# New biomarkers in patients with chronic kidney disease, after kidney transplantation, in kidney donors and in acute myocardial infarction

Inga Strand Thorsen

Thesis for the degree of Philosophiae Doctor (PhD)
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UNIVERSITY OF BERGEN

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# Scientific environment

This work is part of the Internal Medicine Research Group at Stavanger University Hospital. The project startet in 2013 when the first patients were included at the out-patient clinic. The work for this thesis has been done at the nephrology department at Stavanger University Hospital.

Main supervisor Professor Lasse G Gøransson, Stavanger University Hospital and University of Bergen. Cosupervisor was Inger Hjørdis Bleskestad, Stavanger University Hospital.

We collaborated with Øyvind Skadberg, Grete Jonsson and Cato Brede at department of Clinical Biochemistry, Stavanger University Hospital, Stein Ørn and Cord Manhenke at department of Cardiology, Stavanger University Hospital. Anders Åsberg in the Norwegian Renal Registry and Oslo University Hospital, Anders Hartmann, Anna Reisæter, Thor Ueland, Pål Aukrust and Kristian Heldal at Oslo University Hospital.

As a PhD candidate, I have been affiliated with the Department of Clinical Medicine, Medical Faculty, University of Bergen. I received funding as a doctoral research fellow from the Western Norway Regional Health Authority (Helse Vest).

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Stavanger, 2023

Inga Strand Thorsen

# Abbreviations and definitions

| | |
|---|---|
| ANOVA | analyses of variance |
| AUC | area under the curve |
| AKI | acute kidney injury |
| BMI | body mass index |
| CaSR | calcium sensing receptor |
| CITP | c-terminal telopeptide of type I collagen |
| CKD | chronic kidney disease |
| CKD-EPI | chronic kidney disease epidemiology collaboration |
| CKD-MBD | chronic kidney disease-mineral bone disorder |
| CMR | cardiac magnetic resonnans |
| CNI | calcineurin inhibitor |
| CTGF | connective tissue growth factor |
| CV | cardiovascular |
| CVD | cardiovascular disease |
| CyA | cyclosporine |
| DEXA | dual x-ray absorptiometry |
| DGF | delayed graft function |
| DOCA | mineralcorticoid deoxycorticosterone acetate |
| DMP1 | osteocyte protein dentin matrix protein 1 |
| eGFR | estimated glomerular filtration rate |
| EIA | enzyme immunoassay |
| ELISA | enzyme-linked immunosorbent assay |
| ESRD | end stage renal disease |
| $FePO_4$ | fractional excretion of phosphate |
| FGF | fibroblast growth factor |
| FGF23 | fibroblast growth factor 23 |
| FGFR | fibroblast growth factor receptor |
| GBCA | gadolinium-based contrast agent |
| GFR | glomerular filtration rate |

| | |
|---|---|
| HD | hemodialysis |
| iFGF23 | intact fibroblast growth factor 23 |
| iPTH | intact parathyroid hormone |
| KDIGO | kidney disease improving global outcome |
| LV | left ventricular |
| LVH | left ventricular hypertrophy |
| LVEDVI | left ventricular end diastolic volume index |
| LVESVI | left ventricular end systolic volume index |
| LVEF | left ventricular ejection fraction |
| MAPK | mitogen-activated protein kinase |
| MBD | mineral bone disorder |
| MDRD | modification of diet in renal disease |
| MGP | matrix gla protein |
| MMF | mycophenolatmofetil |
| MR | mineralcorticoid receptor |
| MRA | mineralcorticoid receptor antagonist |
| mTOR | mammalian target of rapamycin |
| NFAT | nuclear factor of activated T cells |
| NGAL | neutrophil gelatinase associated lipocalcin |
| NO | nitric oxide |
| NRR | Norwegian Renal Registry |
| NSF | nephrogenic systemic fibrosis |
| OUS | Oslo University Hospital |
| PCI | percutaneous coronary intervention |
| PD | peritoneal dialysis |
| PINP | amino terminal propeptide of type I procollagen |
| PIIINP | amino terminal propeptide of type III procollagen |
| PLC$\gamma$ | phospholipase C-gamma |
| PTH | parathyroid hormone |
| PWV | pulse wave velocity |
| RAAS | renin–angiotensin–aldosterone system |

| | |
|---|---|
| RCT | randomized controlled trial |
| RH | Rikshospitalet |
| ROS | reactive oxygen species |
| SD | standard deviation |
| sKlotho | soluble Klotho |
| SGLT2 | Sodium-glucose co-transporter 2 |
| STEMI | ST-elevation myocardial infarction |
| SUS | Stavanger University Hospital |
| $T_{50}$ | Calcification propensity score |
| TGF-β | transforming growth factor-β |
| UACR | urine albumin to creatinine ratio |
| UPCR | urine protein to creatinine ratio |
| VDR | vitamin D receptor |
| VSMCs | vascular smooth mussle cells |
| 25(OH)D | 25 hydroxy-vitamin D |
| $1,25(OH)_2D$ | 1,25 dihydroxy-vitamin D |

# Abstract

Background

Chronic kidney disease (CKD) affects many people, and the incidence and prevalence is increasing worldwide. Patients with kidney disease have increased mortality and morbidity, and cardiovascular disease is the main reason for the increased risk of death and disability, with both accelerated arteriosclerosis and heart failure. Chronic kidney disease leads to disturbed mineral metabolism, reduced bone density and calcificalion of vessels and soft tissue.

Patients with end-stage renal disease (ESRD) need renal replacement therapy (RRT). RRT means dialysis or kidney transplantation. If the patients are old and frail, a conservative approach is an alternative. Dialysis is given as either hemodialysis or peritoneal dialysis. Kidney transplantation is the form of RRT with best survival and quality of life. After transplantation the patients need immunosuppression for the rest of their lives, and they have increased risk of infections, cancer and cardiovascular disease (CVD) when comparing with the general population. Some patients with ESRD do not meet the requirements of kidney recipients due to comorbidities, high body mass index or other factors, these patients receive dialysis for many years. The waiting list for kidney transplantation is increasing, and living kidney donors is necessary to keep the waiting list stable. A living donor must undergo a medically unnecessary procedure, and donor safety is a concern in the short and in the long term. A potential donor undergo a thorough screening, and any known risk factor for kidney or cardiovascular disease leads to rejection of the donor. Earlier studies claimed that kidney donors did not have increased risk of kidney disease or death when comparing with the general population. Newer studies matching donors with equally healthy controls suggest increased long-term morbidity and mortality.

Objectives

Kidney function is a term that describes the kidney's ability to filter urine. Nephrologists use creatinine and glomerular filtration rate (GFR), as well as

albuminuria to assess kidney function in everyday practice. The standardized tests are inaccurate, and new, improved methods to assess and predict outcome in patients with kidney disease would be welcome. In this thesis, we have explored several new biomarkers in different patient-categories with an aim to increase the knowledge about these biomarkers in different settings. The included biomarkers in this thesis are neutrophil gelatinase associated lipocalcin (NGAL), soluble Klotho (sKlotho), intact fibroblast growth factor 23 (iFGF23), intact parathyroid hormone (iPTH), 25-hydroxy-vitamin D (25(OH)D) and collagen markers.

Methods

Study 1 was a cross-sectional observational single-center study that included patients with CKD stages 3-5, long-term kidney donors and healthy controls with an aim to establish the levels of NGAL, sKlotho, iFGF23 and vitamin D in these groups. Study 2 was a national prospective, observational cohort study of all first-time kidney transplant recipient in Norway from October 2007 to October 2012 that explored the association between vitamin D status 10 weeks post kidney transplantation and long-term graft- and patient survival. Study 3 was a single center observational study including patient with a first-time myocardial ST-elevation infarction investigating the time-dependent changes in iFGF23 the first year post myocardial infarction (MI), and the relationship between iFGF23 levels and infarct size, left ventricular (LV) remodeling and systemic biomarkers of inflammation and collagen turnover following acute MI. Study 4 was a national, prospective observational cohort study that included 132 kidney transplant recipients from November 2012 to October 2013, exploring the relationship between vitamin D, sKlotho and iFGF23 measured 8 weeks and 1 year post-transplant, and long-term graft- and patient survival.

Results

Study 1 showed increased levels of NGAL in kidney donors compared with healthy controls, which may reflect renal hyperfiltration or the reduced kidney function seen after kidney donation. iFGF23 and sKlotho did not significantly differ between donors and controls. As expected, in the patients with CKD, iFGF23 levels increased,

sKlotho levels decreased and NGAL levels increased when estimated glomerular filtration rate (eGFR) declined.

In study 2, we found that vitamin D sufficiency 10 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.

In study 3, we found that iFGF23 levels were significantly decreased prior to, and at 2 days following revascularization in subjects with a first-time MI. iFGF23 levels normalized on day 7 in patients without heart failure. There was a gradual increase in iFGF23 levels, above normal, during one-year follow-up in patients with signs and/or symptoms of heart failure during the acute phase of the MI.

In study 4 we found that patients with vitamin D deficiency (<30 nmol/L) at 8 weeks post-transplant showed reduced graft- and patient survival compared with the patients having higher vitamin D levels in the early post-transplant phase. sKlotho levels increased and iFGF23 levels decreased from 8 weeks to 1 year post transplant. sKlotho and iFGF23 levels did not influence graft- and patient survival.

Conclusion

In patients with CKD, biomarkers can help us estimate the risk of CKD progression, assess cardiovascular risk and determine metabolic abnormalities. The pathophysiology in CKD is complex, and involves many processes during its progression. A single biomarker is probably insufficient to meet all of those expectations, but maybe a panel of biomarkers could be useful in the future. Improved understanding in all aspects of CKD development and CVD risk is likely to enable the development of novel therapeutic interventions capable of reducing the risks associated with CKD, and to optimize the treatment for the individual patient.

# Sammendrag

**Nye markører på nyreskade hos nyretransplanterte, nyre donorer, hos pasienter med nyresvikt og hos pasienter med akutt hjerteinfarkt**

Bakgrunn

Kronisk nyresvikt affiserer mange mennesker. Både insidens og prevalens øker i hele verden. Pasienter med nyresykdom har økt sykelighet og dødelighet, og hjerte- kar sykdom er hovedårsaken til dette, med både økt arteriosklerose og hjertesvikt. Kronisk nyresvikt fører til endret mineralmetabolisme, redusert beintetthet og kalsifisering av kar og bløtvev.

Pasienter med ende-stadium nyresvikt trenger nyreerstattende behandling. Nyreerstattende behandling betyr dialyse eller nyretransplantasjon. Hos gamle og skrøpelige kan man velge en konservativ tilnærming. Dialyse gjennomføres enten som hemodialyse eller peritoneal dialyse. Nyretransplantasjon er den formen for nyreerstattende behandling med best overlevelse og livskvalitet. Etter transplantasjon, må pasientene stå på immundempende medisiner resten av livet, og de har økt risiko for infeksjoner, kreft og hjerte- kar sykdom sammenlignet med resten av befolkningen. Noen pasienter med ESRD kan ikke transplanteres som følge av komorbiditet som for eksempel høy body mass index, aktiv kreftsykdom eller alvorlig hjerte- og karsykdom. Ventelisten for nyretransplantasjon øker, og levende nyredonorer er nødvendig for å holde ventelisten stabil. En levende giver må gjennom et kirurgisk inngrep som ikke er medisinsk nødvendig, og donorens potensielle risiko er viktig både på kort og lang sikt. En potensiell donor går gjennom en grundig screening. Dersom man avdekker risiko for hjerte- kar sykdom eller nyresvikt blir donoren avslått. Tidligere studier fant at nyredonorer ikke hadde økt risiko for nyresykdom eller død når man sammenlignet med den generelle befolkningen. I nyere studier hvor man sammenligner donorer med en like frisk kontrollgruppe, ser man en økt risiko for hypertensjon, nyresvikt og noe redusert levetid etter lengre oppfølgingstid.

Mål

Nyrefunksjon er nyrens evne til å filtrere urin. Nefrologer bruker kreatinin, GFR og albuminuri til oppfølging av pasienter med nyresvikt i vanlig klinisk praksis. Disse metodene er unøyaktige og nye, bedre metoder til evaluering av nyrefunksjon og til å forutse sykdomsprogresjon hadde vært nyttig. I denne avhandlingen har vi sett på flere nye biomarkører i ulike pasient grupper for å øke kunnskapen om disse biomarkørene i ulike sammenhenger. Biomarkørene som er inkludert i avhandlingen er NGAL, sKlotho, iFGF23, iPTH, 25(OH)D og kollagen markører.

Metode

Studie 1 var en enkelt-senter, tverrsnitts observasjonsstudie som inkluderte pasienter med nyresvikt i stadium 3-5, nyredonorer som hadde donert nyre for >5 år siden samt friske kontroller. Målsetningen var å fastsette nivåene til biomarkørene NGAL, sKlotho, iFGF23 og vitamin D i disse ulike gruppene. Studie 2 var en prospektiv, kohort observasjonsstudie som inkluderte alle førstegangs nyretransplanterte i Norge fra oktober 2007 til oktober 2012. Studien undersøkte sammenhengen mellom D vitamin målt 10 uker etter transplantasjonen og langtids nyre- og pasient overlevelse. Studie 3 var en enkelt-senter observasjonsstudie som inkluderte pasienter med førstegangs ST-elevasjons hjerteinfarkt. Studien så på utviklingen av iFGF23 og infarktstørrelse, venstre ventrikkel remodellering og systemiske inflammasjons- og kollagen markører gjentatte ganger i løpet av det første året etter hjerteinfarktet. Studie 4 var en nasjonal, prospektiv observasjonsstudie som inkluderte 132 nyretransplanterte i perioden november 2012 til oktober 2013. Målet var å kartlegge sammenhenger mellom vitamin D, sKlotho og iFGF23 målt 8 uker og 1 år etter transplantasjon, med langtids nyre- og pasientoverlevelse.

Resultat

Studie 1 viste økt NGAL nivå hos nyredonorer sammenlignet med friske kontroller. Dette skyldes hyperfiltrasjonen etter nefrektomi, men det kan også ha sammenheng med redusert nyrefunksjon. Konsentrasjonen av iFGF23 og sKlotho var lik hos

donorer og kontroller. Hos pasientene med nyresvikt økte iFGF23 og NGAL mens sKlotho falt etter hvert som nyresvikten økte.

I studie 2 fant vi en sammenheng mellom normal vitamin D 10 uker etter nyretransplantasjon og bedre nyre- og total overlevelse, bedre nyrefunksjon og mindre fibrose i nyrebiopsi tatt etter 1 år, sammenlignet med de transplanterte med vitamin D mangel eller utilstrekkelig vitamin D nivå. Assosiasjonene mellom vitamin D nivå og overlevelse vedvarte etter korrigering for kjente risikofaktorer.

I studie 3 fant vi redusert iFGF23 nivå i forkant av og 2 dager etter revaskularisering hos pasienter med akutt hjerteinfarkt. iFGF23 nivået ble normalisert etter 7 dager hos pasientene som ikke utviklet post-infarkt hjertesvikt. iFGF23 steg til over normalen i året som fulgte i pasientgruppen med tegn til hjertesvikt.

I studie 4 fant vi at pasientene med vitamin D mangel hadde signifikant dårligere både nyre- og total overlevelse sammenlignet med de andre 2 gruppene 8 uker etter transplantasjon. iFGF23 nivå falt signifikant mens sKlotho nivå økte signifikant fra 8 uker til 1 år etter transplantasjon. iFGF23 og sKlotho nivåene var ikke signifikant assosiert med langtids nyre- og total overlevelse.

Konklusjon

Hos pasienter med kronisk nyresvikt kan biomarkører hjelpe oss til å vurdere risiko for progresjon av nyresvikten, kardiovaskulær risiko og metabolske forstyrrelser. Patofysiologien ved kronisk nyresvikt er kompleks, og involverer mange prosesser etterhvert som nyresvikten progredierer. Bruk av en enkelt biomarkør er sannsynligvis utilstrekkelig, men i framtiden kan man tenke seg bruk av et panel med biomarkører. Forbedret forståelse av alle aspekter innenfor utviklingen av kronisk nyresvikt og utvikling av hjerte- og kar sykdom vil hjelpe til i prosessen med å utvikle nye terapeutiske strategier for å redusere risikoen for progresjon og bedre prognosen for sykelighet og dødelighet assosiert med kronisk nyresvikt. Dette vil kunne bidra til optimalisering av den enkeltes behandling.

# List of Publications

1 Thorsen IS, Bleskestad IH, Jonsson G, Skadberg Ø, Gøransson LG. Neutrophil Gelatinase-Associated Lipocalin, Fibroblast Growth Factor 23, and Soluble Klotho in Long-Term Kidney donors. Nephron Extra. 2016 Oct 12;6(3): 31-39. PMID: 27920796

2 Thorsen IS, Bleskestad IH, Åsberg A, Hartmann A, Skadberg Ø, Brede C, Ueland T, Pasch A, Reisaeter AV, Gøransson LG. Vitamin D as a risk factor for patient survival after kidney transplantation: A prospective observational cohort study. Clin Transplant. 2019 May;33(5)e13517 PMID: 30844090

3 Thorsen IS, Gøransson LG, Ueland T, Aukrust P, Manhenke CA, Skadberg Ø, Jonsson G, Ørn S. The relationship between Fibroblast Growth Factor 23 (FGF23) and cardiac MRI findings following primary PCI in patients with acute first time STEMI. Int J Cardiol Heart Vasc. 2021 Feb 19;33:100727 PMID: 33665349

4 Thorsen IS, Bleskestad IH, Åsberg A, Jonsson G, Skadberg Ø, Heldal K, Gøransson LG. Klotho and Fibroblast Growth Factor 23 are independent of vitamin D, and not associated witk graft- and patient survival after kidney transplantation. Transplantation Direct, accepted July 2023, in process of being published.

# Contents

# 1. Introduction

**Background**

CKD is common, and in Norway the prevalence is 10.2% according to data from the second Health Study of Nord-Trondelag (HUNT II) (1). The same prevalence has been reported from the US (2) and the prevalence is increasing all over the world (3, 4).

In Norway, the main causes of ESRD are hypertensive nephropathy, diabetic nephropathy and glomerulonephritis, in this order (5). In the US, the main cause of ESRD is diabetic nephropathy, with hypertensive nepropathy in second place (6). Glomerulonephritis and "unknown" are more common causes for CKD in Asia and sub-Saharan Africa (7), these differences are related to the burden of disease moving from infections to lifestyle-related diseases and higher life expectancy in developed countries (8). In Norway, a cross-sectional study 10 years apart (HUNT 2 and HUNT 3) found a stable CKD prevalence at the two different time-points (11.3% vs 11.2%). They found a decrease in blood pressure, more physical activity and lower cholesterol levels the second time, most likely due to improved treatment strategies. In contrast, the prevalence of diabetes and obesity increased moderately with time (9).

