significantly associated with graft loss, table 6.

Age, BMI, vitamin D level, donor status and time in dialysis were significantly associated with the combination of graft failure and death as the dependent variable. Sex, eGFR, calcium, phosphate, iPTH, diabetes, vascular morbidity and rejection episodes were not significantly associated with graft loss and death, table 6.

The results in the Cox model remained unchanged whether vitamin D was treated as a continuous or a categorical variable.

Vitamin D level at ten weeks post-transplant was associated with graft failure and death in all three Cox models.

Vitamin D status and vascular disease

NGAL was not significantly different between the groups of patients with vitamin D insufficiency, deficiency and sufficiency, respectively (p=0.39).

Mean T_{50} was 207 minutes (±74min), with significantly higher values in the vitamin D sufficient group of patients compared with the patients with vitamin D deficiency and insufficiency (p=0.037), the patients with vitamin D deficiency having lower values than the vitamin D insufficient patients.

Median aPWV at ten weeks was 9.5 (4.1-24.9) m/s, with no significant difference between the vitamin D groups of patients (p=0.276).

Vitamin D status and one-year follow-up

One year post-transplant follow-up laboratory samples were analyzed in 710 (93%) of the 762 transplant recipients. Seventeen patients were dead or had lost their graft before one-year follow-up, and 35 declined to attend the one-year routine follow-up investigation. Mean serum creatinine levels in the 710 patients with vitamin D data at one-year was 121±40 µmol/L at ten weeks post-transplant, and 122±47µmol/L one-year post-transplant, p=0.496. In the group of patients with vitamin D deficiency (137 patients) creatinine increased non-significantly from 117±43µmol/L to 125±61 µmol/L, p=0.064, and in the group with vitamin D insufficiency (322 patients) the creatinine increased from 120±37 µmol/L to 122±43 µmol/L, p=0.201. In contrast, patients with vitamin D sufficiency (251 patients) had a significant decrease in creatinine, from 124±36 µmol/L to 120±43 µmol/L, p=0.028. Creatinine increased ≥ 20% from week

10 to one-year post-transplant in 106 individuals (15%), 26 patients (20%) in the group with vitamin D deficiency, 55 patients (17%) with insufficiency and 23 patients (9%) with vitamin D sufficiency, p=0.004. The levels of iPTH, phosphate, calcium and albumine were not significantly different ten weeks and one-year post-transplant in the different groups of patients (data not shown).

In the one-year protocol biopsies, interstitial fibrosis was scored into four categories according to the Banff classification, score zero to three. There was significantly more interstitial fibrosis, i.e. biopsies with fibrosis scores two and three compared with scores zero and one, in patients with vitamin D deficiency/insufficiency compared with the group of patients with vitamin D sufficiency (p=0.022).

Discussion

Vitamin D sufficiency (25(OH)D >50 nmol/L) ten weeks post-transplant was associated with better graft- and overall patient survival, better kidney graft function and significantly less interstitial fibrosis in the kidney grafts, compared with patients with vitamin D deficiency or insufficiency. Furthermore, the association between vitamin D levels and all outcomes persisted in adjusted analysis with predetermined conventional risk markers.

25(OH)D deficiency is common in patients referred for kidney transplantation[26]. Prevalence varies across cohorts, reflecting different supplementation policies, sunshine exposure and co-morbidity. After a successful transplantation and restored kidney function, many transplant recipients remain 25(OH)D insufficient[27].

The prevalence of cerebrovascular disease before transplantation was slightly higher in the group of patients with vitamin D insufficiency. The prevalence of peripheral vascular- or coronary disease, aPWV or T_{50} as non-invasive measurements of aortic

stiffness and functional calcification resistance were not significantly different in the groups of patients. Higher aPWV and lower T_{50} at ten weeks post-transplant were however associated with death and graft failure supporting vascular disease as a risk factor. In general, the differences in baseline characteristics cannot explain the increased mortality and incidence of graft failure in patients with reduced vitamin D levels post-transplant. Unfortunately, delayed graft function is not registered in the NRR, thus we have not been able to include this possible confounder in the multivariate analyses.