Regardless of the cause, CKD is characterized by progressive destruction of renal parenchyma and loss of functional nephrons, which may ultimately lead to ESRD (10). In 1990, CKD was the 27$^{th}$ leading cause of death, rising up to 18$^{th}$ place in 2010 (7). Globally, CKD is one of the leading causes of death and disability (11). Cardiovascular (CV) mortality is 10 to 30 times higher in individuals with ESRD than in the general population when matched for age, sex and ethnicity (12, 13), and death seems to be a far more likely outcome than progression to ESRD in all stages of CKD. A 25-34 year old patient with ESRD has a 500-1000 times increased mortality when comparing to a healthy 25-34 year old, corresponding to an 85 year old in the general population (14). The high death rates in CKD patients might reflect accelerated atherosclerosis and heart failure (15).

**Chronic kidney disease**

CKD is defined as abnormalities of kidney structure or function, present for ≥3 months, with implications for health. A CKD diagnosis requires one of two criteria documented for ≥3 months: either eGFR <60 ml/min/1.73 $m^2$ or markers of kidney damage, including albuminuria.

In 2002, a working group founded by the American National Kidney Foundation issued treatment guidelines where CKD was defined from stage 1 to 5, they also introduced a new system for stageing kidney disease, which has been widely adopted (16):

| Stage | eGFR (/ml/min/1.73$m^2$) | Description |
|---|---|---|
| 1 | ≥90 | Kidney damage with normal or ↑ eGFR |
| 2 | 60-89 | Kidney damage with mildly ↓ eGFR |
| 3a | 45-59 | Mildly to moderately ↓ eGFR |
| 3b | 30-44 | Moderately to severely ↓ eGFR |
| 4 | 15-29 | Severely ↓ eGFR |
| 5 | <15 (or dialysis) | Kidney failure |

For near-normal kidney function, additional signs of kidney damage are required, and findings should be present for more than 3 months. Kidney damage is defined as pathological abnormalities or markers of damage, including abnormalities in blood, urine tests or imaging studies, and could alse be summarized in a shorter version (16):

| eGFR (ml/min/1.73m$^2$) | ≥90 | 60-89 | 30-59 | 15-29 | <15 |
|---|---|---|---|---|---|
| Normal to mildly increased albuminuria | No CKD | No CKD | Stage 3 | Stage 4 | Stage 5 |
| Moderately to severely increased albuminuria | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |

Previously, there had been no commonly accepted definition of CKD, thus this new classification represented a paradigm shift in nephrology, shifting the focus from end-stage to less advanced disease, enabling more prevention (17).

The CKD-guidelines were updated in 2012, and they were adapted by Kidney Disease Improving Global Outcome (KDIGO) which are international kidney disease quality guidelines. The definition of CKD was not changed, but a classification of CKD was added to the guidelines. CKD is classified based on cause, eGFR category, and albuminuria category, with the inclusion of albuminuria being the biggest change (18). The 2012 KDIGO guidelines a model including eGFR categories, albuminuria categories and prognosis/ risk prediction in a red-orange-yellow-green “traffic light” color scheme was presented. This model is used in clinical practice today, figure 1.

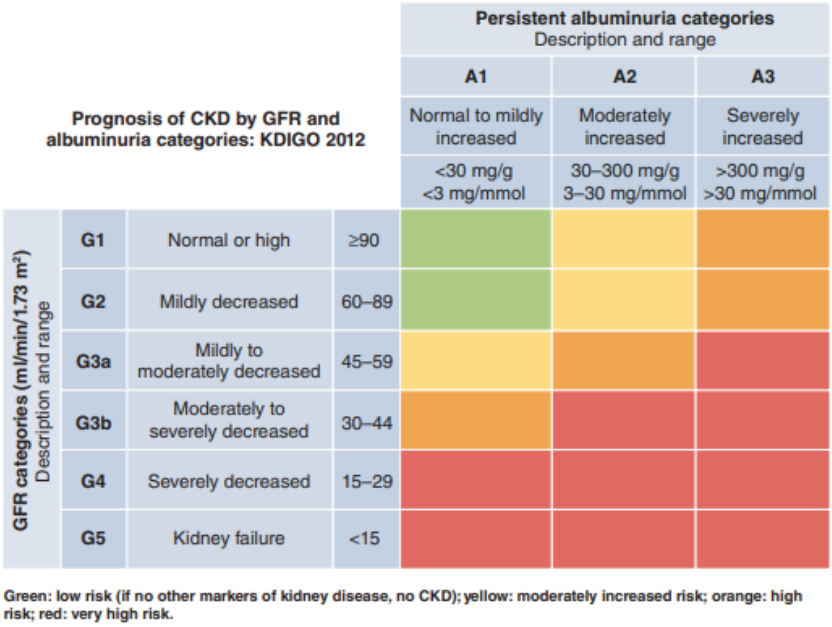



**Figure 1.** Traffic light model showing the prognosis of CKD based on eGFR and albuminuria category. *Reprinted with permission from Elsevier/ International Society of Nephrology (2).*

Ageing is an unavoidable process of life, and the kidneys are also affected by this process. Renal mass decreases between 30 and 80 years of age, with the steepest decline after 50 years (19, 20). With longer life expectancy, the proportion of people over 60 years is increasing much faster than any other age group. This may influence the epidemiology of nephrology (20). In a large study on patients CKD stages 3-5, the incidence of both ESRD and death was inversely related to eGFR among patients of all ages. However, the relative frequency of these outcomes in patients with similar eGFR, varied considerably by age, where the older patients were more likely to die than to develop ESRD, and the younger patients were more likely to develop ESRD than to die (21).

Recently, it has been suggested that using the same levels of eGFR to define CKD in all patients, may classify many older people as having a disease, exposing them to

various investigations and hospital appointments, when the reality is that they have a normal, physiological age-related eGFR decline (22, 23). This trend is clearly demonstrated in figure 2, showing a clear reduction in eGFR with increasing age. All eGFR measurements done at the lab of biochemistry in Stavanger University Hospital (SUS) in 2018 are shown. Each person is included only once, and 108 993 individuals are included. Each person's highest eGFR is shown. The horizontal lines show the accepted CKD stages.

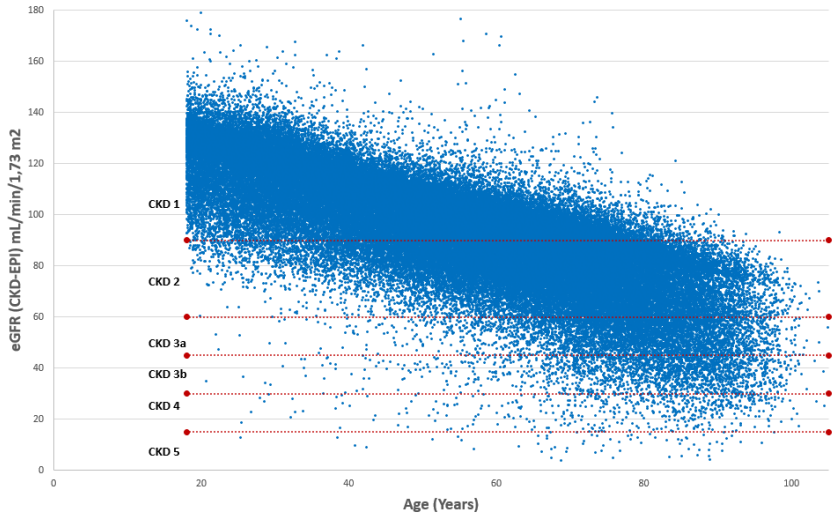



**Figure 2.** The relationship between eGFR and age. *The figure is made by Øyvind Skadberg, and is printed with his permission.*

It the future, the CKD definition may include age-specific tresholds for eGFR. This would reduce the CKD prevalence substantially. Perhaps, in younger people, an age-related threshold could lead to earlier identification of CKD (23).

A large recent population-based study in Tromsø, Norway, found that healthy middle-aged women had lower eGFR than healthy men indicating a sex difference in kidney function, where more healthy women had CKD stage 3a. However, the women had a slower eGFR decline over time (24). These sex differences and the age-

related GFR loss seen in healthy people have relevance in the discussion whether the CKD definition should be age- and sex adjusted (24).

The progression of established CKD is variable and depends on several risk factors. Non-modifiable factors are race, age, sex and genetics, and the rate of progression is often faster in males (25) and older individuals (26). Often we, as nephrologists, cannot predict if an individual with CKD will progress slowly or rapidly. We focus on optimizing modifiable risk factors. The most modifiable risk factors are hypertension (27) and glycaemic control in patients with diabetic nephropathy (28). Proteinuria/ albuminuria is a reliable marker of disease severity and a strong predictor of CKD progression, (29-31), figure 3. The association between albuminuria and progression of CKD is independent of the underlying cause of CKD (32).

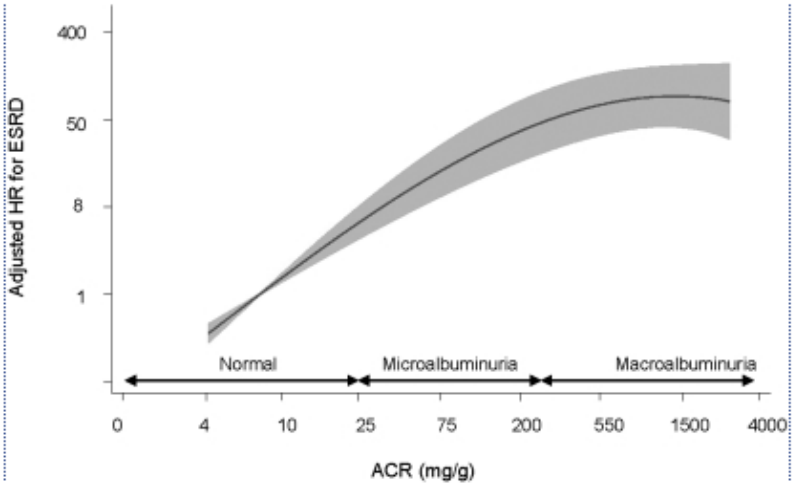



**Figure 3.** Hazard ratio with 95% confidence interval for ESRD associated with urine albumin excretion. *Reprinted with permission from JASN/ Clinical Epidemiology (31).*

Nephrologists focus on renoprotection in patients with CKD. Randomized controlled trials (RCTs) suggest that renin-angiotensine II-aldosterone blockers in the form of ACE inhibitors or angiotensin receptor blockers may slow CKD progression, and their effects include reduction in proteinuria (33, 34). ACE inhibitors and angiotensin receptor blockers are well-established in the standardized care of CKD patients.

Sodium-glucose co-transporter 2 (SGLT2) inhibitors are a relatively new class of antidiabetic drugs that lower glucose levels by preventing glucose reabsorption in proximal tubules in the kidneys. This leads to a reduction in blood glucose levels, and and increased glucose excretion in the urine (35). Many studies have shown that SGLT2 inhibitors are beneficial in diabetic kidney disease, to prevent the progression of CKD (36, 37). SGLT2-inhibitors have an additional effect of weight loss which is very beneficial for most patients with type 2 diabetes mellitus (38). SGLT2 inhibitors also lower blood pressure, reduce albuminuria and have beneficial cardio-protective effects (39, 40). SGLT2 inhibitors are effective in the treatment of patients with heart failure, and the use have been widely implemented by cardiologists worldwide, and SGLT2 inhibitors are included in the international AHA/ACC/HFSA Heart Failure guidelines from 2022 (41, 42). The mechanisms behind the beneficial effects of SGLT2 inhibitors are complex and still emerging, but clinical trials are focused on evaluating renoprotective effects of SGLT2 inhibitors in nondiabetic CKD patients. The antidiabetic effect of SGLT2 inhibitors decreases as eGFR declines, and in trials SGLT2 inhibitors were not indicated in diabetic patients with eGFR <45 ml/min/1.73m$^2$. However, accumulating evidence indicate that the nephron-protective and cardio-protective effects remain even at lower eGFRs, even though the anti-diabetic effects are limited. The indications for SGLT2 inhibitors are broadening, and results from recent trials show the same effects in non-diabetic CKD patients with reduced CKD progression, reduced albuminuria and reduced risk of CVD death (43-46). In October 2022 refund for CKD patients without diabetes was approved in Norway, and prescription-rates are increasing.

Recent studies have shown that the novel nonsteroidal mineralcorticoid receptor antagonist (MRA), Finerenone, reduce the aldosterone-mediated proinflamatory

effects that are involved in fibrotic remodeling processes, and has the potential to slow progression of CKD (47). In the FIDELIO-DKD trial patients with diabetes kidney disese who were treated with finerenone had a lower risk of CKD progression and death from renal causes than the patients included in the placebo arm. The finerenone patients also had lower risk of death, CV events, cerebral events and hospitalization for heart failure. Albuminuria was also reduced in the finerenone group (48). In the FIGARO trial patients with earlier stages of CKD were included. Finerenone reduced risk of death, CV events, cerebral events and hospitalization for heart failure (49). Finerenone has just been approved for use in Norway in patients with CKD stages 3 and 4 as well as diabetes mellitus type 2, but there is no refund yet. If Finerenone will be added to the renoprotective regime of most CKD patients in the future is yet to be determined, but Finerenone was included in the KDIGO guidelines for patients with diabetes and CKD in 2022 (50). Additional new drugclasses are expected to follow, and the field of renoprotection will most likely expand in the years to come.

**Renal replacement therapy**

Patients with CKD stage 5 who progress to ESRD have to choose between RRT and conservative treatment. RRT consists of kidney transplantation or dialysis. Kidney transplantation is the treatment of choice for patients with ESRD, with lower morbidity and mortality compared with patients remaining in dialysis (51-53), but transplantation is not an option for all patients with ESRD.
Patients in dialysis are treated either with hemodialysis (HD) or peritoneal dialysis (PD). HD is usually a hospital based dialysis treatment, where patients come to the hospital 3 times a week for 4-5 hours. A few patients administer HD at home, with regular appointments at the hospital. In HD, the patient's blood is filtered through an artificial kidney in a dialysis machine extracting excess water, waste products and electrolytes. HD-patients need an available acess point to the bloodstream via a permanent catheter, an AV fistula or an AV graft. PD, on the other hand, is done

daily, at home. The patients have a permanent peritoneal catheter operated into their abdomen, and infuse dialysis fluids into the abdominal space. The peritoneum functions as a filter, with an exchange of excess water, electrolytes and waste. The two forms of dialyses are equal in quality, and the choise of modality is based on several factors; patient wish, comorbidity, travelling time to the hospital, tradition of the nephrology department.

In Norway, there were 526 new patients in renal replacement therapy in 2021, 66.6 % were male and the median age was 67.3 years. 51 patients received a preemptive transplantation (before starting dialysis treatment). 155 started with PD and 321 started with HD (5). The number of new patients in RRT have been increasing since the 1980s, but are relatively stable from 2008-2021, with a slight decrease the last two years, figure 4.

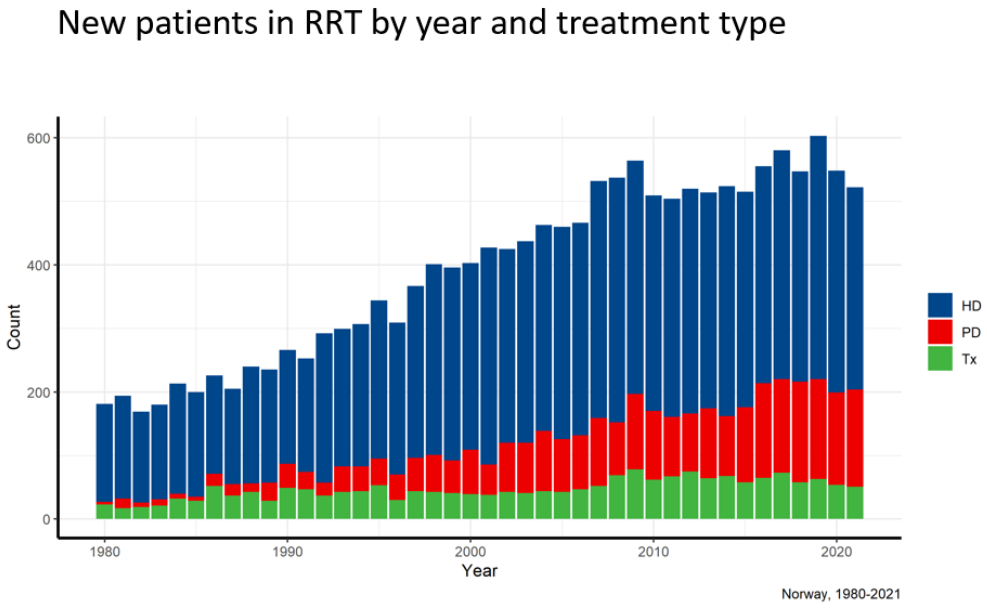



**Figure 4.** New patients in RRT, first treatment modality indicated by colour.
*Reprinted with permission from The Norwegian Renal Registry (NRR) (5).*

**Kidney transplantation**

Kidney transplantation has been a story of succes since the first sucessful transplantation in Boston, USA, in 1954 between two monozygotic twins. In Norway, the first transplantation was performed only two years later. Immunosuppression included steroids and total body irradiation, and the patient lived for 30 days. The first transplantation with a family kidney donor was performed in 1963, when a mother gave a kidney to her son. Azathioprine and steroids were used as immunosuppressive therapy, and the patient lived for 22 years with a functioning kidney graft. The donor (his mother) died when she was 92 years old (54).

Later in the 1960s, polyclonal anti-lymphocyte antibody preparations were used to prevent graft rejection (55). The development of immunosuppressive agents was crucial for organ transplantation to become a successful option for patients with end-stage organ diseases (56). Over time, a variety of agents that block different steps in the body's natural immune response have been incorporated into the practice of organ transplantation. But all immunosuppression agents pose a significant risk of opportunistic infections, neoplasias and other complications (56). Improvements in immunosuppressive treatment and infection control are the main causes of the reduction of short-term complications post-transplant (57).

In 1983, cyclosporine A (CyA) revolutionized the immunospressive treatment in kidney transplant recipients. The immunosuppressive effects of CyA was discovered in 1972 in Switzerland after a swiss microbiologist discovered a new fungus (Tolypocladium inflatum) when he was on vacation on Hardangervidda, in Norway, with his wife. He broght a sample back to his lab in Novartis (former Sandoz) in Basel, Switzerland for testing. The discovery was done during a screening program of fungus extracts (58). CyA showed superior immunosuppressive abilities with a selective action on T-lymphocytes, supressing antibody and cell-mediated immunity and chronic inflammation, without severe toxic or CV side effects (59).

Tacrolimus and CyA are both calcineurin inhibitors (CNIs), and CNI-based regimes are the cornerstone of the maintenance immunosuppression in kidney transplant

recipients (60). Tacrolimus is a macrolide with immunosuppressive properties which inhibits the cellular and humoral immune response by various mechanisms, with a central effect on calcineurin inhibition. Tacrolimus was introduced in renal transplantation in the late 1990s, and a prolonged-release formula gives a more stable drug exposure (61). Evidence does exist that tacrolimus may be associated with better renal function than CyA (60), fewer acute rejection episodes and improved long-term graft survival (62). Tacroliumus is associated with lower lipid levels (63), and reduced need for antihypertensive and lipid-reducing drugs (62). On the other hand, tacrolimus has other side effects, such as increased incidence of diabetes mellitus, neurological and gastrointestinal side effects, therefore the choice of CNI should be individualized after considering the risks and benefits of each drug (64).

The antiproliferative agents used in kidney transplantation are azathioprine, mycophenolatmofetil (MMF) and mammalian target of rapamycin (mTOR) inhibitors. They inhibit purin based synthesis required for T- and B-cell proliferation (56). The formerly used Azathioprin act by blocking all cells in mitosis, preventing rejection, but the drug comes with side effects on bonemarrow and cell linings in the gut causing anemias and diarrhea (59). With long-term use, the drug is consideres co-mutagenic, and there is a high risk of developing skin cancer (56). MMF is an inhibitor of inosine monophosphate dehydrogenase, which leads to a cytostatic effect on T and B cells and suppresses the formation of antibodies by B-cells (56). Side effects from MMF are mostly gastrointestinal, but some patients develop leukopenia (65). MMF was shown to be effective in preventing acute rejection when added to a CNI-based regime (60). Substituting azathioprine with MMF reduced the incidence of rejection from 35% to 16% respectively (66), and MMF is shown to be associated with longer graft survival than azathioprine (67). mTOR inhibitors are also antiproliferative drugs that inhibit the proliferation and differentiation of T and B cells and the production of antibodies, and the proliferation of non-immune cells such as fibroblasts and smooth muscle cells (68). mTOR inhibitors were approved in the late 1990s, and have been suggested as an alternative to CNI, because of CNI nephrotoxicity with longterm use. Unfortunately, mTOR inhibitors are not free of

side effects either, potentially causing anemia, thrombocytopenia, proteinuria, non-infectious pneumonitis, reduced wound healing and increased cholesterol, and data on long-term outcomes are conflicting (56, 69).

Induction treatment is used to provide intense immunosuppression in the early post-transplant period to prevent acute rejection (60). Induction treatment with monoclonal antibodies (basiliximab, daclizumab, alemtuzumab or anti-thymocyte globulin) has been shown helpful in preventing acute rejection episodes and reducing CNI dosages (69).

Steroids have anti-inflammatory, immunosuppressive and immune modulatory effects (56). Steroids bind to glucocorticoid receptors and inhibit the production of various pro- inflammatory cytokines (70). Steroids are used in the standard immunosuppression regimen, but concerns about side effects such as weight gain, diabetes, osteoporosis and hyperlipidemia have prompted increasing interest in steroid-free or low- dose steroid immunosuppression with a few trials indicating that with a potent combination of CNI and MMF, steroids could be withdrawn or the dose could be reduced in patients with a low immunological risk (69).

Belatacept is a fusion protein that binds to the CD80 and CD 86 molecule in antigen presenting cells and prevents their interaction with the CD28 molecule of T-lymphocytes, blocking co-stimulation (71). It is used to prevent acute cellular rejection, and an RCT showed similar patient and graft survival compared with a CyA based regime, with improved renal function (72). Belatacept is administered as an intravenous infusion every four weeks, for some patients this is preferable from an adherence perspective. But, the use of Belatacept is associated with post-transplant lymphoproliferative disease in Epstein Barr negative recipients receiving an Epstein Barr positive kidney (73).

The standard immunosuppressive regimen in Norway, includes the use of basiliximab and methylprednisolone induction. The maintenance immunosuppression includes a CNI in combination with MMF and prednisolone. Initially, after being introduced, tacrolimus was the preferred CNI to patients under 50 years, and CyA to older

patients, patients with high body mass index (BMI) or impaired glucose tolerance. From 2012, tacrolimus was the preferred CNI to all patients. mTOR og Belatacept is used instead of CNI in a few patients due to side effects and individual considerations.

Introduction of CyA significantly improved patient and graft survival (60). In the nineties early rejection episodes and graft loss were the major challenges in kidney transplant patients. With the use of newer immunosuppressive drugs, acute rejection episodes have been reduced from 50% to 20%. However, long-term graft survival has not improved similarily in the same time period (74, 75). Overall half-life of a kidney is around 15 years (76). Currently, the strategy for immunosuppression focuses on reducing adverse effects and improving renal function to prolong graft survival (60). Improvement of long-term graft survival is one of the major challenges in the field of kidney transplantation (77). Individually tailored immunosuppression regimes are considered a gold standard at the moment, with regimes fitted to the patients according to tolerance, side effects and compliance (69). Patients are not equal concerning the chances of rejection, risk of graft loss and occurrence of side-effects. Immunosuppressive strategies require a careful balance between too much and too little. With too much immunosuppression, the patients are at risk of infections, malignancies and nephrotoxicity, and with too little immunosuppression there is risk of rejection and graft loss (56).

In Norway, transplantation is offered to all patients considered to profit from it, if no contraindication in the form of severe comorbidity exists. There is no strict upper age limit, and patients over 80 years have been transplanted. In a 5-year follow-up study on Norwegian transplant recipients >70 years, there was no difference in graft survival when censored for death with functioning graft. Overall 5-year survival was lower in the older recipients, but this is as expected with higher age (78). We have one transplantation center in Norway, Oslo University Hospital (OUS), Rikshospitalet (RH). It is the largest solid organ transplant center in Europe.

The Norwegian Renal Registry (NRR) contains data reported annually since 1980 by all nephrology units in Norway on all patients receiving RRT. From 2018, all patients with CKD 5 were included in the annual reports. The NRR was formally constituted in 1994 as a collaboration between the Norwegian Renal Association and OUS, RH. Written informed consent is obtained from each individual patient before any information is sent to the registry. In 2012 the registry acquired status as a national quality register for all patients receiving RRT. The reporting to NRR is closely monitored, and the coverage for individuals and annual data are >99.9% and 96-98%, respectively.

In 2021, 231 kidney transplantations were performed in Norway. 16% were retransplantations. Preemptive transplantation was performed in 26% of all first transplantations in 2021. The 144 non-preemptive, first transplant recipients had been in dialysis for a median of 2.05 years, ranging from 7 days to 11.4 years (5).

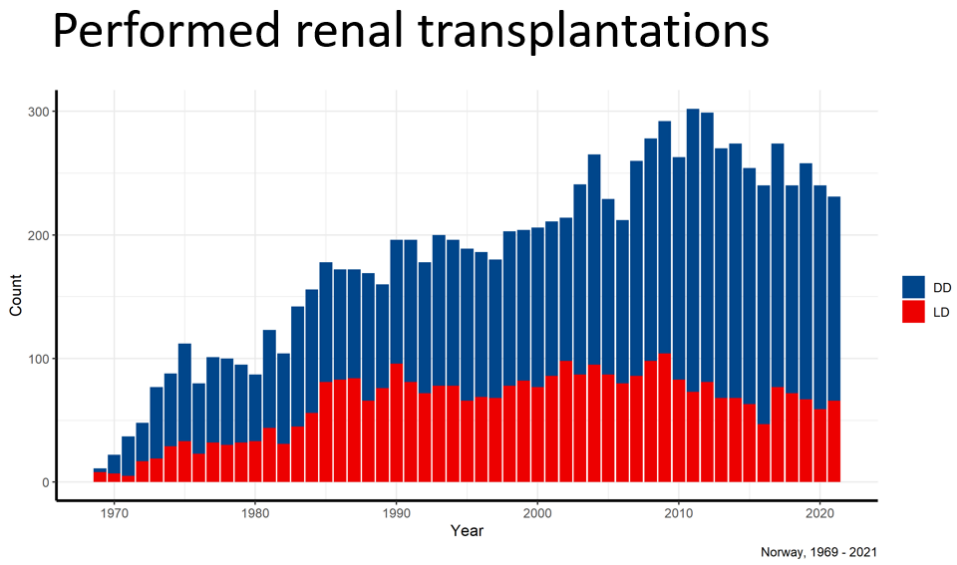



**Figure 5.** Renal transplantations performed in Norway, donor type indicated by colour. *Reprinted with permission from the NRR (5).*

**Living kidney donors**

The short- and long-term effects of a unilateral nephrectomy on living donors have been important issues for more than 60 years. A living donor must undergo a medically unnecessary procedure, and safety-concerns have always been important. In Norway, living donors have been used for decades, and have been instrumental for keeping the waiting list stable. At the end of 2021, 391 patients were on the waiting list for a deceased donor kidney transplantation. The list is increasing year by year, and in 2021 the median waiting time for a deceased donor transplantation was 18 months for a first transplant and 14 months for a retransplant. To keep the recipient waiting list as short as possible, to minimize time before transplantation, it is important to keep the numbers of living donor transplantations as high as possible (5).

Early studies claimed that kidney donors neither had increased risk of mortality or kidney failure compared with the general population. However, donors are selected from a group of very healthy individuals thoroughly screened for conditions such as hypertension, kidney disease and CVD. Kidney donors do not mirror the general population. Newer studies match donors with equally healthy controls. Some studies found increased long-term morbidity for kidney donors, with an addional risk of hypertension, metabolic derangements particularly involving calcium homeostasis, proteinuria and CKD-development (79-83), whereas other studies have not found increased risk of CVD and death when comparing kidney donors and matched controls (84-86). A recent Norwegian study with long-term follow-up and matched controls found increased risk of ischaemic heart disease in kidney donors (87). This leads us into a difficult ethical dilemma and may influence the selection process of living kidney donors.

If kidney donors have increased morbidity and mortality after donation, this must be addressed in consultations prior to kidney donation. Providing this information to a potential donor is complicated, and demands an individual approach and enough time. The information provided could benefit from standardization, and for many donors it would be beneficial to receive written information describing potential long-

term risks after donation in addition to the information given by their evaluating physician (88). All potential donors in Norway receive a booklet called “Til deg som har blitt bedt om å gi en nyre” with updated and thorough information about kidney donation. The brochure has information about possible risks and complications, and is used in the consultations before the donation. Fortunately, the number of donors with complications, both short- and long term are very low. The potential donors are thoroughly screened for comorbidities and risk factors, and donation is only recommended to potential donors if they are healthy in the pre-donation screening, and appear well-informed as well as motivated to take this possible risk. The donors are followed by the nephrology departments across the country on a regular basis post-transplant, with regular reporting to the NRR. The follow-up is regulated, and it is based on the declaration of Istanbul on organ trafficking and transplantation tourism, and it is described in a national guide from the Norwegian health directorate (89, 90).


**Figure 6.** Front page of the brochure Provided to all kidney donors in Norway. *Printed with permission from nephron.no*

**Patient survival in RRT**

Mortality is increased in patients with reduced kidney function, and CKD is a strong predictor of premature death (12, 14). The relative risk of death in patients with CKD stage 5 is six times higher than in the general population (91). The hazard ratio for death is much higher than what can be explained from traditional risk factors like diabetes, lipids and hypertension. A total of 577 patients with CKD 5 in Norway died during 2021, 79 had never started RRT, 312 were in dialysis and 140 transplanted. Dialysis treatment was terminated and followed by death in 46 patients. Median age of death was 75 years, ranging from 23 to 96 years. Infections and cardiac complications were the most frequent causes of death, followed by malignant tumors (5).

Observational studies in kidney transplant recipients have shown that the risk of death was 68% lower after a successful transplantation compared with those remaining in dialysis (53). But, life expectancy among kidney transplant recipients remain inferior to age-matched controls from the general population. In the nineties, a renal transplant patient of 30 years had approximately the same CV risk as a 70-year-old person in the general population (91). And, a female transplant recipient between 40 and 44 years have a projected lifespan that is over 10 years shorter than a 40-44 year old female from the general population (92). The reasons for this are many, but it is very important to explore and identify the mechanisms behind the increased mortality. CVD is the number one cause of death, the second is the development of malignant disease, and the third is death from infection (57).

In Norway, patient survival is best in patients receiving a preemptive kidney transplant, followed by transplant recipients in PD and then transplant recipients in HD. The differences in survival manifests shortly post-transplantion, and continues in long-term patient follow-up, figure 7.

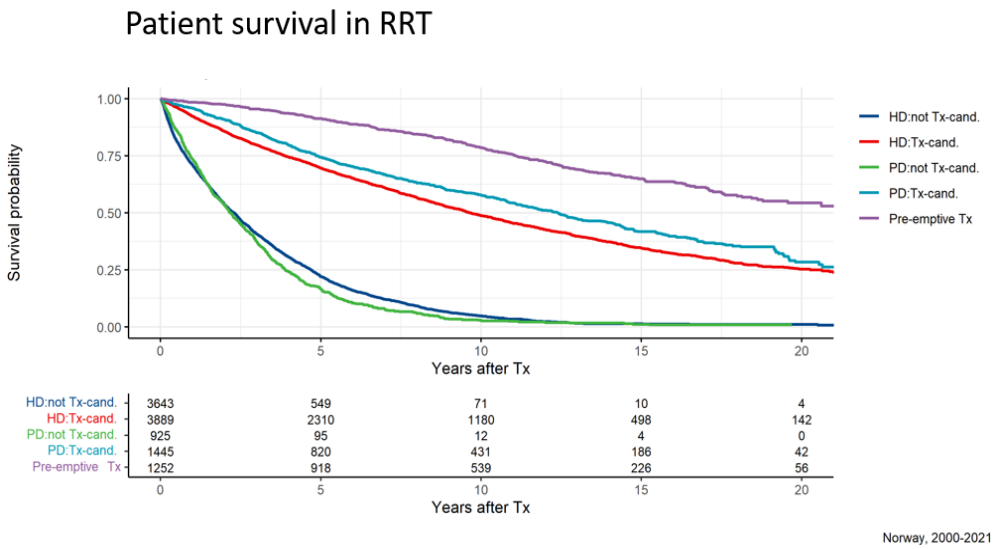



**Figure 7.** Patient survival in RRT in Norway from 2000-2021, showing better survival in transplant recipients compared with patients remaining in HD/PD *Reprinted with permission from the NRR (5).*

**Methods to evaluate kidney disease**

Kidney function is a term that describes the kidney's ability to filter urine, the GFR. The volume of plasma cleared of an exogenous filtration marker eliminated exclusively by glomerular filtration (e.g. inulin or iohexol) per unit time is the GFR. The measurement of plasma clearance is time consuming, and repeated blood samples are necessary to calculate the clearance curve accurately. This of course, is not done regularly in the individual patient because it is time-consuming and expensive (93).

As a consequence, GFR is typically estimated by endogenous filtration markers (creatinine and/ or Cystatin C). The estimation of GFR is usually sufficient for clinical decision making, but at the early stages of CKD, the measured GFR is more accurate than the estimated GFR.

The most common endogenous filtration marker is creatinine. Creatinine is freely filtered by the glomerulus, and is secreted by the renal tubules. Creatinine is a breakdown product of dietary meat and creatine phosphate found in skeletal muscle, and its production in the body depends on muscle mass (94). The Creatinine Clearance (CrCl) rate approximates the calculation of GFR since the glomerulus filters creatinine freely (95). Serum creatinine and various formulas based on this cheap and available analyte, remains the cornerstone of the assessment of kidney function. The relationship between creatinine and GFR is non-linear, and under the influence of several variables. Numerous formulas to estimate GFR have been published over the years (96). The Cockcroft Gault formula gained worldwide acceptance from 1976 despite being based on only 249 patients (97). In 1999, the Modification of Diet in Renal Disease (MDRD) study presented the new MDRD formula, based on 1628 patients with CKD (GFR <60 ml/min/1.73m$^2$). The MDRD formula included urea, albumin, race, age and sex (98). The MDRD formula was widely accepted for many years, and the main concern was the formulas' inaccuracy in patients with GFR >60 ml/ min/1.73m$^2$. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula was developed as an alternative, using the highest available standards. The CKD-EPI formula also included creatinine,

age, race and sex, and the CKD-EPI formula provides a more thorough estimate in patients with high GFRs (99). After a debate in the nephrology environment around the world, as well as in Norway, the CKD-EPI has been widely accepted. The CKD-EPI formula replaced the MDRD formula at SUS in January 2012.

There are several available GFR estimating equations with advantages and disadvantages. Recently a race-free CKD-EPI eGFR equation was developed (100). The recognition that race is a social and not a biological entity has called for the removal of race in clinical algorithms. As a response the National Kidney Foundation and the American Society of Nephrology created a task force that recommend to replace the older equations with a newer formula without race, CKD-EPI 2021, in clinical laboratories (100). The new formula performs similarly to the old CKD-EPI equation in transplant recipients (101), but in CKD patients the prevalence of CKD is slightly lower when using the new equation compared with the old CKD-EPI formula (102). The new equation has not implemented into routine care in laboratories in Norway at this time, but it will most likely be implemented in the future.

Cystatin C is another endogenous filtration marker that can be used to measure kidney function. Cystatin C is a protein synthesized at a constant rate by all nucleated cells. Cystatin C is not dependent on factors such as age, sex, race and muscle mass, and in certain groups of patients Cystatin C may be more useful that creatinine. After measuring Cystatin C, a Cystatin C based GFR is estimated, and the CKD-EPI formula is commonly used. Cystatin C has not been included as a routine laboratory test in most hospitals due to high costs and lack of evidence of added benefit. Cystatin C is a beneficial supplementary analysis in patients with high muscle mass, in children, the elderly and in individual with conditions affecting muscle composition (103). Cystatin C has not been measured in the present studies and will not be mentioned more.

In addition to blood tests measuring kidney function, nephrologists monitor their patients' proteinuria. The gold-standard for quantification is a 24-hour urine collection. An excellent correlation between 24 hour samples and single voided urine

samples has been found, preferably the first morning specimen, but a random specimen is acceptable (104), and is widely used in clinical practice. 24 hour urine collection is burdensome for the patients to perform, and errors due to incomplete collection is common.

KDIGO classifies normal to mildly increased albuminuria as urine albumine to creatinine ratio (UACR) <3mg/mmol (<30 mg/g), moderately increased albuminuria is UACR 3-30 mg/mmol (30-300 mg/g), and severely increased albuminuria is UACR >30 mg/mmol (>300 mg/g). Normal to mildly increased proteinuria is classified as urine protein to creatinine ratio (UPCR) <15 mg/mmol (<150 mg/g), moderately increased proteinuria as UPCR 15-50 mg/mmol (150-500 mg/g), and severely increased proteinuria as UPCR >50 mg/mmol (>500 mg/g). UPCR >3 g/day is indicative for nephrotic proteinuria (18).