Serum creatinine decreased during the first year after transplantation and there were fewer patients with significant fibrosis in the one-year protocol biopsy in patients with vitamin D sufficiency compared with the other groups. Berchtold et al showed that high iPTH, low vitamin D and T_{50} were associated with interstitial fibrosis and vascular lesions in the kidney grafts, independently of eGFR[28], supporting our findings. Others have found an association between low 25(OH)D levels three months post-transplant and a higher risk of rejection, increased interstitial fibrosis, lower eGFR and graft failure[29-31]. Low 25(OH)D levels measured on average six years post-transplant were in another study independently associated with increased mortality and a higher annual decline in eGFR[32]. It is a matter of debate if vitamin D is just a marker for increased mortality and graft failure in these patients, or if low vitamin D triggers a cascade of events leading to the actual findings. Due to the design of our study it is unfortunately not possible to investigate a cause-effect relationship. From prior studies it is however known that vitamin D deficiency increases the levels of FGF23 and decreases the levels of Klotho. High FGF23 levels are associated with increased mortality and cardiovascular death, and low Klotho with ageing and mortality[33]. Vitamin D is a pleiotropic hormone that affects most

tissues through the vitamin D receptors which are widely distributed throughout the body, interfering amongst other with the immune system[34,35]. The VITA-D study randomized adult kidney transplant recipients with vitamin D deficiency to treatment with oral vitamin D3 or placebo. With 1 year follow-up they did not find improved short-term outcome, possibly the opposite on the kidney grafts [36]. To our knowledge, no other randomized study exploring the effect on these parameters and hard end-points of vitamin D substitution in kidney transplant recipients have been performed, and follow-up time more than 1 year would be preferable.

Studies on FGF23 and Klotho signaling pathways have revealed that high affinity binding of FGF23 to target cells depend on a receptor complex consisting of FGF receptors and Klotho[37]. A Klotho independent FGF23 signaling pathway, a calcineurin and nuclear factor of activated T-cells (NFAT) signaling, has recently been identified in the parathyroid glands as well as in the heart[38-40]. Calcitriol prevents cardiac hypertrophy by blocking the NFAT signaling in cardiac myocytes in uremic rats by selectively blocking FGF23 effects on the heart and thereby reducing hypertrophic growth of cardiac myocytes[41]. Low vitamin D levels are associated with disturbances in the FGF23/Klotho axis which may explain why low vitamin D levels are associated with reduced graft survival and increased morbidity and mortality. The associations between vitamin D and FGF23 and Klotho should be further explored in kidney transplant patients.

A brief intervention study with paricalcitol two to four weeks prior to scheduled abdominal aneurysm repair resulted in a significant reduction in CD4+ T-helper cells, and reduction in CD3+T-cells, as well as reduction in IL-2, 4 and 10 in the aneurysm wall samples[42]. These findings support that paricalcitol as a vitamin D receptor agonist may have effects on local inflammation. Downregulation of the inflammatory

response may also explain the association between vitamin D levels in kidney transplant patients and the risk for interstitial fibrosis, graft failure and death. Another intervention study with paricalcitol post-transplant found a reduction in PTH but not in vascular health, or influence on allograft gene expression[43].

The potential effects of nutritional vitamin D supplementation represent an important question. In the general population and in patients with CKD, preliminary intervention studies suggest that restoring 25(OH)D levels with nutritional supplementation might reduce the risk of mortality[44,45]. Adequate powered randomized studies are needed to explore the effects of vitamin D supplementation in these patients.

Strengths and limitations

The investigated cohort of transplanted patients, represent the whole Norwegian cohort, without any selection bias with only one transplantation center. The NRR shows close to 100% national patient and data coverage. Routine laboratory samples were analyzed in the same laboratory both ten weeks and one-year post transplant (OUS-RH), and all the renal biopsies where performed and examined in the same department. The median follow-up period of 83 months is longer than in most other studies, and very few patients did not meet at the one-year investigation. We found a significant association between vitamin D levels 10 weeks post-transplant and death, vitamin D levels and graft loss showed a trend towards a significant association with a near-significant p-value. We believe that this may be due to a power problem, and that the association would be more significant with a higher number of included patients. Unfortunately, delayed graft function is not registered in the NRR, thus we have not been able to include this possible confounder in the multivariate analyses. The study is however, an observational study, and the conclusion is hypothesis

generating. Most patients were Caucasians, and the results cannot be extrapolated to transplanted patients with other ethnicities.

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Table 1

Baseline characteristics 10 weeks post-transplant:

	All patients	**25(OH)D <30 nmol/L**	**25(OH)D 30-50 nmol/L**	**25(OH)D >50 nmol/L**	**p-value**
	n=762	n=148 (19%)	n=351 (46%)	n=263 (35%)	
Males/ Females	515/ 247	95/ 53	242/ 119	178/ 85	0.62
Age (years)	57 (16-82)	54 (17-80)	57 (19-82)	57 (16-77)	0.042
BMI (kg/m²)	25.6±4.1	26.0±4.6	25.5±4.0	25.5±3.9	0.448
Deceased donor, n, %	495 (65%)	107 (72%)	239 (68%)	149 (57%)	0.001
Preemptive Tx, n (%)	224 (29%)	28(18%)	99 (28%)	97 (37%)	0.001
Dialysis modality, HD/ PD	386/ 152	88/ 32	173/ 79	125/ 41	0,306
Months of dialysis	9 (0-90)	11 (0-64)	10 (0-85)	6 (0-90)	0.000
eGFR 10 weeks (ml/min/1.73m²)	59.3±20.1	63.9±23.5	59.3±20.2	56.6±17.4	0.002
Creatinine (µmol/L)	121.1±38.7	117.3±43	121.2±38.6	123.5±35.8	0.306
Calcium (mmol/L)	2.37±0.15	2.37±0.18	2.37±0.14	2.37±0.15	0.972
Phosphate (mmol/L)	0.89±0.39	0.89±0.23	0.89±0.52	0.90±0.20	0.805
NGAL (ng/mL)	244.4±92.9	250.3±103.9	239.5±95.9	247.9±81.8	0.390
iPTH (pmol/L)	11.1 (1.6-202)	12.8 (3.8-202)	11.4 (1.6-59)	9.8 (2.9-48)	0.000
Albumine (g/L)	42.0±3.4	41.5±3.6	41.9±3.4	42.6±3.1	0.003
Hemoglobin (g/dL)	12.2±1.4	12.2±1.4	12.3±1.4	12.4±1.3	0.312
Systolic BP (mmHg)	134 (92-209)	134 (97-183)	136 (94-209)	132 (92-190)	0.052
Diastolic BP (mmHg)	85 (52-117)	84 (52-105)	85 (56-117)	85 (56-109)	0.297
Total cholesterol	6.3±1.5	5.9±1.7	6.3±1.5	6.5±1.4	0.005

(mmol/L)					
LDL (mmol/L)	3.9±1.2	3.7±1.3	3.9±1.2	4.1±1.2	0.003
HDL (mmol/L)	1.5±0.5	1.3±0.5	1.5±0.5	1.5±0.4	0.000
HbA1c (%)	5.9±0.94	5.9±1.05	5.9±0.95	5.8±0.86	0.229
aPWV (m/s)	10.3±3.4	10.4±3.7	10.1±3.6	10.0±3.0	0.276
T_{50} (min)	207±74	206±81	200±70	217±74	0.037
Vascular morbidity, n (%)	195 (26%)	45 (30%)	103 (29%)	47 (18%)	0.002
Cerebro vascular disease (incl TIA)	54 (7%)	21 (14%)	24 (7%)	9 (3%)	0.001
Peripheral vascular disease	88 (12%)	20 (14%)	42 (12%)	26 (10%)	0.571
Coronary disease	126 (17%)	25 (17%)	69 (20%)	32 (12%)	0.047
Malignancy, n (%)	65 (9%)	8 (5%)	33 (9%)	24 (9%)	0.315
Ecchocardiography verified left ventricular hypertrophy, n (%)	108 (14%)	22 (15%)	51 (15%)	35 (13%)	0.880
Diabetes, preTx, n (%)	137 (18%)	37 (25%)	65 (19%)	35 (13%)	0.012

Median and range for age, dialysis duration, iPTH and blood pressure, mean±SD for other normally distributed variables. ANOVA was used on numeric variables, chi square on categorical variables.