There is a strong correlation between UACR and UPCR, especially when UACR >300 mg/g or UPCR >200 mg/g. The association is less accurate for lower UACR. UPCR measures the total urine protein excretion, including albumin, light chains and other globulins. UPCR also has the advantage of measuring fragmented albumin, a byproduct of tubular processing, not detected by UACR. UACR only accounts for the albumin concentration (105). In patients with certain diseases such as light chain disease, UACR and UPCR do not correlate, with a much higher UPCR due to light chains (13). There appears to be no concensus concerning the golden standars for evaluating proteinuria, but both UACR and UPCR should be used as a screening tool initially. After that, in early nephropathy UACR is of the greatest interest, especially with hypertensive or diabetic nephropathy.

Both albuminuria and proteinuria are important disease markers of CKD (31, 106), used at time of CKD-diagnosis, and during follow-up, to monitor the effects of therapy and the rate of progression. Both eGFR <60 ml/min/1.73m$^2$ and albumin/creatinine ratio ≥30 mg/g are significantly associated with increased risk of all-cause and CV mortality independently of each other and other traditional risk factors (13).

**Biomarkers**

The above presented standard laboratory tests for kidney disease are inaccurate. New and improved methods to assess and predict outcome in patients with CKD would be welcome in the field of nephrology. A biomarker is defined as a parameter of structural, biochemical, physiological or genetic change that indicates the presence, severity or progress of a disease (107). An ideal biomarker is stable, undergo little degradation and show minimal variability (diurnally and longitudinally). The analysis should be accurate, reproducible and affordable. The information obtained from the biomarker should add to or improve existing tests, improve patients management or risk assessment (108). The below presented biomarkers will be discussed in more detail in at least one of the four studies presented in this thesis.

*Neutrophil gelatinase-associated lipocalcin (NGAL)*

NGAL is a protein belonging to the lipocalin family (109). NGAL was originally isolated from the supernatant of activated neutrophilic granulocytes (110). NGAL is also produced in other types of tissue, like the kidneys (109). The function of NGAL is largely unknown, but NGAL increases in cells under "stress" for instance during infections, inflammation, degeneration and neoplastic transformation (111). Initially NGAL was a marker of acute cellular stress, with no relation to kidney injury or kidney failure. It has later been shown that NGAL increases in plasma and urine in patients with acute renal failure and after aortocoronary bypass surgery (112). High NGAL levels is also associated with worse prognosis in patients with CKD (113-115) and after kidney transplantation (116-118). One study found that increased NGAL levels 1 week after donor nephrectomy was associated with eGFR <60 ml/min/1.73m$^2$ 6 months postoperatively (119). The hypothesis called the "forest fire theory" suggests that the increase in NGAL in CKD patients is the result of a sustained production of inflamed but vital tubular cells, whereas reduction of kidney function is a general loss of functional cells and nephrons (120). NGAL may

represent a time-real biomarker of ongoing kidney damage, and NGAL has been named the “troponin of the kidney” (121).

*Klotho*

Ever since its discovery in 1997, Klotho has been linked to reduced kidney function. Klotho was originally identified as an anti-ageing protein in a mouse model of human ageing, but at a later stage it has been shown to possess a number of biological functions (122-124). Klotho is expressed in different organs, but mostly in kidney tissue, especially in the distal tubuli cells, in the parathyroid glands, and in the choroid plexus in the brain (123, 125, 126). Klotho deficient mice and CKD subjects have similar phenotypes, suggesting a close link between Klotho and CKD (127, 128). Klotho also act as a co-receptor for the fibroblast growth factor receptor (FGFR), and the tissue specific Klotho expression determines the organ specificity for FGF23 (123) . In animal studies Klotho has kidney protective abilities, importance in the calcium-phosphate homeostasis, anti-ageing and CV protective properties (122, 129). Klotho levels are quickly down-regulated in the presence of albuminuria (130), inflammation (131) and acute kidney injury (AKI) (132, 133). Serum levels of Klotho progressively decrease with age (134). Klotho has been reported to be closely associated with reduced kidney function (135-138), but other studies have reported stable Klotho levels in CKD stages 2-4 (139). Low levels of Klotho are associated with increased vascular calcification (140), and increased risk of CV events and mortality in patients with ESRD (141).

*Fibroblast growth factor 23 (FGF23)*

FGF23 is a member of the fibroblast growth factor (FGF) superfamily, a family of 22 structurally related polypeptides with many functions (142). Being in the FGF family is not defined by similarities in cellular actions, but by the presence of a 102 amino-acid long protein that mediates FGF binding to a particular family of cell surface receptors, called fibroblast growth factor receptors (FGFR) (143). The FGFs can be classified as intracellular (intracrine) FGFs, canonical (paracrine) FGFs and hormone-like (endocrine) FGFs by their mechanism of action (144).

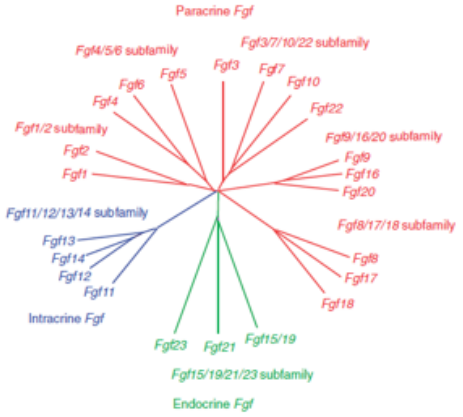



**Figure 8.** Phylogenetic analysis divide the 22 FGFs into seven subfamilies, and three categories (different colors) based on their mechanism og action. *Reprinted with permission from Oxford University Press (142)*

The intracrine FGFs act as FGFR-independent intracellular molecules that regulate the electrical exitability of neurons via voltage gated sodium channels. Intracrine FGFs are not secreted extracellularly (142, 145). The paracrine FGFs mediate biological responses by binding to and activating cell surface tyrosine kinase FGFRs with heparin/ heparin sulfate as a necessary cofactor for local signaling (142). The paracrine FGFs act as local paracrine signaling molecules with effects on embryonic development, cell proliferation and migration in neighbouring cells, acting from a small distance (142, 144).

The endocrine FGFs mediate biological effects in a FGFR dependent manner, but they bind to the heparin/heparin sulfate coreceptor with very low affinity. This reduced binding enables endocrine FGFs to function over long distances as endocrine hormones (142, 146). The endocrine FGFs use Klotho as a coreceptor to activate FGFRs (143). FGF23 binds to FGFRs using αKlotho as a coreceptor (123), and

FGF19 and FGF21 use βKlotho (147) as their coreceptor (142, 148). FGFRs are widely distributed, but the tissue specific patterns of Klotho/ βKlotho expression determine the specific function of the endocrine FGFs (146). FGF19 modulates cholesterol/ bile acid synthesis, FGF21 controls glucose and lipid homeostasis, and FGF23 is a potent regulator of phosphate and vitamin D metabolism (146).

FGF23 was initially discovered as a factor causing phosphate waisting in an inherited form of rickets, autosomal dominant hypophosphatemic rickets (149). FGF23 is secreted from bones after phosphate intake, and acts on the kidney to inhibit phosphate reabsorption in the urine. The goal is to maintain phosphate balance in the body (150). The high expression of Klotho in the kidney determines FGF23s' ability to target the kidney, and establishes an FGF23 driven bone/renal axis which allows FGF23 to function as a phosphatonin (146).

Intact FGF23 (iFGF23) is a small polypeptide of 251 amino acids, and is enzymatically cleaved into inactive c-terminal and n-terminal fragments. iFGF23 appears to be the biologically active form, and there is a strong correlation between iFGF23 and cFGF23 in patients without kidney failure (108). The c-terminal of the FGF23 molecule is responsible for binding to the FGF receptor (FGFR), with Klotho as an obligate co-receptor (151). In the kidney, this binding leads to an inhibition of the NaPi-2a and NaPi-2c channels, resulting in reduced absorption of phosphate (148). FGF23 further inhibits 1-α-hydroxylase (Cyp27b1) in the kidney and stimulates 24-hydroxylase (Cyp24a1), leading to a reduction in the concentration of 1,25 dihydroxy-vitamin D ($1,25(OH)_2D$) (148). Thus FGF23 is a phosphaturic, anti-vitamin D hormone.

FGF23 also acts directly on the Klotho-expressing parathyroid gland where it inhibits parathyroid hormone (PTH) production (146, 151). But surprisingly, both high Klotho and high FGF23 is associated with hyperparathyroidism (152, 153).

FGF23 regulates phosphate metabolism in the kidneys, intestine and parathyroid glands by down-regulating the sodium-phosphate co-transporter in the proximal tubule and the gut and by decreasing PTH secretion and production via a Klotho-

dependent pathway (154). FGF23 levels rise quickly as renal function declines, to increase phosphate excretion (155). At a certain point, the mechanism is overloaded, and increased levels of FGF23 are associated with increased mortality in HD patients (156), in patients with CKD stages 2-4 (157) and in kidney transplant recipients (158). Increased FGF23 combined with reduced GFR predicts rapid progression towards ESRD (159), and in kidney transplant patients it might predict increased risk of death and allograft loss (158).

In the heart, there is no Klotho expression, and FGF23 levels are very low in a normal heart (154, 160). But recent evidence show that cardiomyocytes are able to produce FGF23 in a strained heart (161), and FGF23 production increases in cardiomyocytes in rodent models with acute MI (162). In situations with high levels of circulation FGF 23, such as advanced CKD, FGF23 levels can reach 1000 times above the normal range (155). In these situations, when FGF 23 levels in the heart are high (locally produced and/ or due to CKD), there is evidence that FGF23 can act directly on cardiomyocytes in a Klotho independent, fibroblast growth factor receptor 4 (FGFR4) mediated signaling pathway. This binding activates the phospholipase C-gamma (PLC$\gamma$)/ calcineurin/ nuclear factor of activated T cells (NFAT) signaling axis, which is known to induce left ventricular hypertrophy (LVH) and hypertrophic growth of cardiac myocytes in response to other stimuli (154, 160, 163). The local production of FGF23 with local effects, gives FGF23 a paracrine ability in addition to its' endocrine activities (164).

Elevated levels of FGF 23 are associated with LVH (160) and myocardial fibrosis (154). There is a debate on wether FGF23 has a direct effect on blood vessels or not. Some studies report a link between circulation FGF23 and arterial stiffness (165), general atherosclerosis (166) and coronary artery disease (167), but others failed to find an association between blood vessel disease and FGF23 (168, 169).

The endothelial dysfunction seen in CKD is characterized by an imbalance between nitric oxide (NO) availability, upregulation of NO synthase and reactive oxygen species (ROS) (170). The imbalance between ROS production and antioxidant

defences leads to a condition called oxidative stress (171). Klotho possesses antioxidative properties and increase resistance to oxidative stress, which could contribute to Klothos anti-ageing properties (172). Klotho deficient mice show reduced NO synthesis in endothelial cells (173), and Klotho overexpression improves endothelial function (174). An in vitro study demonstrated the expression of Klotho on human coronary artery endothelial cells, confirming the role of Klotho in modulating FGF23 effects on oxidative stress (170). The study showed that FGF23 activates FGFR1, which in turn stimulates the secretion of Klotho, enhances NO release and increased ROS detoxification. ROS formation is also increased by FGF23, but the ROS formation is counterbalanced by the increased NO release. When blocking Klotho, the FGF23 stimulated NO synthesis is lost, this leads to enhanced ROS formation instead of ROS degradation, and reduced NO availability. In contrast to its effects on ROS formation, FGF23 stimulated NO release and ROS detoxification requires Klotho. With CKD comes Klotho deficiency, and FGF23 will then primarily promote oxidative stress and endothelial dysfunction (170). In a mouse model, it was found that high levels of FGF23 inhibits endothelial relaxation in the mouse aorta due to increased superoxide, which inhibits NO bioavailability. This supports that high levels of FGF23 contributes in CVD (175).

In CKD, vascular calcification is very frequent, and with high levels of circulation FGF23 it is conceivable to imagine that there might be a local Klotho-independent FGF23 effect on blood vessels (164). The evidence of this is inconclusive at best, most studies did not find a direct association between FGF23 and blood vessels (176, 177), but others have found a possible link where FGF23 impairs endothelial vasodilatation by inhibiting NO availability, causing endothelial dysfunction (175). There is controversy regarding the expression of Klotho in vascular tissue (178). It has been suggested that the different expression profiles of Klotho in vessels are dependent of the origin of the vessels studied, as well as inconsistency in the methods used to detect Klotho (178). Figure 9 illustrates the many potential effects of FGF23.

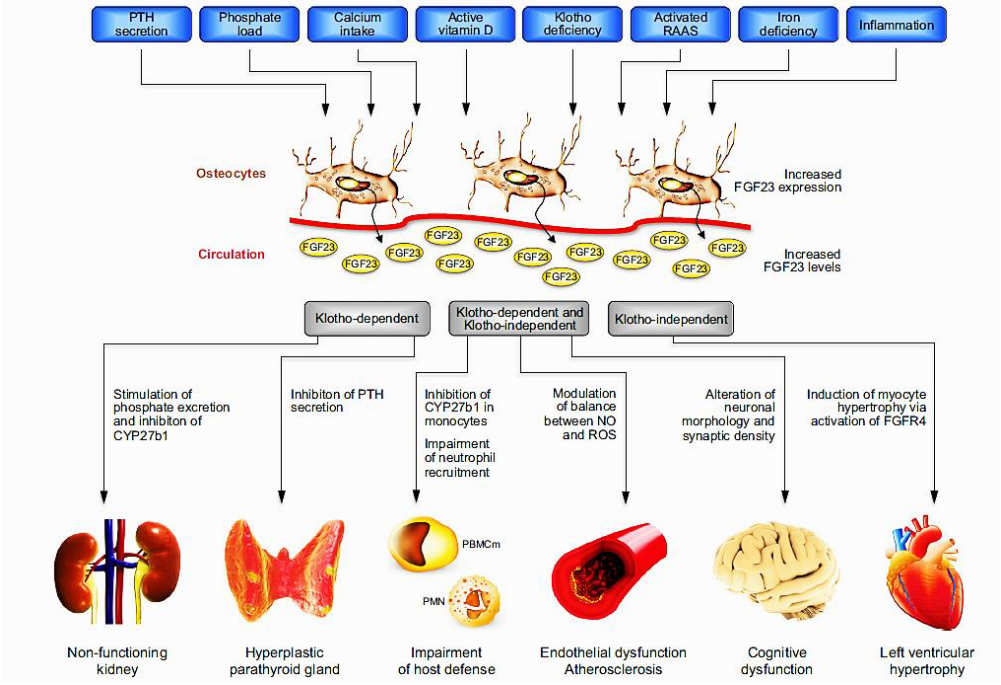



**Figure 9.** Renal and extrarenal effects of FGF23 in CKD. Bone and kidney are the main sources of FGF23. In CKD FGF23 is excessively increased by several factors, including chronic phosphate load, calcium intake, secondary hyperparathyroidism, active vitamin D therapy, Klotho deficiency, activated renin–angiotensin–aldosterone system (RAAS), iron deficiency, and inflammation. FGF23 regulates mineral metabolism in the kidney and parathyroid gland by utilizing Klotho as a permissive co-receptor. It also interferes with the immune system directly and/or Klotho-dependent mechanisms by acting on polymorphonuclear leukocytes and macrophages via activation of FGF-receptors FGFR2 and FGFR1, respectively. Experimental data suggest that FGF23 impacts on the vasculature and brain via both Klotho-dependent and Klotho-independent mechanisms. Finally, the heart has been identified as an off-target organ in which FGF23 stimulates cardiac growth in the absence of Klotho. CYP27b1 1α-hydroxylase; PBMCm, peripheral blood mononuclear cell monocyte, ROS reactive oxygen species, NO nitric oxide. *Reprinted with permission from Springer Nature (179).*

*Parathyroid hormone (PTH)*

PTH is produced by four small parathyroid glands that are located in proximity to the thyroid gland. PTH is a single chain peptide composed of 84 amino acids. Intact and fragmented hormone is present and secreted by the parathyroid gland. The intact hormone represents a smaller fraction, but its portion is increased when calcium levels are low and decreased when calcium levels are high.

The main role of PTH is to regulate serum calcium levels, through effects on bone, kidney and intestine. When serum calcium levels are decreased, the calcium sensing receptor (CaSR) in the parathyroid gland is inactivated, leading to the release of preformed PTH within minutes. Increased PTH synthesis takes hours, and parathyroid hyperplasia is established within days of prolonged inactivation of CaSR, leading to increased PTH production (180). PTH then increases serum calcium by promoting the release of calcium from bone into the bloodstream, stimulating the kidneys to reduce calcium elimination in the urine while promoting phosphate elimination in the urine, and stimulating the kidneys to convert vitamin D from the inactive to the active form, which in turn increases the absorption of calcium from food in the intestine. Negative feedback mechanisms ensure that these actions are reversed when calcium levels are normalised (181).

In the bloodstream PTH has a short lifespan, with a halflife of less than 5 minutes due to fast uptake and cleavage in the liver and the kidneys. The fragments are referred to as C-terminal fragments and are variably sized, missing from 6 amino acids to half the molecyle. The c-terminal fragments have a longer half-life, exists in higher concentrations and are eventually cleared by the kidneys. In CKD, these fragments typically accumulate, hence the elevated PTH levels (182).

Secondary hyperparathyroidism (SHPT) is a major complication in patients with CKD, and the hyperfunction in the parathyroid glands develop early in CKD progression. The changes are related to proliferation of the parathyroid cells, including the downregulation of vitamin D receptors (VDRs), CaSRs and the cyclin-

dependent kinase inhibitor, as well as the upregulation of transforming growth factor-α in resected glands from hemodialysis patients (183).

The optimal PTH levels in patients with CKD is not known, but the KDIGO guidelines from 2017 suggest that in predialytic CKD patients with PTH levels rising persistently above the upper normal limit, modifiable factors such as hyperphosphatemia, hypocalcemia, high phosphate intake and vitamin D deficiency should be evaluated and treated. Vitamin D analogs and calcitrol should be reserved for those with severe and progressive hyperparathyroidism. In dialysis patients it is suggested to maintain PTH levels in the range of 2-9 times the upper normal limit. If PTH lowering treatment is needed, calcimimetics, calcitriol or vitamin D analogs are recommended. In patients with severe hyperparathyroidism and failure to respond to pharmacological treatment, parathyroidectomy should be considered (184).

*Vitamin D*

Vitamin D was discovered in 1922 when it was discovered that heated, oxidized cod-liver oil could prevent rickets in rats (185). Vitamin D is a steroid hormone with many effects. One of the most important roles of vitamin D is to maintain skeletal calcium homeostasis. This regulation is mediated by promoting calcium absorption in the intestine, by increasing the number of osteoclasts to promote bone resorption, and allowing proper functioning of parathyroid hormone to maintain serum calcium levels within narrow limits (186). Vitamin D3 (cholecalciferol) is primarily produced from 7-dehydrocholesterol in the skin by exposure to sunlight, only a small amount comes from dietary sources or supplements (187). The formation of the active vitamin D metabolite, $1,25(OH)_2D$ or calcitriol, requires a two-step hydroxylation, the first step takes place in the liver with the formation of 25(OH)D which is the major circulating form of vitamin D. 25(OH)D is biologically inactive, and must undergo a second hydroxylation in the kidneys where $1,25(OH)_2D$ is formed (188), figure 10. Increased PTH secretion stimulate and enhance $1,25(OH)_2D$ production. $1,25(OH)_2D$, in turn, inhibits the synthesis and secretion of PTH, providing a negative feedback regulation

of 1,25(OH)$_2$D (189). The levels of vitamin D are associated to the immune response and may have protective effects against CVD, cancer and infections (190). Vitamin D deficiency can result in lower bone mineral density and an increased risk of reduced bone density (osteoporosis) or bone fracture (191).