BMI=body mass index, Tx=transplantation, HD= hemodialysis, PD=peritoneal dialysis, eGFR=estimated glomerular filtration rate, NGAL=neutrophil gelatinase-associated lipocalin, iPTH=intact parathyroid hormone, LDL=low-density lipoprotein, HDL=high-density lipoprotein, BP=blood pressure, HbA1c=glycosylated hemoglobin, T50=calcification propensity score, aPWV= arterial pulse wave velocity, TIA= transitory ischemic attack

Table 2

Renal diagnosis before transplantation

Diagnosis	All patients	25(OH)D <30nmol/L	25(OH)D 30-50 nmol/L	25(OH)D >50nmol/L
	n=762	n=147	n=351	n=264
	n (%)	n (%)	n (%)	n (%)
Hypertensive nephropathy	171 (22%)	34 (23%)	82 (23%)	51 (19%)
Glomerulonephritis	137 (18%)	28 (19%)	68 (19%)	41 (16%)
Polycystic kidney disease	134 (18%)	16 (11%)	63 (18%)	55 (21%)
IgA nephropathy	86 (11%)	14 (10%)	32 (9%)	40 (15%)
Diabetic nephropathy	83 (10%)	23 (16%)	43 (12%)	17 (6%)
Pyelonephritis/interstitial nephritis	38 (5%)	5 (3%)	15 (4%)	18 (7%)
Tubulointerstitial nephritis	27 (3%)	5 (3%)	7 (2%)	15 (6%)
Hereditary kidney disease (Alport, Cystinosis, Fabry etc)	12 (2%)	3 (2%)	7 (2%)	3 (1%)
Renal malformations	7 (1%)	3 (2%)	3 (1%)	1 (0.5%)
Wegeners granulomatosis	16 (2%)	2 (1%)	7 (2%)	7 (3%)
Myelomatosis/Amyloidosis	9 (1%)	1 (1%)	5 (2%)	3 (1%)
SLE	6 (1%)	2 (1%)	2 (1%)	2 (1%)
Cortical or tubular necrosis	7 (1%)	2 (1%)	4 (1%)	1(0.5%)
Uncertain etiology	12 (2%)	2 (1%)	6 (2%)	4 (1%)
Other	21 (3%)	8 (6%)	7 (2%)	5 (2%)

Table 3

Causes of death

Diagnosis	All patients n=118	25(OH)D <30nmol/L n=25	25(OH)D 30-50 nmol/L n=67	25(OH)D >50nmol/L n=26
Cardial events	27	6	19	2
Infection	37	9	19	9
Cerebrovascular events	5	0	1	4
Malignancy	29	4	18	7
Perforation of colon	3	0	2	1
Other (pulmonary embolus, haemorrhage, mesenteric infarction, suicide, accident, unknown)	17	6	8	3

Table 4

Kaplan-Meier estimates of survival with functioning graft

	% alive with functioning graft after 1 year (±SE)	**% alive with functioning graft after 5 years (±SE)**	**Estimated time to 75% survival**
All patients (n=762)	97.1±0.6	80.1±1.3	93 months
25(OH)D <30 nmol/L (n=148)	97.3±1.3	76.4±3.3	74 months
25(OH)D 30-50 nmol/L (n=351)	95.7±1.1	78.9±2.0	88 months
25(OH)D >50 nmol/L (n=263)	99.2±0.7	85.9±1.9	>117 months

Table 5

Hazard ratios with 95% confidence intervals in the unadjusted and the adjusted Cox regression models

Model	**Combined end-point (graft failure and/ or death)**		**Graft failure**		**Death**	
Unadjusted	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
Vit D >50 nmol/L	Reference		Reference		Reference	
Vit D 30-50 nmol/L	1.7	1.2-2.5	1.1	0.6-2.1	1.8	1.1-3.1
Vit D <30 nmol/L	1.8	1.2-2.8	1.7	0.8-3.3	2.3	1.5-3.6
Adjusted*	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
Vit D >50 nmol/L	Reference		Reference		Reference	
Vit D 30-50 nmol/L	1.6	1.0-2.7	1.3	0.4-3.8	1.7	1.0-3.1
Vit D <30 nmol/L	1.6	1.1-2.4	1.5	0.6-3.3	1.8	1.2-2.9