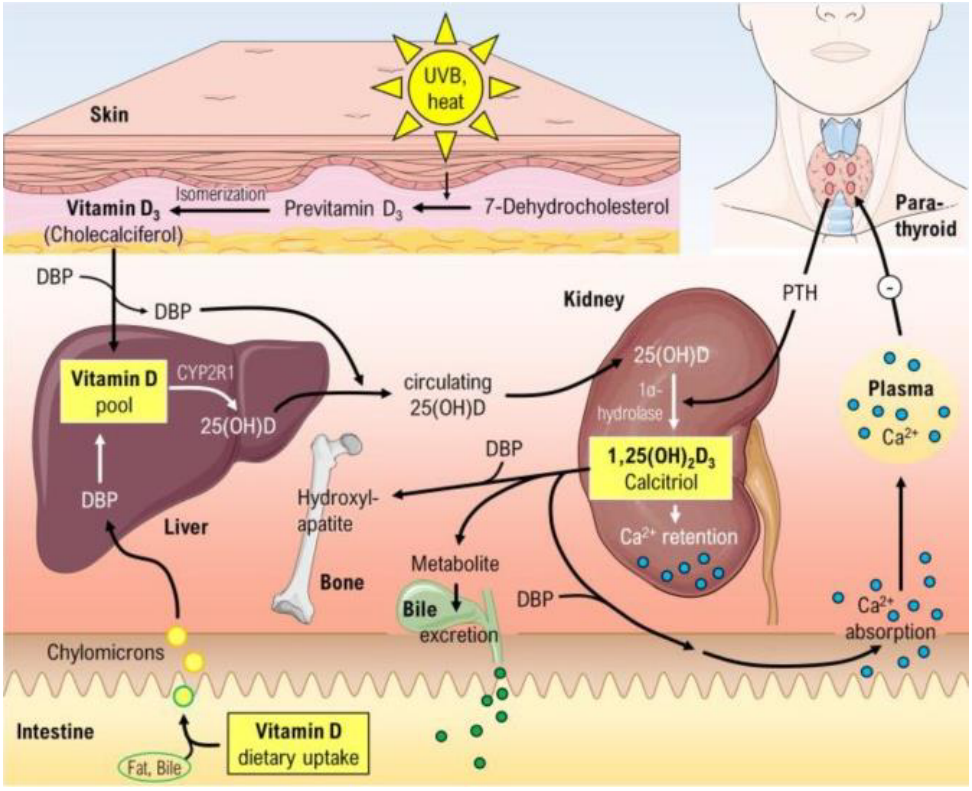



**Figure 10.** Overview of the vitamin D metabolism. 25(OH)D, Calcidiol; 1,25(OH)$_2$D$_3$, Calcitriol; CYP2R1, cytochrome P450 2R1; DBP, Vitamin D binding protein. *Reprinted with permission from MDPI (Open Access) (192).*

Serum levels of 25(OH)D is regarded as a marker of vitamin D body stores, and is used to evaluate vitamin D status (193). What represents an optimal 25(OH)D level is controversial. Levels <25 nmol/l are in the general population associated with muscle weakness, bone pain, fractures and elevated PTH levels. The Institute of Medicine (IOM) concluded in their 2011 report that a level >50 nmol/l would protect 97.5% of

the population against clinical outcomes like fractures (194, 195). The KDIGO guidelines suggest using the same treatment strategies recommended for the general population on CKD patients (184). There is an ongoing debate whether 50 nmol/l is sufficient in both the general population and in CKD patients in particular. The second step in the formation of active vitamin D takes place in the kidneys and this process is disturbed in patients with CKD (196). Many studies use different reference ranges, so care must be given when considering the results.

Solar UV-B radiation is the major source of vitamin D in humans, and vitamin D status is influenced by season, latitude, time spent outdoor and exposure to sunshine. Concerns about skin cancer and increased focus on sun protection have influenced vitamin D levels in the general population (194). This is especially applicable in kidney transplant recipients who are recommended to be careful with sun exposure due to increased risk of skin cancer because of their immunosuppressive treatment (197).

A large number of observational studies indicate that vitamin D has a number of effects beyond the regulation of mineral metabolism. The VDR was discovered in 1969, and it was early established that the VDR is a cell nucleus-localized receptor. The almost universal presence of VDRs in the body, figure 11, may explain why 25(OH)D deficiency is associated with numerous diseases such as diabetes (198), CVD (199), cancer (200), respiratory tract infections including COVID 19 (201) and overall mortality (202).

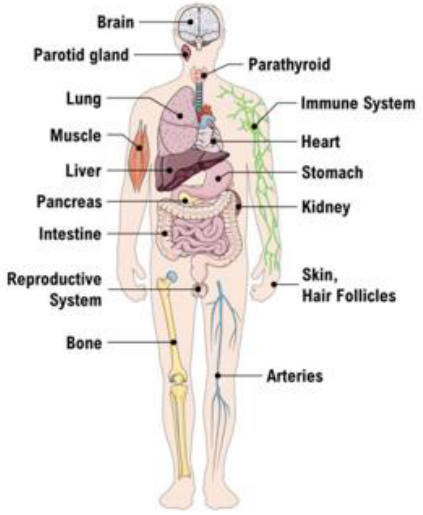



**Figure 11.** Tissue distribution of the Vitamin D receptor. *Reprinted with permission from MDPI (192)*

On one hand, supplementary vitamin D has not shown a protective effect against the development of skin cancer (203), CVD, death, or stroke (204). On the other hand, it

is suggested that vitamin D has renoprotective abilities (205). In mice, it has been shown that vitamin D supresses the renin–angiotensin–aldosterone system (RAAS), and attenuates RAAS-induced fibrinogeses in the kidneys (206). Vitamin D has been shown to reduce glomerulosclerosis and proteinuria in animal models (207) and in human studies on patients with IgA (208) and diabetes nephropathy (209). There is an ongoing debate on vitamin D and extra-skeletal health, and weather it is a causality or just an association. Observational studies do not meet the criteria to establish a cause- and effect relationship, and low levels of vitamin D may reflect bad news or poor health in general (210).

*Phosphate*

Phosphate homeostasis is maintained by a balance between dietary intake, intestinal absorption, mobilization from bone and renal excretion (211). Phosphate regulation is coordinated by a complex crosstalk between bone, intestines, kidneys and parathyroid glands. Dysfunction in this system leads to an increased risk of CV morbidity and mortality (212). Hyperphosphatemia occurs when eGFR declines, and controlling phosphate load by reducing dietary phosphate intake is an important early intervention in the treatment of CKD (212). In early CKD, phosphate levels are normal due to increased fractional excretion of phosphate in the urine. Recently it has been shown that high dietary phosphate load in early CKD increases phosphaturia, which in turn leads to a faster progression of CKD. This acceleration in CKD progression might be mediated through renal tubular injury (213). To maintain normal levels of phosphate in early CKD, elevated FGF23 levels are necessary to counteract phosphate retention and to reduce phosphate reabsorption (155). High phosphate levels have been shown to increase blood pressure in subjects with (214) and without CKD (215), suggesting that high phosphate levels may contribute to hypertension in CKD patients as well as in the general population. Hyperphosphatemia is associated with LVH (216). High phosphate triggers vascular calcification through a complex process where vascular smooth muscle cells (VSCMs) synthezise increased levels of collagen, leading to deposition of collagen-rich extracellular matrix which increase vascular tissue mineralization (217, 218).

*Collagen markers*

Type I and III collagens are the major structural proteins of the extracellular cardiac matrix in the human heart (219). Amino terminal propeptide of type I procollagen (PINP) and amino terminal propeptide of type III procollagen (PIIINP) are cleaved from procollagen type I and III in the synthesis of collagen type I and III, and they may be used as markers of this process. C-terminal telopeptide of type I collagen (CITP) is a marker of collagen type I degradation, and it increases shortly after MI (220), the increased levels are maintained beyond the subacute healing phase (221).

Connective tissue growth factor (CTGF) is a member of the CCN family of matricellular proteins (222). CTGF is a key signaling and regulatory molecule involved in several different processes, both biological processes such as cell proliferation, wound healing, angiogenesis, and pathological processes such as tumor development and tissue fibrosis (223). CTGF has also been found to be a potential independent predictor of ESRD (224). The underlying mechanisms of CTGF are not completely understood, but the accepted theory is that interactions between different domains in CTGF and other regulatory proteins contribute to the many effects (223).

Transforming growth factor beta (TGF-β) is a multipotent growth factor belonging to the transforming growth factor superfamily (225). TGF-β is found in all tissues, but is particularly abundant in bone, lung, kidney and placental tissue. TGF-β is produced by many parenchymal cell types (226). TGF-β is a multifunctional peptide with effects on cell differentiation, proliferation, apoptosis and matrix production. TGF-β also antagonize many immune responses, and is a potent negative regulator of immune responses (227). TGF-β can be a proto-oncogene and a tumor suppressor, depending on cell context and tumor stage (228). Renal fibrosis is characterized by excessive deposition of extracellular matrix that replaces functional renal parenchyma and leads to CKD. Elevated TGF-β levels is highly associated with the progression of renal fibrosis (229). TGF-β is also involved in healing after MI, where TGF-β inhibits the expression of FGF23 in cardiomyocytes in the healing phase allowing the scar to mature faster (230).

**Chronic kidney disease- mineral bone disorder**

In 2005, the term chronic kidney disease- mineral bone disorder (CKD-MBD) was introduced to describe a broad clinical syndrome that developes as a systemic disorder of mineral and bone due to CKD. The syndrome is manifested in bone and mineral laboratory abnormalities, histological bone disease, and calcification of vasculature and other soft tissues (231, 232). Several organ systems are involved in the mineral metabolism, including the kidneys, the intestine, the skeleton and the parathyroid glands. Disturbances in the mineral metabolism develop early in CKD (184, 233), and becomes gradually more prominent as eGFR declines towards ESRD (234).

The recognition of mineral bone disorder (MBD) as a risk factor for premature mortality and CVD began with observations about risks related to high phosphate and high calcium (235). For many years, arterial calcification in CKD patients was considered a result of high calcium levels and high calcium phosphate ion products resulting in super saturated plasma (236). Newer studies have shown that arterial calcification is a regulated process where plasma constituents maintain minerals in solution and inhibit their deposition in tissues (237). Many proteins involved in bone metabolism can be expressed in arterial tissues, reflecting changes in the phenotype of vascular smooth muscle cells (VSMCs) (238, 239). When exposed to high phosphate concentrations in culture, human VSMCs differentiate towards osteoblast-like cells (240). Arterial calcification is considered an active process with similarities to bone formation, supporting CKD-MBD as a clinical syndrome (241). Calcium and phosphate disturbances in ESRD are associated with renal osteodystrophy, and an inverse relationship between arterial calcification and bone density/ turnover is seen in uremic patients (242).

Before the discovery of the new biomarkers Klotho and FGF23, the sequential changes CKD-MBD were believed to be the result of decreased functional renal tissue with a reduction in $1,25(OH)_2D$ production and phosphate retention. The ability to filter and excrete phosphate is progressively compromised when kidney

function declines, starting in CKD stage 3, requiring a higher fractional excretion of phosphate in the urine to maintain balance. The fractional excretion of phosphate is controlled by FGF23 and PTH (243). The reduction in vitamin D levels result in a reduced calcium uptake from the intestine and increased PTH levels. The secretion of PTH increases when kidney function declines as a compensatory response to deteriorating mineral metabolism (high phosphate, low $1,25(OH)_2D$, low calcium). PTH levels start to increase when eGFR falls below 45 ml/min/1.73m² (233). This clinical entity is SHPT, and includes the biochemical characteristics of CKD-MBD (244).

Elevation in FGF23 has been found to precede the reduction in vitamin D levels, and to be the strongest independent predictor of reduced active vitamin D levels (155, 159). An increased level of FGF23 has been suggested as the main trigger for MBD, and a sequence has been postulated in which a reduction in Klotho levels precede changes in FGF23 and PTH levels, figure 12 (136).

As FGF23, and then PTH rise in CKD, they exert opposing effects on vitamin D metabolism, PTH stimulates 1-α-hydroxylase, and FGF23 inhibits it (159, 245). FGF23 acts directly on the Klotho-expressing parathyroid glands where it inhibits the production of PTH (146). Rising FGF23 creates a state of vitamin D deficiency that lowers calcium and also elevates PTH (152, 243). In the kidney, PTH increases calcium reabsorption (246). Adding to this physiological spiral in CKD is the reduction in the expression of α-Klotho, the co-receptor of FGF23 (247). It has also been suggested that CKD is a state of Klotho deficiency (247).

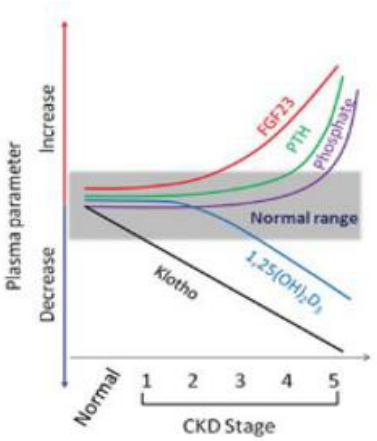



**Figure 12**. Proposed time profile of disturbances in mineral metabolism and prosphate-regulating hormones with CKD progression. Normal range in the grey box. The x-axis represents decline in kidney function. *Reprinted with permission from Oxford University Press (245)*

**Renal osteodystrophy**

From the 1940s the term renal osteodystrophy has been used to describe the abnormalities in bone morphology that follows CKD: osteitis fibrosa, osteomalacia, osteoporosis and osteopetrosis (232, 248). Renal osteodystrophy is an important cause of morbidity and reduced quality of life in patients with CKD. Osteoporosis is a term used to describe fragile bones in the general population, and is evaluated with dual x-ray absorptiometry (DEXA). Renal osteodystrophy is used to describe fragile bones prone to fractures in patients with CKD. Renal osteodystrophy comes from a combination of bone turnover (assessed by bone biopsy), bone density (assessed by DEXA) and bone architecture (249). To diagnose renal osteodystrophy a bone biopsy is the gold standard. This is not used widely in clinical practice due to its invasiveness and lack of interpretation abilities (231, 248).

The main component in renal osteodystrophy is the abnormal bone turnover. Bone disease with high turnover (or osteitis fibrosa) is associated with hyperparathyroidism, and bone disease with low turnover (or adynamic renal osteodystrophy) is associated with PTH-resistance or oversuppression of PTH release (250). Adynamic renal osteodystrophy is most prevalent, and is seen in CKD stages 3-5 and in dialysis populations (250, 251). The abnormal bone turnover starts early in CKD, and there are many variables to consider such as age, sex, race, the use of steroids, nutritional status and time with CKD (249).

Renal osteodystrophy is associated with increased levels of PTH, and PTH levels have been used as a surrogate marker of bone turnover, together with levels of calcium, phosphate and alkaline phosphatase to diagnose, treat and monitor renal osteodystrophy (231). The specificity of PTH as an indicator of bone health has been questioned, especially in the early days due to the lack of specificity in the many assays used (249).

After the introduction of CKD-MBD as a clinical entity, renal osteosystrophy was reserved for the bone histological features included in CKD-MBD.

**Mineral metabolim after kidney transplantation**

A successful kidney transplant restores kidney function and alters many of the abnormalities of MBD developed in the course of CKD prior to transplantation. It is debated whether the changes are completely reversed or just altered (252). The reality is that biochemical parameters of mineral metabolism often remain impaired in kidney transplant recipients, with persistent hyperparathyroidism, hypophosphatemia and hypercalcemia (252).

A decrease in serum calcium is usually seen immediately post-transplant. This is most likely due to the abrupt cessation of calcium or vitamin D supplements together with the reduced ability to mobilize calcium from bone as a result of PTH reduction (253). The hypocalcemia is usually mild. The "normal" kidney transplant recipient presents with calcium levels that increase to a plateau after 3-6 months before slowly decreasing (253). However, severe hypocalcemia and hungry bone disease may be seen after parathroidectomy (254). Hypercalcemia is present in 13% of long term kidney transplant patients with eGFR >45 ml/min/1.73m$^2$ (255). Severe hypercalcemia can cause AKI in the graft because of vasoconstriction and reduced graft perfusion (254). In most cases, the hypercalcemia is mild and asymptomatic, and can be managed with adequate fluid intake and avoidance of medications that increase calcium levels, such as tiazid diuretics, calcium supplements and vitamin D supplements in cases with adequate vitamin D levels (254).

Hypophosphatemia is observed in up to 90% of transplanted patients shortly post-transplant. The lowest levels are shown after about 1 month, after which the levels return to normal (256). The hypophosphatemia is usually self-limiting and asymptomatic. Hyperphosphatemia may be seen in patients with delayed graft function (DGF), or in transplant recipients with severely reduced graft function and a low eGFR post-transplant (254).

In patients with ESRD, FGF23 levels may be up to 1000 times higher than in healthy individuals (156), and the levels are reduced by >95% immediately post-transplant. Studies have demonstrated that FGF23 levels decline to levels comparable to CKD

patients matched with the same eGFR within 1 year post-transplant (256-258). Studies have found reduced FGF23 levels as early as the fifth (259) and seventh (260) day post-transplant, suggesting a renal elimitation of FGF23. Another study on 18 kidney transplant recipients found that FGF23 levels decreased by 89% from before transplantation to 3 months post-transplant, FGF23 levels remained stable throughout the rest of the study period which remained until 1 year post-transplant (257). One study on 52 kidney transplant recipients found that FGF23 levels were elevated before transplantation, but decreased to normal levels within 3 months post-transplant as eGFR normalized. Vitamin D levels remained low and PTH levels remained elevated post-transplant (258). However, one study reported elevated FGF23 levels 3 months post-transplant (261), and other studies have showed persistently elevated FGF23 levels in transplant recipients 1 year post-transplant (256, 262). Even in long term kidney transplant patients (>10 years post-transplant) with a well-functioning graft the levels of FGF23 have been found increased compared with normal controls (263). Elevated FGF23 is associated with decreased post-transplant eGFR (264, 265), increased risk of graft loss (158), as well as cardiovascular (266) and all-cause mortality (158, 266) in the long-term kidney transplant recipients.