*adjusted for the following variables: Sex, age, BMI,10 week eGFR, iPTH, calcium, phosphate, albumine, diabetes, vascular comorbidity, time on dialysis, rejection and donorstatus (living vs deceased donor). All adjustment variables were set to their empirical means when computing the adjusted survival curves.

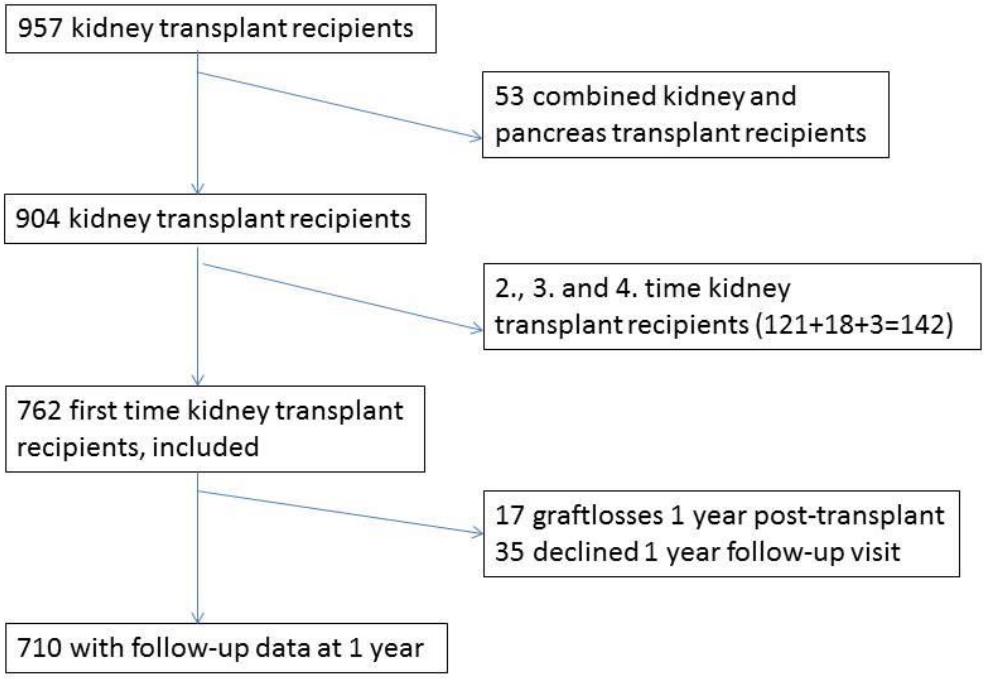
Table 6

P-values and hazard ratios with 95% confidence intervals in the Cox regression models

	Combined end-point (graft failure and/ or death)			**Graft failure**			**Death**		
Variables	**p-value**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**
Sex	0.664	0.92	0.637-1.333	0.469	0.75	0.339-1.644	0.943	0.99	0.660-1.471
Age	0.000	1.05	1.032-1.070	0.368	0.99	0.955-1.017	0.000	1.08	1.059-1.106
BMI	0.018	0.95	0.902-0.990	0.818	0.99	0.902-1.085	0.023	0.94	0.0894-0.992
eGFR	0.080	0.99	0.982-1.001	0.046	0.98	0.957-1.000	0.519	1.00	0.986-1.007
PTH	0.083	1.01	0.999-1.024	0.520	0.96	0.941-1.031	0.016	0.97	1.003-1.027
Calcium	0.927	0.95	0.316-2.851	0.529	0.44	0.034-5.673	0.970	1.02	0.323-3.241
Phosphate	0.473	1.13	0.816-1.550	0.566	1.16	0.701-1.916	0.499	1.14	0.780-1.664
Vitamin D	0.022	0.99	0.977-0.998	0.149	0.99	0.962-1.006	0.038	0.99	0.976-0.999
Albumine	0.102	0.96	0.911-1.008	0.184	0.93	0.840-1.034	0.247	0.97	0.916-1.023
Diabetes	0.147	0.74	0.487-1.113	0.443	0.71	0.291-1.716	0.035	0.63	0.403-0.967
Vascular morbidity	0.183	0.78	0.536-1.126	0.659	1.23	0.495-3.042	0.135	0.74	0.498-1.099
Time in Dialysis	0.046	1.01	1.000-1.022	0.998	1.00	0.973-1.028	0.047	1.01	1.000-1.024
Donor-	0.049	0.62	0,386-	0.587	0.78	0.325-	0.026	0.53	0.302-