One study found that Klotho levels decreased initially post-transplantat in the post-operative phase, with a gradual increase over the next 12 months of follow-up. Klotho levels were highest at 12 months post-transplant, with a higher average than before transplantation (262). Another study found that Klotho levels at 7 days and 1 month post-transplant were similar to Klotho levels pre-transplant, but the levels started increasing at 4 months post-transplant (260). Klotho levels after ≥1 year post-transplant was significantly lower in transplant recipients when comparing with healthy controls, but their eGFRs were also differing (267). Another study on long term kidney transplant recipients (>10 years post-transplant) with a well-functioning graft found a non-significant decline of Klotho (263), this could be an expected decline due to 10 years of ageing in the long period of follow-up.

Reduced serum levels of vitamin D are prevalent in CKD patients and in kidney transplant recipients (233, 257, 258, 268). Most renal transplant recipients have overt

vitamin D deficiency, a condition that may be associated with DGF (269), decline in graft function (190), and vitamin D levels usually remain low for a prolonged period post-transplant (270). Vitamin D defeciency is found in both summer and winter time, and seasonal variation does not explain the deficiency (271, 272). Many transplant recipients benefit from vitamin D supplementation if serum levels are low.

SHPT in advanced CKD results from multiple stimuli, including hypocalcemia, hyperphosphatemia, low vitamin D levels and skeletal resistance to PTH, which in turn results in continuous stimulation of PTH synthesis and secretion (254). The cell turnover in a normal parathyroid gland is very low, the VDRs, CaSr, FGF23 receptors and Klotho receptors are downregulated in diffuse or nodular hyperplastic parathyroid glands. An increase in gland mass prior to kidney transplantation is therefore believed to regress very slowly, and may partially explain why hyperparathyroidism, in some transplant recipients, persists for years. Another possibility is the development of a parathyroid adenoma prior to transplantation, PTH levels will remain high for a long time, and parathyroidectomy may be indicated (255, 273). This was confirmed in a histological study where the parathyroid glands of transplant recipients with persistent SHPT were examined. The study revealed that SHPT post-transplant depends on the pattern of hyperplasia before transplantation. They found restoration of VDR and CaSR expression, and with diffuse hyperplasia there was a strong tendency for SHPT-regression. In recipients with at least one nodular hyperplastic gland, they found very low SHPT reversibility, and surgical intervention should be considered (183). Elevated levels of PTH is seen in as many as 25-60% of transplant recipients after 1 year (274-276). A norwegian study found elevated PTH levels in 52% of kidney transplant recipients with normal kidney function >1 year post-transplant (255). Studies suggest that hyperparathyroidism is associated with vasoconstriction and tubulointerstitial calcification, which again could lead to graft loss (275, 277).

CKD results in renal osteodystrophy, and transplant recipients are, as mentioned earlier, at risk of bone disease before transplantation. In the first 12 months post-transplant the medication is intense, and the bone loss is the most pronounced (254,

278). If kidney function is normalized, markers of bone turnover and DEXA scans may return to normal levels with time (279), but the risk of hip fractures is still increased (280). A bone biopsy study, in transplant recipients with good graft function and minimal doses of corticosteroids, found that there was an elevated incidence of alterations in bone turnover when measured 2-5 years post-transplant (281). As kidney transplant recipients grow older, the long-term influence of immunosuppressive medication together with age related osteopenia and osteoporosis dispose kidney transplant recipients to bone related disease, and this should be addressed regularly by the nephrologist following the patient (254).

**Mineral metabolism after kidney donation**

Living kidney donors provide a unique setting to evaluate the effects of nephrectomy in otherwise very healthy indivuduals. The short-term risk of kidney donation is well established, with a 0.03% risk of mortality and less than 1% risk of morbidity (86). Immediately following a unilateral nephrectomy, renal blood flow increases by approximately 40%. This is associated with glomerular hypertrophy and an increase in renocortical volume. Adaptive hyperfiltration by the remaining kidney is maintained at a constant level for at least 6-8 years after donation (282, 283), and donors usually present with a rapid increase in GFR after donation (284), before stabilizing after a few months (285).

The question of changes in the mineral bone metabolism after living kidney donation is a matter of debate, and the results are somewhat concerning (286). In the first days after nephrectomy, results may be influenced by postoperative changes, iv hydration and post-operative inflammation. Within the first few days, donors present with transient dilution hypocalcacemia and SHPT. Urinary phosphate resorption decreases (287).

In a more chronic stage, 6-12 months post kidney donation, donors have lower eGFR and I,25 (OH)D compared with pre-donation levels (287, 288), with unchanged 25(OH)D (287). PTH levels increase (289), and one study showed 23% higher PTH at 6 months post donation. Higher PTH results in decreased plasma phosphate levels and renal tubular reabsorption of phosphate, the same study presented 5.4% lower phosphate 6 months after donation (290). In a study on 9 donors and no controls, FGF23 levels remained unchanged 6 months after donation (291), but in another study on 21 donors, FGF23 was increased after 1 year (288). In an even larger cohort of 198 individuals, FGF23 and PTH were elevated 5 years post donation (292). One study found a significant reduction in Klotho post kidney donation, but there was not a similar increase in the kidney recipients. The patients were only followed for 5 days post operatively (293). Another study found a rapid drop in Klotho day 1 post donation, and Klotho levels remained lower than baseline at 1 year post follow-up

(294). Different studies have shown circulating Klotho levels that were lower than pre-donation, but higher than immediately post donation at 6 and 12 months after donation (287, 288).

The understanding of long-term adaptations to kidney donation is still under investigation, and living kidney donors are essential to keep the waiting lists for a kidney graft under control. Healthy kidney donors after donation is key when recruiting potential donors, and long-term follow-up is necessary as a surveillance tool to establish the potential long-term health risks for the donors. The results from this follow-up will influence the selection and screening process initiated before kidney donation, which is essential to maintain living kidney donation as a safe first-choice for patients with ESRD (295).

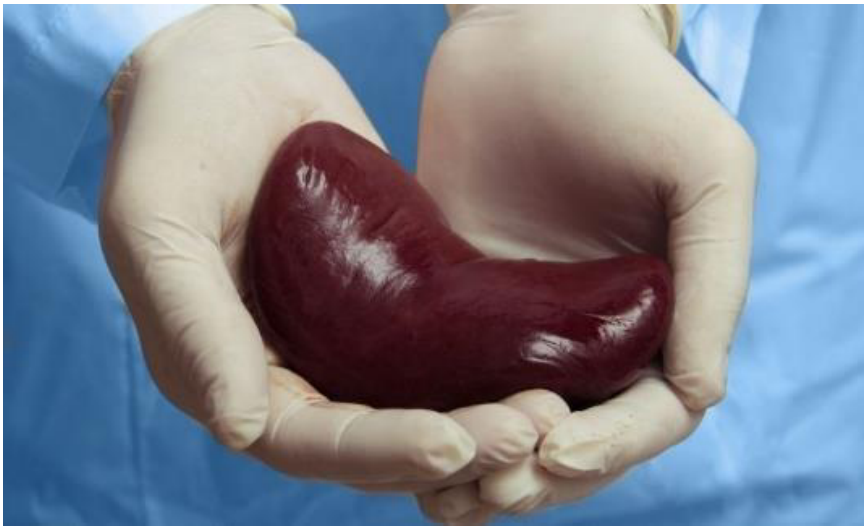


**Figure 13.** A kidney from a living kidney donor being transplanted. *Published with permission from Dr. Francisco Salcido-Ochoa, www.franciscokidneycentre.com*

**Cardiovascular disease**

CVD is a general term for diseases that involve the heart and/ or blood vessels. It is usually associated with a build-up of fatty deposits inside the arteries and an increased risk of blood clots. The traditional risk factors for CVD are smoking, high blood pressure, high cholesterol, diabetes, inactivity, obesity, a family history of CVD, age and male gender. The main types of CVD are coronary heart disease, stroke and transitory ischaemic attacks, peripheral arterial disease and aortic disease. Coronary heart disease occurs when the flow of oxygen-rich blood to the heart muscle is blocked or reduced. This puts strain on the heart, and can lead to angina, MI or heart failure. According to the WHO, CVDs are the number 1 cause of death globally; more people die annually from CVDs than from any other cause of death (296).

**CVD in CKD**

CVD and CKD are closely interrelated, and disease in one organ system causes dysfunction of the other, often leading to failure of both organs. Many studies focus on CVD in patients with ESRD, but recently large studies have demonstrated that CVD risk increases very early in the history of CKD, probably as early as eGFR <75 ml/min/1.73m$^2$ when creatinine levels may be within the normal range (13). It is shown that there is an inverse relationship between eGFR and the hazard rate of CV events (297), figure 14.

CVD is the most common cause of death in patients with CKD (14). 50% of all patients with CKD stages 4 and 5 have CVD (298), and CV mortality accounts for 40-50% of all deaths in patients with CKD stages 4 or 5, compared with 26% in controls with normal eGFR (14, 299).

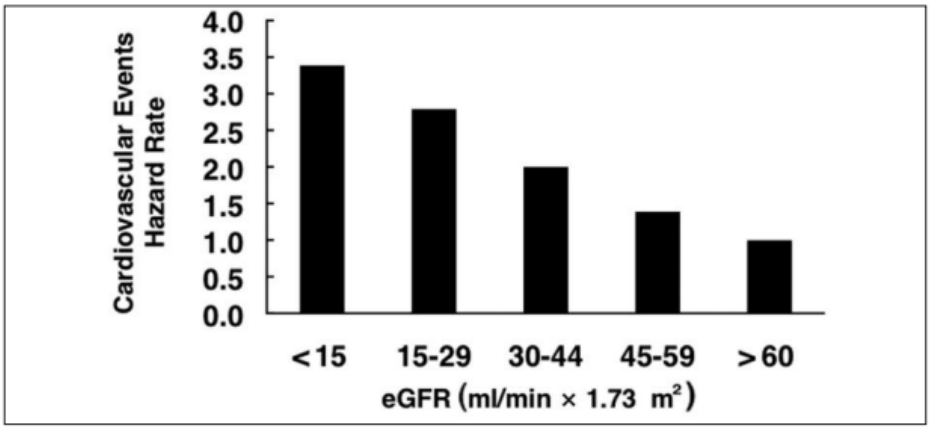



**Figure 14.** The inverse relationship between eGFR and hazard rate of CV events
*Reprinted with permission from Eur Rev Med Pharmacol Sci (297).*

The risk of CVD is higher than the risk of developing ESRD in patients with CKD, and CKD should be considered an important risk factor for CVD (14, 300). CV risk is increased both by decreased renal function and albuminuria (301), and albuminuria is a marker of CV risk and mortality independent of eGFR (302). Early detection of proteinuria, leads to more aggressive intervention, which may reduce CV risk (303). The 2021 ESC guidelines in CVD prevention added albuminuria to renal function as a CV risk stratification (304), as a tool to better identify patients that should be treated with RAAS inhibitors and more recently SGLT2 inhibitors (305).

CKD patients often presents with the traditional CVD risk factors of age, hypertension, diabetes mellitus and hypercholesterolemia. These traditional risk factors are common, but they do not explain the increased risk of CVD in CKD. Standard CVD-modifying drugs like statins have, unfortunately, failed to improve CV mortality in CKD as much as they do in the general population (306). This suggests that there might be other explanatory mechanisms for CVD in patients with CKD. Arteriosclerosis, arterial stiffening and abnormal cardiac structure appear to dominate, and CKD patients are influenced by additional “CKD-risk factors” like anemia, mineral metabolism abnormalities, proteinuria, malnutrition, volume overload, inflammation, high phosphate and time in dialysis (307, 308).

In the 1980s and 90s, echocardiographic studies identified common changes in cardiac structure and function in patients with ESRD. The changes included increased LV mass, LVH, LV dilatation, LV systolic dysfunction and myocardial fibrosis. These changes are termed uremic cardiomyopathy, and the severity of uremic cardiomyopathy as measured by LV mass is a powerful predictor of CV mortality (309, 310). These structural changes appear early in the course of CKD (311).

Myocardial fibrosis has been suggested as the main cause of uremic cardiomyopathy, and in the 1990s a post-morten study found myocardial fibrosis in 91% of patients with ESRD without significant coronary lesions. The amount of fibrosis correlated with time in HD (312). In the early 2000s another study comparing myocardial biospies in HD patients with reduced left ventricular ejection fraction (LVEF) and other patients without CKD but with cardiomyopathy found high levels of myocardial fibrosis and cardiomyocyte hypertrophy in the dialysis patients. The amount of fibrosis was a prognostic marker of CV mortality within 3 years (313). Studying myocardial fibrosis in ESRD is challenging, since myocardial biopsies are not without risk, especially in patients with ESRD and comorbidities (314).

Arterial calcification is a common complication of CKD, and the presence and extent of arterial calcifications are independently predictive of CVD beyond conventional risk factors (241). Vascular calcification is an active cell-regulated process in which ectopic deposition of calcium-phosphate salts occurs in blood vessels or cardiac valves (212).

Media calcification is associated with stiffening of the blood vessels, and it occurs in arteries of any size (212). Media calcification is associated with ageing in the general population. It is significantly more common in patients with metabolic disorders, such as metabolic syndrome, CKD or diabetes mellitus (237, 241). Media calcification may be called media sclerosis or Mönckeberg's media sclerosis, named after Möckenberg, a German pathologist who first described the non-atherosclerotic pattern of calcification (240). Media calcification is organized along the elastic lamellae, and is associated with VSMCs (238, 315) which makes up the majority of

the cells in the media. In uremic conditions, VSMCs are reprogrammed into distinct osteoblast-like cells with a secretory phenotype, secreting typically bone-associated proteins (316, 317). As a result, the CV calcifications seen in CKD are accelerated, and even children with CKD may have significant vascular calcifications (318). High phosphate levels also promote media calcification and stiffening of arterial walls (212), contributing to hypertension and LVH (240), figure 15.

Intima calcification is calcification of the innermost layer of vasculature (212). Intima calcification is a progressive feature of atheriosclerosis that occurs when minerals are deposited within atheriosclerotic plaques in the intima of the arterial wall (237). It is associated with the development of plaques and occlusions. It is frequently seen with age, hypertension, diabetes, dyslipidemia and smoking in the general population. It is a discontinuous process that involves the endothelialium, as well as monocytes, macrophages and VSMCs in lipid-rich regions (315), figure 15.

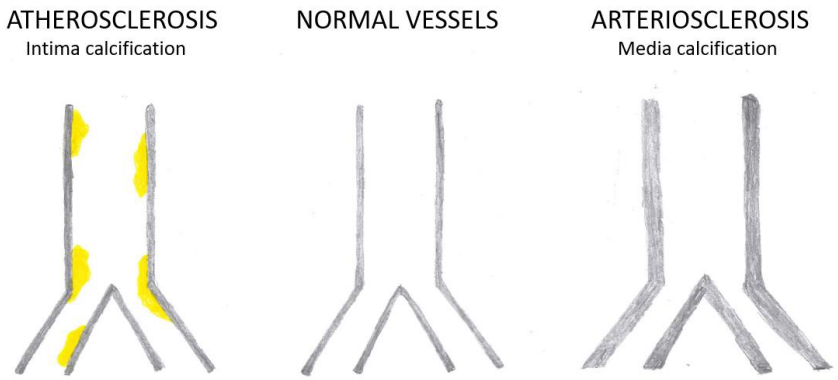



**Figure 15.** Schematic illustration of vascular calcification showing the difference between intimal and medial calcification. Normal vessel in the middle. *My own illustration.*

Atherosclerosis is an inflammatory process where the immune system interacts with risk factors to initiate, proliferate and activate arterial plaques (319). Stable plaques are characterized by a chronic inflammatory infiltrate, and vulnerable plaques are characterized by an active inflammation involved in the thinning of the fibrous cap, predisposing the plaque to rupture (320).

As recently as 20 years ago, vascular calcification was viewed as a passive process with deposition of calcium and phosphate, where high levels was a disposing factor (321). More recently, it has been established that vascular calcification is an actively regulated, multifactorial process involving several factors in the so called “uremic milieu” as well as the traditional risk factors (322, 323).

A combination of media and intima calcification is seen in CKD patients, but in young CKD patients the calcification is almost exclusively medial (237). In clinical care, the two forms of calcification are indistinguishable, and general CVD-prevention is advocated to CKD patients just like to the general population (stop smoking, physical activity, treatment of obesity, controlling lipids, blood pressure and glucose levels). In addition, controlling calcium and phosphate levels, avoiding oversuppression of parathyroid activity and adynamic bone are important preventive measures (237). Patients with CKD often have severe calcification in their arterial vascular tree, figure 16.

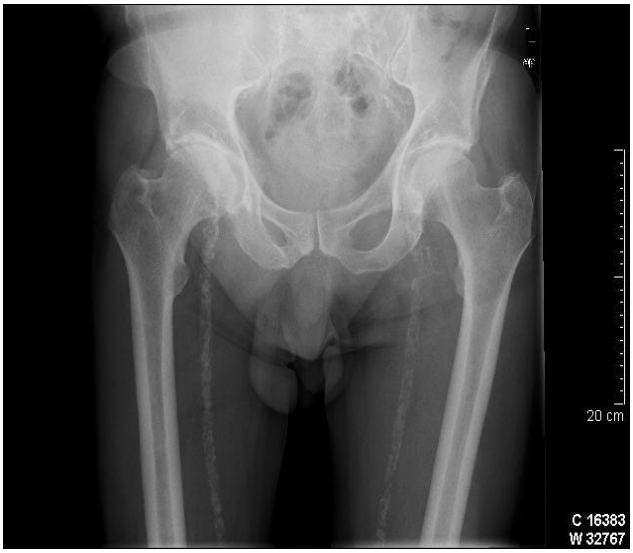



**Figure 16.** X-ray showing extensive diffuse calcification of the arterial vascular tree in both lower limbs. *Archive photo.*

A severe form of vascular calcification in CKD is uremic calcific arteriolopathy, or calciphylaxis, caused by calcium deposits in the dermo-hypodermic arterioles (324). Calciphylaxis leads to necrosis of the skin, and has a high mortality rate (325). The mechanicm behind it is unclear, but high calcium-phosphate product in combination with an active cellular process is the most likely explanation (326).

Calcification of the heart valves are also common in CKD, and the rate of progression is often very fast. There is an association between high phosphate levels, hypertension, hyperparathyroidism and hyperlipidemia and the development of aortic stenosis (327). The most common heart valve requiring intervention is the aortic valve (328).

LVH is present in 1 of 3 patients with CKD, and in 70-80 % of patients with ESRD (14). Patients with CKD have characteristic LVH changes in the myocardium with myocardial fibrosis, collagen deposits and cardiac hypertrophy which together represents uremic cardiomyopathy (329). Many factors contribute to LVH in CKD,

such as systolic hypertension, arterial stiffness and LV overload and volume overload (329, 330).

In addition to the structural changes seen in CVD in CKD, altered mineral metabolism contribute to increased CV mortality. The risk factors include dysregulated phosphate metabolism, hyperparathyroidism, low vitamin D levels, elevated FGF23 levels and reduced Klotho levels (143). Recently, mineralcorticoid receptor (MR) activation has emerged as an additional mediator for CV injury and CKD progression (331).