status Rejection	0.877	1.08	0.997 0.434-2.661	0.035	0.30	1.889 0.097-0.921	0.513	1.47	0.928 0.461-4.704

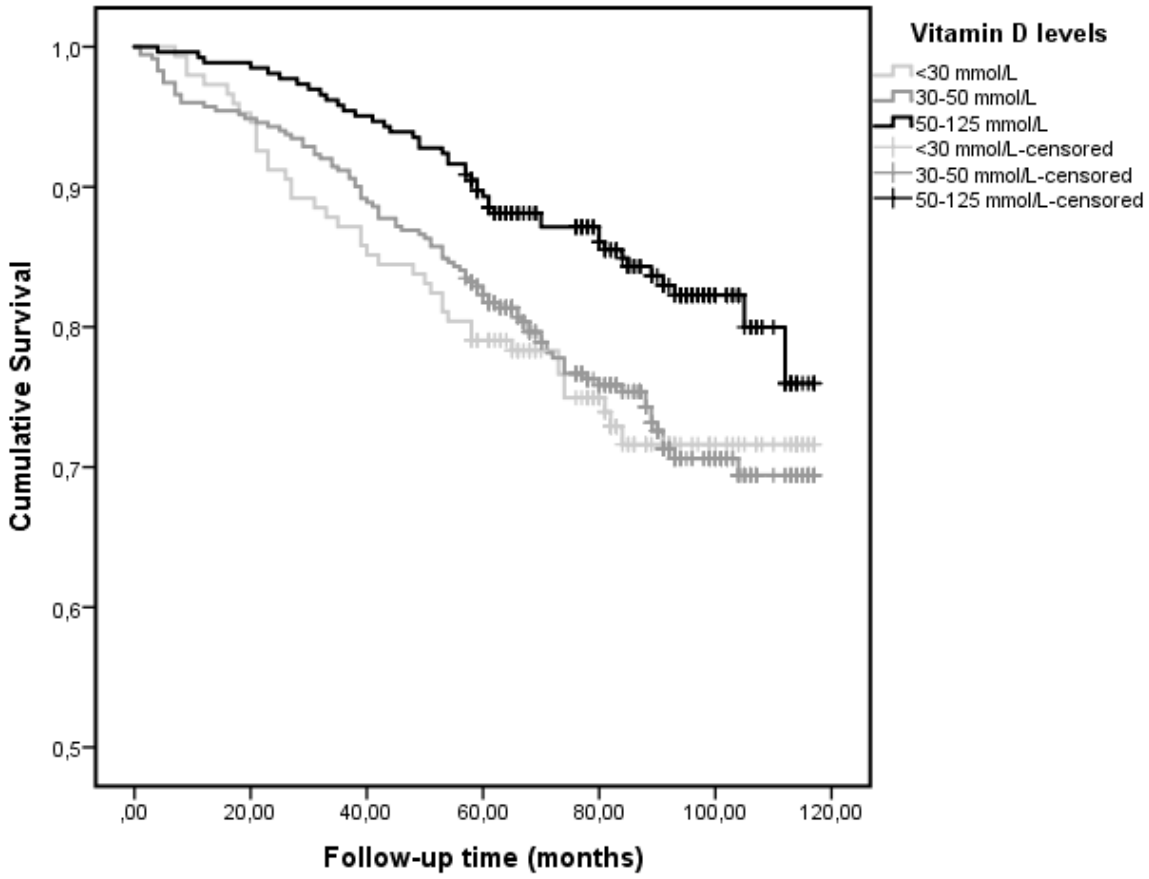
Figure 1:



Flowchart of patients

Figure 2:

Kaplan-Meier plot showing overall survival in patients with vitamin D insufficiency, deficiency and sufficiency

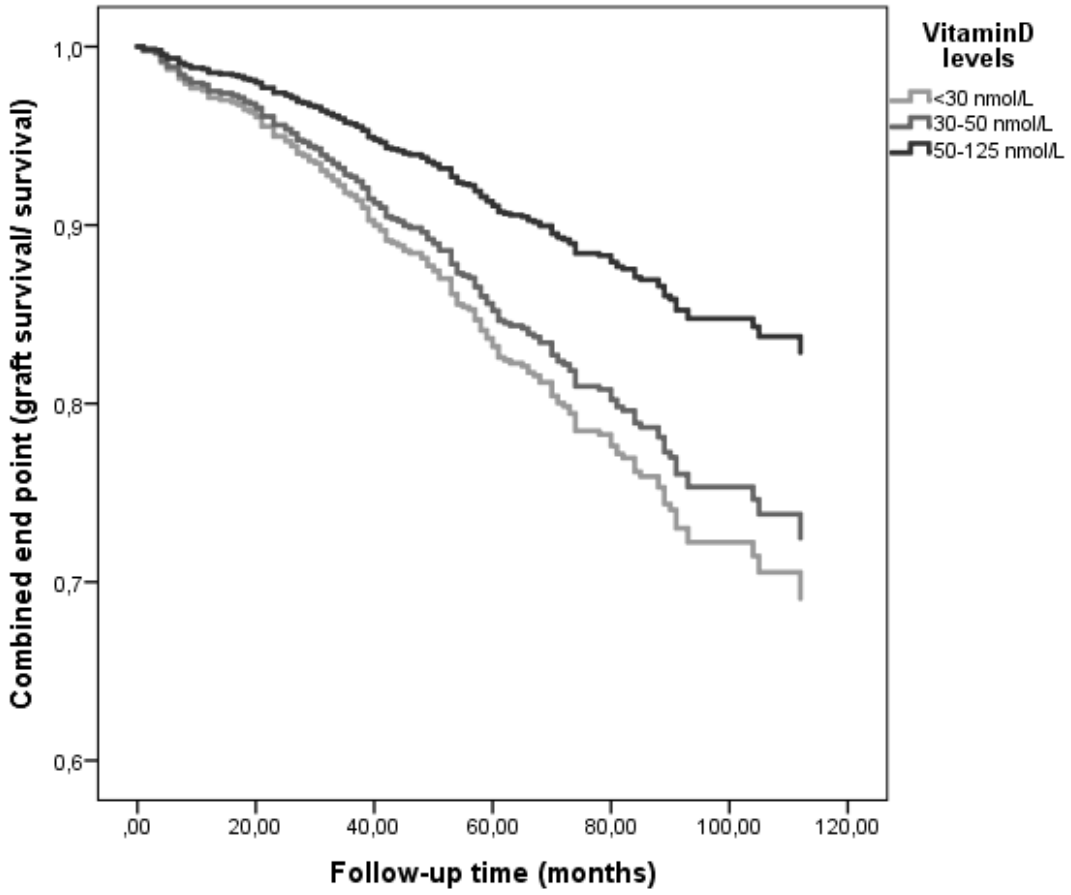


Number at risk:

VitD	0	20	40	60	80	100	End
<30	148	143	133	123	91	32	0
30-50	351	336	323	291	192	80	0
>50	263	261	255	236	168	60	0
Total	762	740	711	650	451	172	0

Figure 3:

Adjusted* cox survival curves showing graft survival in patients with vitamin D insufficiency, deficiency and sufficiency



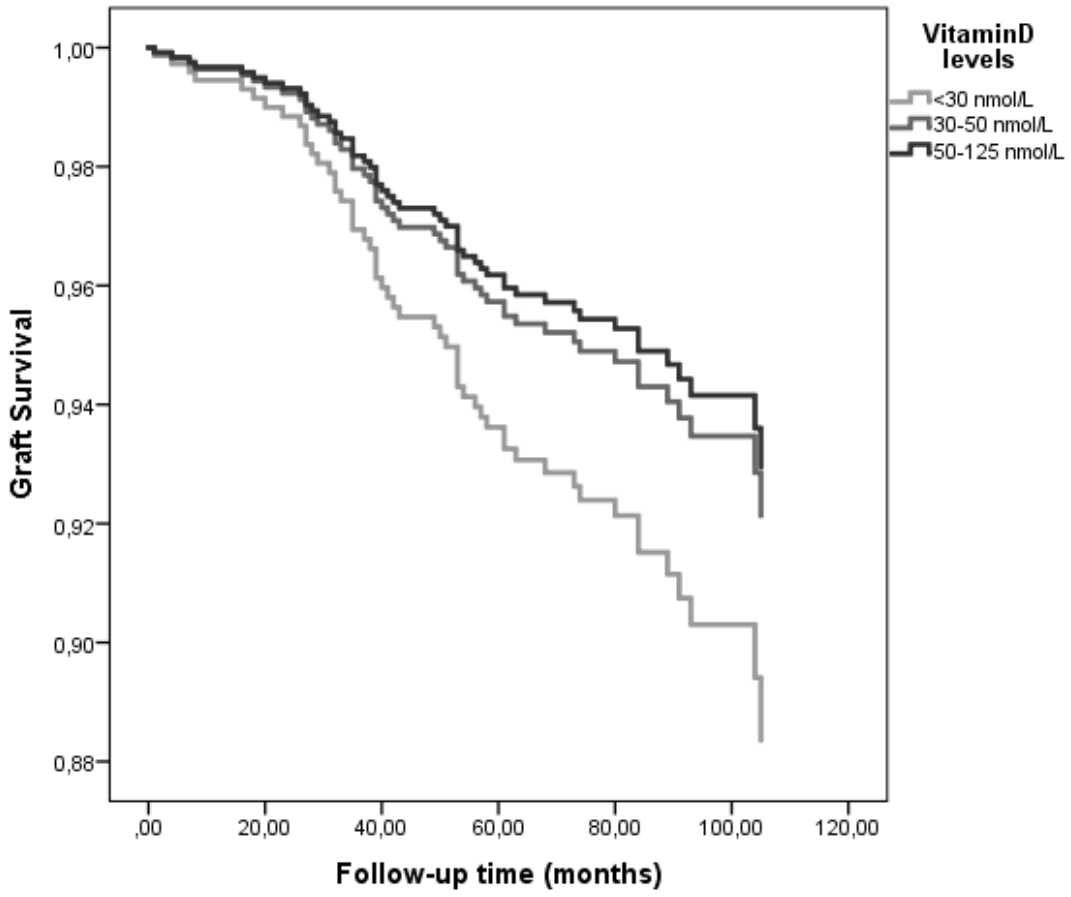
Number at risk

VitD	0	20	40	60	80	100	End
<30	148	141	126	113	81	26	0
30-50	351	333	313	279	183	75	0
>50	263	260	250	226	161	58	0
Total	762	734	689	618	425	159	0

*adjusted for the following variables: Sex, age, BMI,10 week eGFR, iPTH, calcium, phosphate, albumine, diabetes, vascular comorbidity, time on dialysis, rejection and donorstatus (living vs deceased donor). All adjustment variables were set to their empirical means when computing the adjusted survival curves.

Figure 4:

Adjusted* cox survival curves showing death-censored graft survival in patients with vitamin D insufficiency, deficiency and sufficiency



Number at risk

VitD	0	20	40	60	80	100	End
<30	148	141	126	113	81	26	0
30-50	351	333	313	279	183	75	0
>50	263	260	250	226	161	58	0
Total	762	734	689	618	425	159	0

*adjusted for the following variables: Sex, age, BMI,10 week eGFR, iPTH, calcium, phosphate, albumine, diabetes, vascular comorbidity, time on dialysis, rejection and donorstatus (living vs deceased donor). All adjustment variables were set to their empirical means when computing the adjusted survival curves.