High FGF23 is associated with poor clinical prognosis, and adverse events such as all-cause and CV mortality, CV events and heart failure (156, 332-336) in the general population, as well as in patients with CKD (337). FGF23 has a complicated relationship with its coreceptor Klotho, where Klotho deficiency increase FGF23, and high FGF23 exaberate Klotho deficiency via low vitamin D (247). To determine the exact role of Klotho and FGF23 in CVD is challenging, because Klotho primarily functions as a membrane-bound receptor in tissues which are not usually sampled in human experiments. Cleaved and alternatively spliced forms of Klotho circulate, but their role is not yet established (338). Current methods to measure circulating Klotho have significant limitations due to assays and sample instability, and it is difficult to draw any conclusions about Klotho's role in CVD due to differing results (139, 339). However, it has been shown that reduced Klotho is associated with the presence of coronary artery disease independently of other CV risk factors (340). Klotho expression is stimulated by VDR activators that bind to elements in the Klotho gene promoter that is vitamin D responsive (341). Klotho expression is suppressed by aldosterone, angiotensine II, FGF23, inflammation, oxidative stress, uremic toxins, hypoxia and TGF-β (342). Klotho reduces hypertension, have renoprotective abilities, attenuates tissue fibrosis, reduces CKD progression and attenuates vascular calcification (245, 342). The kidney is the main source of Klotho, and it is not surprising that Klotho levels are low in CKD patients (247, 343).

FGF23 is related to LVH and diastolic dysfunction (160, 335, 344, 345). In children on HD with preserved EF, elevated FGF23 was associated with lower left atrial global longitudinal strain, which is an early indicator of cardiac remodeling (346)

Experiments show that FGF23 elevate intracellular calcium levels in isolated cardiomyocytes as well as increase contractility in ventricular muscle strips from mice and primary cardiac myocytes (160). Klotho-expressing cells respond to FGF23 with activation of the Ras/ mitogen-activated protein kinase (MAPK) cascade (151). In myocytes, FGF23 activates FGFR4 and subsequently the PLCɤ/ calcineurin/ NFAT signaling pathway. This stimulus induce hypertrophic growth of cardiac myocytes (154, 160, 347), figure 17.

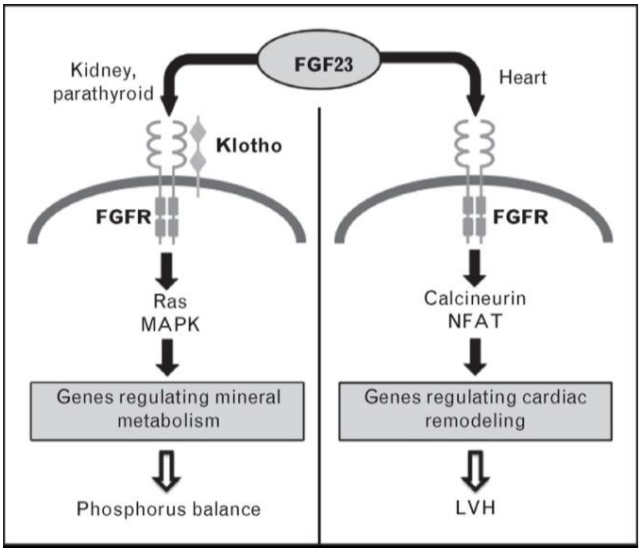



**Figure 17.** Schematic overview of FGF23 signaling in 'classic' target cells (kidney/ parathyroid) and cardiac myocytes. Left: In the kidney and parathyroid glands, FGF23 signaling requires FGFR and the coreceptor klotho. FGF23–klotho binding to FGFR stimulates autophosphorylation of the receptor tyrosine kinase and induces signaling mainly through the Ras/ MAPK pathway. Right: In the heart, binding of FGF23 to FGFR on cardiac myocytes stimulates receptor activation independent of klotho, which is not expressed in cardiac myocytes, and signals primarily through the PLCɤ/ calcineurin/ NFAT pathway. *Reprinted with permission from Wolters Kluwer Health, Inc.* (348).

These findings described above have been supported by animal models with elevated FGF23, such as FGF23 injections in mice, FGF23 injections in klotho deficient mice, high phosphate diet in mice, and renal ablation in rats, showing the development of LVH. Taken together, this supports that the heart may be capable of directly responding to circulating FGF23 (154, 160, 348, 349). Mice lacking FGFR4 do not develop LVH when injected with FGF23, but mice with high FGFR4 expression and normal FGF23 show increased PLCɣ/ calcineurin/ NFAT signaling and development of LVH, supports that FGF23/ FGFR4 signalling acts as a pro-hypertrophic signaling pathway in the heart (154).

A recent study on mice with cardiorenal syndrome, where mice were subjected to MI for 12 weeks to induce cardiorenal syndrome, showed that the elevated FGF23 levels caused by MI promoted renal fibrosis and decreased Klotho levels in the kidneys (350).

Another potential pathway contributing to LVH is the FGF23 effects on renal handling, where FGF23 upregulates the natrium chloride cotransporter in the distal tubule by promoting natrium reabsorption with volume expansion and hypertrophy (351). Mice exposed to mineralcorticoid deoxycorticosterone acetate (DOCA) displayed elevated FGF23 levels, hypertension and end-organ damage (352, 353). These changes were reversed by the administration of the MRAs spironolactone and eplerenone (353, 354).

Several CVDs such as hypertension, calcified heart valves and heart failure are associated with elevated PTH (355, 356). Increased PTH is also associated with increased myocardial electric vunerability, a trigger for cardiac arrhythmias (357). The sustained activation of the parathyroid glands in ESRD patients induces parathyroid hyperplasia, and elevated PTH levels lead to high phosphate and hypercalcemia, increasing the CV risk in these patients (358). Chronic hypercalcemia is related to calcification of cardiac valves and vessels (359). Parathyroidectomy has been shown to reduce the risk of arrythmias (360) and reduce cardiac hypertrophy (361) in patients with primary hyperparathyroidism. At the cellular level, PTH-

dependent pathways participate in pathophysiological processes involved in cardiomyocyte function, such as contraction, proliferation and apoptosis (362). Cardiomyocytes incubated with PTH show an increase in cellular contractions per minute and premature cell death (363). Both clinical and experimental studies suggest that PTH is associated with cardiac remodeling and hypertrophy implicating that high PTH has damaging effects on the heart (364). Hyperphosphatemia is an independent risk factor in the progression of coronary artery calcification in patients established in peritoneal dialysis (365).

Studies have shown that vitamin D have a role in cardiac remodeling (364). In a rat model of hypertension, treatment with paricalcitol (active vitamin D analog) inhibited the development of LVH, without influencing the blood pressure (366). In a similar model, paricalcitol has been used to prevent the progression of pre-existing LVH and the development of heart failure (367). In a different model, with surgically induced MI in mice, paricalcitol reduced the development of heart failure by reducing infarct size, inflammation and fibrosis (368). VDR also inhibits NFAT genes which can explain the cardioprotective properties provided by VDR activator administration (369). Many studies show an association between low vitamin D and CV events and mortality, but they fail to confirm the clear trends found in animal models. The studies show associations and cannot prove a cause-and-effect relationship (199, 370). In metanalysis and reviews it has been suggested that low vitamin D is a marker of poor health in general, as an alternative to a risk factor for CVD (371, 372).

Patients with CKD are in a state of “chronic inflammation”, where an inverse relationship between eGFR and inflammatory cytokines exist (373). This chronic inflammatory state also contributes to the increased morbidity and mortality seen in patients with CKD (295). In CKD patients without CVD manifestations (known coronary heart disease, heart failure, arrhythmias or severe hypertension), increased FGF23 and decreased Klotho levels were associated with cardiovascular remodeling, measured by echocardiography, pulse wave velocity (PWV) and calcification score (374).

After kidney transplantation the risk of cardiovascular events remain elevated, despite the partial or complete resolution of uremia-associated metabolic derangements (323). Studies have demonstrated progressive coronary arterie calcification and aorta calcification in stable kidney transplant recipients (375). Persistent hyperparathyroidism is associated with hypercalcemia (376) which induces vasoconstriction and vascular calcification (377). Low-grade systemic inflammation is an established complication to kidney transplantation, where the graft continuously stimulates the innate and the adaptive immune responses (323). This leads to production of inflammatory cytokines which increase VSMC differentiation leading to mineralization (378). Inflammation is intertwined with oxidative stress which also promotes vascular calcification (323, 379). Immunosuppressive drugs have a role in the calcification process due to their effects on carbohydrate and lipid metabolism. Steroids, CNIs and mTOR inhibitors are major determinants of post-transplant diabetes mellitus (380). mTOR inhibitors are linked to hypercholesterolemia (381). Experimental data suggest that immunosuppression directly affects VSCMs and endothelial cells by inhibiting NO synthase, which promotes atherosclerosis and induce VSMC calcification (382, 383). Kidney transplant recipients are also subjected to the traditional risk factors for CVD, including diabetes, hypertension, dyslipidemia and old age which contribute to vascular calcification (323). A recent study found an inverse relationship between bone mineral density and abdominal aortic calcification in kidney transplant recipients examined within 6 months post-transplant, supporting the existence of a bone-vascular axis post-transplant, which may help us target the increased risk of CVD post-transplant if addressed properly (384).

Aldosterone is important in blood pressure control and extracellular volume regulation. Aldosterone raises blood pressure by actions on the vasculature and central nervous system through MR- dependent and independent mechanisms (331). The MR is located in many tissues, endothelial cells, VSMCs, adipocytes, cardiomyocytes (385), as well as in the kidney's distal tubule, collecting duct, podocytes and mesangial cells (386). In diabetes and CKD, MR activation promotes detrimental effects including podocyte injury and albuminuria, reduced renal blood

flow and increased expression of proinflammatory cytokines, which can lead to progression of CKD (386-388).

Patients with primary aldosteronism have increased risk of developing CVD (389) and kidney disease (390), and these outcomes can be mitigated by MR antagonist therapy (389, 391). A recent study showed that serum aldosterone concentrations are inversely correlated with eGFR and proteinuria, and associated with progression of CKD (392). This was independent of diabetes, suggesting that MR agonist therapy should also be investigated as a renoprotective method in patients without diabetes (392).

In VSMCs the effect of MR stimulation by aldosterone is the triggering of an osteogenic cascade leading to calcification (393). Spironolactone has been shown to reduce this calcification in mouse models (394). Vascular calcification in CKD is also a result of reduced phosphate elimination followed by hyperphosphatemia and precipitation of calcium-phosphate (395). Hyperphosphatemia is associated with upregulated aldosterone synthase, which is the terminal enzyme in the aldosterone biosynthesis pathway, leading to elevated aldosterone levels as a result of high phosphate levels (394, 395). Aldosterone also upregulates bone expression of FGF23 (353), leading to additional effects on the heart, kidneys and vasculature (331).

Obesity is a traditional risk factor for CVD, and the incidence is increasing worldwide (396). Aldosterone levels are elevated in obese individuals, caused by a leptin-mediated upregulation of aldosterone synthase (397) contributing to hypertension. In a mouse model spironolactone reduced the leptin-mediated endothelial dysfunction (397). Leptin also stimulates FGF23 synthesis in bone in mice (398, 399), suggesting leptin as a possible FGF23 regulator in humans.

In conclusion, an intricate interaction between several factors interact to influence the development of CVD in patients with CKD.

**Methods to evaluate CVD in CKD.**

In the following section, the methods used to evaluate CVD in the studies included in the thesis are presented. Evaluations such as ecchocardiography and computer tomography of the coronary arteries have not been used in these studies and will not be presented in detail, even though they are important clinical tools in the evaluation of CVD.

*Pulse wave velocity (PWV)*

PWV is the velocity at which the blood pressure pulse propagates through the circulatory system. PWV is the gold standard as a non-invasive measure of arterial stiffness. Measurement of carotis to femoral PWV is recommended (400), and the measurement predicts future CV events and all-cause mortality independently of conventional risk factors (401). Calcification of central arterial vessels contribute to elevated PWV. PWV is recognized as an indicator of target organ damage and a useful additional test in the investigation of hypertension (402).

*Calcification propensity score* ($T_{50}$)

$T_{50}$ is a blood test evaluating the anti-calcifying buffering system capacity, where the ability to resist calcification by measuring the calcification inhibitory forces present in individual blood samples is tested. Short $T_{50}$ is consistent with accelerated transformation and low calcification resistance, whereas delayed transformation reflects intact calcification resistance (403).

*Coronary angiography*

Invasive coronary angiography is the gold standard in the diagnosis of coronary artery disease (404). The procedure access the coronary circulation of the heart using a catheter, and is used for diagnostic and interventional purposes. Through injection of contrast agent and illumination with x-rays, the procedure allows the recognition of occlusion, stenosis, restenosis, thrombosis or aneurysmal enlargement of the arteries (404). The patient is awake during the procedure. The phycisian has the ability to inflate a balloon or insert a device (stent) that will improve blood flow to the artery (404). In study 3, all patients presented with ST-elevation myocardial infarction (STEMI). Acute percutaneous coronary intervention (PCI) was performed according to international guidelines (405), the details of the PCI procedure itself with regards to choise of catheters, guidewires, balloons, stents and concommittant therapies were a clinical decision made by the operator.

Although individuals with CKD have worse prognosis in the setting of an MI, they are less likely to receive an early invasive strategy as recommended by international guidelines (406). Studies have shown that invasive management was associated with lower in-hospital mortality with both STEMI and NSTEMI in all patients with CKD when compared with a conservative strategy (407). When choosing an invasive strategy, measures to prevent AKI should be taken. Current guidelines recommend low-volume iso- or hypo-osmolar contrast materials during the catheterization of individuals with CKD. Adequate hydration before and after the intervention is also important to prevent AKI (408, 409).

*Cardiac MRI*

Cardiac magnetic resonance (CMR) is one of the best tools available for the assessment of myocardial function and damage following an MI. CMR visualizes LV-morphology and function with high reproducibility and accuracy (410). Contrast enhancement techinques allow the exact description of infarct size and shape, depth

and location. Large infarcts with prolonged obstruction in the infarct-related artery are characterized by dark, central zones surrounded by hyperenhanced regions, and reperfused infarcts with less dysfunction have uniform signal hyperenhancement (410, 411). CMR is accepted as the gold standard in assessment of LV-remodelling, due to CMR's 3D imaging technique and its ability to accurately define the deformed ventricle (412).

Contrast based CMR improves views of the heart muscle and blood flow, and uses gadolinium-based contrast agents (GBCAs), where the contrast is distributed in extracellular fluid. The contrast allows the visualization of scar tissue and oedema following MI. This is an accurate, non-invasive method that can visualize very small infarcted areas. Contrast-enhanced CMR is also used to evaluate diseases like cardiomyopathy and myocarditis (413). In an acute setting, contrast MRI in combination with cine MRI can be used to distinguish between acute MI (hyperenhanced and with contractile dysfunction), injured but viable myocardium (not hyperenhanced but with contractile dysfunction) and normal myocardium (not hyperenhanced and with normal function) (411).

Free gadolinium ions are toxic, and ligands are used to create chelates for safe use in humans. Based on the formula the GBCAs are classified as linear or macrocyclic (414). There has been concern regarding cerebral depositions after repeated GBCA adminitrations, with studies suggesting that linear compounds have higher likelihood of deposition than macrocyclic compounds. As a preventive measure, the European Medicines Agency suspended the marketing authorization for linear GBCA types (except for hepatic imaging) (415). At SUS, the contrast agent Dotarem is used for CMR, this is a macrocyclic compound.

Imaging studies using CMR have redefined uremic cardiomyopathy in CKD as a forsiktighetdistinct phenotype consisting of prognostically significant changes including LVH, left atrial dilatation, diastolic dysfunction and myocardial fibrosis. Initially, standard MRI contrast in the form of gadolinium was used in CKD and dialysis patients (416). Reports on the potential toxic effects of gadolinium, and the

association between gadolinium contrast and nephrogenic systemic fibrosis (NSF) in patients with decreased renal elimination were concerning (414). The risk for NSF led to the use of lower doses of contrast and lower-risk gadolinium agents (gadobutrol, gadoteridol, gadoterate). These measures appear to minimize the risk (417). Nevertheless, most nephrologists and radiologists consider eGFR <30 ml/min/1.73m$^2$ as a contraindication for an MRI with gadolinium constrast to avoid the risk of NSF (414), this is also the limit used by the radiology department at SUS for CMR.

Non-contrast native $T_1$ relaxation time is a viable alternative to the use of gadolinium contrast, and has been shown to correlate with cardiac fibrosis in histology samples (418). $T_1$ time reflects the longitudinal recovery time of hydrogen atoms following their excitation. At any given magnetic field strength, each type of tissue has a normal range of values. A significant variation from the normal range represents tissue pathology (419). CMR has a unique ability to provide a non-invasive examination of cardiac structure and function, arterial function, myocardial tissue characterization (T1 mapping and inversion recovery imaging) and myocardial metabolic function (spectroscopy), and is an ideal method to provide insights into the mechanisms leading to uremic cardiomyopathy (420).

Studies on patients with ESRD, showed significantly increased $T_1$ time suggesting that this represents cardiac fibrosis found in uremic cardiomyopathy (419, 420). A study on 134 predialytic patients with CKD stages 2-5 without myocardial ischemia with CMR found an inverse relationship between interstitial myocardial fibrosis (measured by native myocardial $T_1$ time) and eGFR. Exercise tolerance measured by stress echocardiography decreased, LV mass increased and serum biomarkers of fibrosis and heart failure increased as kidney function deteriorated (421). These findings support the hypothesis of very early onset of fibrosis in the development of CKD, as well as the hypothesis of diffuse myocardial fibrosis as a driver of myocardial dysfunction and uremic cardiomyopathy in CKD.

# 2. Aims and objectives

Overall objectives

Paper 1:

To establish the levels of NGAL, sKlotho, iFGF23 and vitamin D in patients with CKD stages 3-5, kidney donors and healthy controls.

Paper 2:

To explore the association between vitamin D status 10 weeks post kidney transplantation and long-term graft- and patient survival.

Paper 3:

To investigate the time-dependent changes in iFGF23 and the relationship between circulating iFGF23 levels and MI size, LV remodeling and systemic biomarkers of inflammation and collagen turnover following acute STEMI.

Paper 4

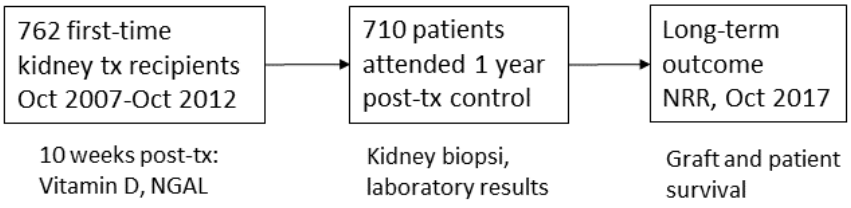
To explore the relationship between vitamin D, sKlotho and iFGF23 measured 8 weeks and 1 year post kidney transplantation and long-term graft- and patient survival.

# Overview over studies 1-4:

**Study 1**: NGAL, iFGF23 and sKlotho in long-term kidney donors

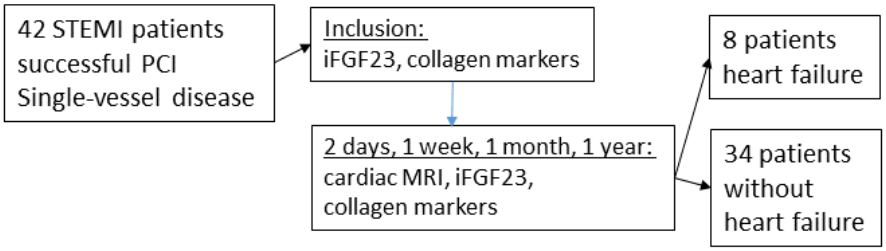

**Cross-sectional single-center study**

**Study 2**: Vitamin D as a risk factor for patient survival after kidney transplantation



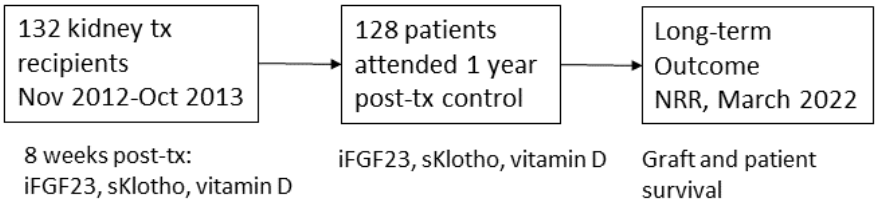

**National, prospective, observational cohort study**

**Study 3**: FGF23 and cardiac MRI findings in patients with acute first time STEMI




**Single-center observational study**

**Study 4**: Klotho and FGF23 are independent of vitamin D, and unlike vitamin D, are not associated with graft- and patient survival after kidney transplantion




**National, prospective, observational cohort study**

**CKD:** chronic kidney disease, **iFGF23:** intact fibroblast growth factor 23, **NGAL:** neutrophil gelatinase-associated lipocalcin, **NRR:** Norwegian Renal Registry, **tx:** transplantation,, **Vitamin D:** 25(OH)vitamin D

# 3. Materials and methods

**Patient populations and study design**

Paper 1:

All patients with CKD, kidney transplant recipients and kidney donors that are followed by the nephrology department at SUS are registered in a clinical database (Nephrobase) which is used in every visit to the department. To identify eligible patients to be includeed in Study 1, we did a search in Nephrobase for kidney donors with eGFR ≥60 ml/min/$m^2$. We included donors with normal eGFR to avoid confounding with regard to the biomarkers, and we included donors with a follow-up >5 years since nephrectomy (422).

We also included patients in CKD stages 3 and 4 in connection with planned appointments at the department in October and November 2013. Patients with CKD 5 were included from the HD unit. Patients with severe comorbidities and a limited life-expectancy, as well as previously parathyroidectomized patients were excluded to avoid confounding. Controls were recruited among collegues and friends who regarded themselves as healthy. Only age and gender were recorded. Patients and controls were included after giving written, informed consent (422).

Study one was a cross-sectional observational single-center trial. 35 kidney donors, 35 healthy controls, 22 patients with CKD stage 3, 18 patients with CKD stage 4 and 20 patients with CKD stage 5 were randomly included.

Paper 2:

All adult first-time single kidney transplant recipients in Norway from October 15, 2007-October 15, 2012 were included. Patients who received a combined transplantation with kidney and pancreas were excluded, as well as second, third and fourth time kidney recipients. We aimed to avoid possible influences by previous kidney grafts or complications due to the pancreas grafts. We ended up with a cohort of 762 first-time kidney recipients, figure 18 (423).

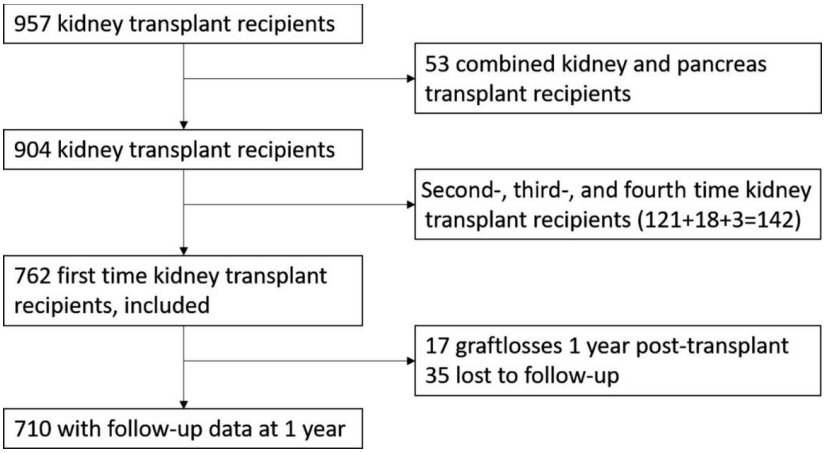



**Figure 18**. Flowchart showing the inclusion prosess in study 2. *Reprinted with permission from Wiley (423).*

Approximately 10 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) PWV investigation. Plasma and serum samples for analysis of 25(OH)D, NGAL, and $T_{50}$ were obtained and stored at −70°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 (423).

Patients with a functioning kidney graft 1 year post-transplant were invited to OUS, RH for clinical examination and a laboratory follow-up. At 1 year follow-up we have data on 710 of the patients. From 2009, a routine protocol kidney graft biopsy was included in the 1 year examination. Long-term outcomes were obtained from the NRR where annual data are collected on the entire Norwegian transplant population. Written informed consent was obtained from all patients before any data or 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 on October 18, 2017 (423).

Study 2 was a national prospective, observational cohort study that included all first-time kidney transplant recipients between October 2012 and October 2017 in Norway.

Paper 3

Study 3 included patients with their first-time STEMI, typical chest pain and ST-elevations on ECG. The patients were recruited if they had acute proximal/ mid-occluded single vessel disease and went through a successful revascularization with a primary PCI defined as TIMI 3 flow, and no significant residual stenosis. Patients were excluded if they had evidence of previous MI based on history, ECG or angiography, or if they had more than single vessel disease on angiography or CMR. Patients were also excluded if they had severe co-morbidities, reinfarction during the first week or contraindications against CMR. A total of 42 STEMI patients fulfilling all these criteria were included (424).

All patients were treated according to standard PCI procedure, with aspirin, clopidogrel, heparin and statins. Post-MI, the patients were treated according to current guidelines with ACE inhibitor/ angiotensin receptor blockers, diuretics and beta-blockers. They were treated with an aldosterone antagonist if indicated, in accordance with the criteria used in the EPHESUS study (424, 425). Patients with LV dysfunction as documented by LVEF of 40 percent or lower, and heart failure as documented by the presence of pulmonary rales, chest radiography showing pulmonary venous congestion. In patients with diabetes who met the criteria for LV dysfunction after acute MI, symptoms of heart failure did not have to be demonstrated, since such patients have an increased risk of CV events similar to that of nondiabetic patients with symptoms of heart failure. In our cohort, 8 patients were treated with an aldosterone antagonist. They all had LVEF <40% and clinical signs of heart failure. We divided the cohort according to the presence or absence of heart failure post MI, using +/- indication for aldosterone antagonist to divide the group. Written informed consent was obtained from all patients before inclusion (424).

Study 3 was a single center observational study including patients with a first-time STEMI due to an occlusion of a single, large coronary artery, successfully revascularized with PCI. The included patients were followed with repeated CMRs and lab tests during a 1 year follow-up period.

Paper 4

A total of 236 patients received a kidney only transplant in the period of November 19, 2012 to October 31, 2013. 170 from a deceased donor and 66 from a living donor. Thirtyfour of 236 were re-transplantations. Approximately 8 weeks post-transplant all patients are examined at the laboratory for Renal Physiology at OUS, RH. Vitamin D levels were measured as a part of the follow-up at week 8 post-transplant. We randomly selected 132 patients based on vitamin D measurement at the 8 week investigation without taking any other information into consideration The aim of the study was to explore the relationship between sKlotho and iFGF23 measured 8 weeks and 1 year post-transplant with long-term graft- and patient survival in a cohort of kidney transplant recipients with deficient and non-deficient vitamin D levels. Patients with a functioning kidney graft 1 year post-transplant are invited to OUS-RH for clinical examination and laboratory follow-up. One hundred and twenty-eight of the 132 patients included attended the 1 year follow-up. In the routine in-depth investigations at both 8 weeks and 1 year post-transplant, vital signs, blood pressure (BP) and blood samples for clinical chemistry were obtained. All patients were examined with aortic (carotid-femoral) PWV using SphygmoCore® (426) at 8 weeks and 1 year.

Plasma samples for analysis of sKlotho and iFGF23 were obtained and stored at −70°C in the Diagnostic and Treatment biobank “Nyrefysiologisk laboratorium” (Biobank nr 266-2005-142234). Plasma samples for analysis of sKlotho and iFGF23 were sent to Stavanger and measured en bloc at Stavanger University Hospital, and the biomarker results were not reported to the treating phycisians (426).

Long-term outcomes were collected from the NRR. Written informed consent was obtained from all patients before any data or biological material was included in the NRR and the biobank at OUS, RH. The patients were followed from time of transplantation until graft failure, death or end of study March 01, 2022 (426).

Study 4 was a national prospective, observational cohort study that included 132 kidney transplant recipients.

**Materials**

Paper 1

Blood samples and urine samples were collected one time per person. Routine hematological and biochemical analyses were performed in the biochemical laboratory at SUS. Urine samples were analyzed for albumine, creatinine, calcium, phosphate in all participants, except the CKD 5 patients, since many HD patients are anuric. The biomarkers were measured en bloc. 25(OH)D, iFGF23 and sKlotho were analyzed in serum, NGAL was analyzed in EDTA plasma.

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 analysis by liquid chromatography coupled with tandem mass spectrometry detection (422, 427). iFGF23 was measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit from Kainos Laboratories Inc. (Tokyo, Japan). sKlotho was measured by a commercially available ELISA kit from IBL (Immuno-Biological Laboratories GmbH, Hamburg, Germany). NGAL was measured with a commercially available ELISA kit with microwells precoated with a monoclonal antibody raised against human NGAL (KIT 036; Bio-Porto Diagnostics, Gentofte, Denmark). The analysis were performed in accordance with the manufacturers' protocols. Freshly thawed samples were measured in duplicate, and the reproducibility of the method was monitored by analyzing 3-5 aliquotes of a serum control with each assay (422).

The fractional excretion of phosphate ($FePO_4$) and calcium ($FeCa_3$) was calculated according to the following formula: $FePo_4$: (urine-phosphate x serum creatinine) x 100/(serum phosphate x urine creatinine). $FeCa_3$: (urine-calcium x serum creatinine) x 100/(serum calcium x urine creatinine).

Paper 2

Blood samples were collected from fasting patients, and routine laboratory samples were analyzed at the biochemical department at OUS, RH. 25(OH)D, $T_{50}$, and NGAL were analyzed in biobanked serum at SUS, Calciscon AG, and OUS, RH, respectively (423).

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-EPI equation (99).

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 at the laboratory of medical biochemistry at SUS (423).

NGAL was analyzed by enzyme immunoassay (EIA) using antibodies from R&D systems (Stillwater, Minnesota, USA). Inter- and intra-assay coefficients of variation are 4.8% and 2.3%, respectively (423).

$T_{50}$ was determined using a method that measures the time of in vitro transformation from primary to secondary calciprotein particles. 40 µL serum was exposed to high and supersaturated concentrations of calcium (35 µL) and phosphate (25 µL) 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 (403, 423).

PWV measurements were done in the morning with patients in a quiet room. PWV 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 on 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 (423, 428).

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 were available in 510 of the 584 kidney transplantations done after 2009 included in this study. Some recipients did not have a functioning graft 1 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. Interstitial fibrosis was scored from zero to three and categorized into two groups with score zero and one vs two and three (423, 429).

Paper 3

The patients were followed with CMR at 4 time-points during the first year following infarction: at 2 days, 1 week, 2 months and 1 year. The CMR examinations were used to assess infarct size and LV volumes and LVEF. Blood samples were drawn at admittance (before revascularization), and at the same time points as the CMRs. The CMR images were ECG-gated and obtained during breath holding. Patients were scanned in a supine position by a 1.5 T whole body scanner (Intera™ R10, Philips Medical Systems, Best, The Netherlands) using a dedicated cardiac coil. Resting LV function was determined with cine images using a steady-state free precession technique. For first-pass perfusion, a turbo field echo sequence with three short-axis slices per heartbeat (prospective triggering) and a selective saturation recovery prepulse was used. Gadolinium-based contrast agent (Omniscan™, Amersham Health, Little Chalfont, UK) was given at a dose of 0.075 mmol/kg. Immediately following the completion of the first-pass imaging, another 0.175 mmol/kg of contrast agent was infused, and 10–15 min following the latter infusion-delayed hyper enhancement images were acquired. All post- processing was performed on the View Forum™ Software (Philips Medical Systems, Best, The Netherlands) in a random, blinded fashion. Assessment of left ventricular end diastolic volume index (LVEDVI), left ventricular end systolic volume index (LVESVI), and LVEF was done by short-axis volumetry. Infarct size was assessed manually with plainimetry on each short-axis slice by delineating the hyper enhanced area from the non-enhanced myocardium (221, 424)

At the time of study 3, the contrast agent Magnevist (gadopentetate dimeglumime) was used as a standard for CMR. This is a lineær compound, and is not used today due to the risk of NSF. The contrast agent used today is Dotarem (gadoterate meglumine), which is a cyclic compound. The amounts of contrast agent is the same before and now, and contrast is given twice during the examination. First for perfusion, 0.075 mmol/kg (0.15 ml/kg), and then the rest of the contrast 0.25 mmol/kg (0.5 ml/kg) minus the perfusion bolus.

Venous blood samples were collected on admission to the hospital, immediately prior to PCI, and 2 days, 7 days, 2 months and 1 year following MI. The pyregen-free blood collection tubes were immediately immersed in melting ice (ethylenediaminetetraacetic acid-containing tubes, plasma) or placed in room temperature (tubes without any additives, serum) and centrifuged within 20 minutes at 2500*g* for 20 minutes to obtain platelet-poor plasma, or centrifuged at 1000*g* for 10 minutes after coagulation (serum). All samples were stored at −50 °C and thawed only once. Biomarkers were analyzed in one run, after the study was completed (424).

Plasma iFGF23 was measured by commercially available ELISA kits from Immutopics International, San Clemente, CA, USA. Freshly thawed samples were measured in duplicates, and the reproducibility of the method monitored by analyzing 4 aliquots of a control with each assay. Serum levels of CTGF were determined by a sandwich ELISA (430). The concentration of transforming growth factor b1 (TGF-b1) was measured by EIA (R&D Systems) (431). Serum 25(OH) vitamin D were 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 analysis by liquid chromatography coupled with tandem mass spectrometry detection (427). In this study, levels of serum 25(OH) vitamin D are termed deficient <30 nmol/l (<12 ng/mL), insufficient at 30–50 nmol/L (12–20 ng/mL), sufficient at >50 nmol/L (>20 ng/mL) (424). PINP, (PIINP) and CITP were analysed using a radioimmunoassay from Orion Diagnostica (Finland). The lower detection limit was 2.0 ng/mL for PINP, 0.20 ng/mL for PIIINP and 0.50 ng/mL for CITP respectively (424).

Paper 4

Blood samples were collected from fasting patients, and routine laboratory samples including 25(OH)D were analyzed at the biochemical department at OUS, RH. iFGF23 and sKlotho were analyzed in biobanked serum at SUS.

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-EPI equation (99, 426). Glycosylated hemoglobin (HbA1c) results were given in % and converted to mmol/mol using the formula: IFCC HbA1c mmol/mol= (10.931*NGSP-HbA1c)-23.524 (432).

25(OH)D, including both 25-OH Vitamin D2 and 25-OH Vitamin D3 was measured in fresh serum samples by reversed-phase liquid chromatography coupled with tandem mass spectrometry detection. 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) (195) , and we pooled insufficiency 30-50 nmol/L (12-20 ng/mL) and sufficiency >50 nmol/L (>20 ng/mL as non-deficient vitamin D levels (≥30 nmol/L) to increase the clinical relevance and the statistical power (195, 426).

iFGF23 and sKlotho were measured in EDTA-plasma by commercially available ELISA kits from Immutopics International (San Clemente; CA, USA) and IBL (Immuno-Biological Laboratoires GmbH, Japan), respectively. Freshly thawed samples were analyzed in duplicates, and the mean value reported. Coefficients of variation between duplicates were <17% for sKlotho and <16% for iFGF23. The intra- and inter-assay variability of the methods were <11% and <12% for iFGF23, respectively, and <13% and <12% for sKlotho, respectively (n=3-6). iFGF23 in EDTA-plasma based on 8 healthy adults measured in the same local laboratory was mean 43.9 pg/ml (±19pg/ml) (424). sKlotho measured by IBL ELISA kit in serum from 142 healthy subjects, was reported to range from 239 to 1,266 pg/ml, with a mean of 562 ± 146 pg/ml, in the original publication on the method (433).

**Statistical methods**

In general:

Descriptive statistics are presented as mean ± standard deviation (SD) when normally distributed, median and range when not normally distributed.

The level of statistical significance is defined as $p < 0.05$. All statistical analysis were conducted using SPSS versions 22.0-26.0.

Paper 1

NGAL, sKlotho and iFGF23 were logarithmically transformed to obtain normal distribution.

The variables were compared between the different groups by one-way analysis of variance (ANOVA). Mann-Whitney U test and Kruskall-Wallis test gave similar results. We used a multiple regression model with the biomarkers NGAL, iFGF23, and sKlotho as dependent variables to explore the possible effects of the independent variables age, sex, 25(OH) vitamin D, phosphate, iPTH, albumin-corrected calcium, creatinine, and the other 2 biomarkers on the dependent variables. NGAL, iFGF23, and sKlotho were used as dependent variables in 3 different models. We excluded nonsignificant variables using a backward selection model. We performed the analysis on the complete study population to ensure an adequate sample size (422).

Paper 2

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, 10-week eGFR, iPTH, calcium, phosphate, albumin, pre-transplant diabetes, combined vascular morbidity (a history of coronary and/or cerebral and/or peripheral vascular disease), time in dialysis, rejection episodes, and donor status (living vs deceased donor) as independent variables measured at 10 weeks post-transplant.

Three different multivariable Cox regression models were performed using death, graft failure, and death-censored graft loss as dependent variables. Sex, age, BMI, 10-week eGFR, iPTH, calcium, vitamin D, phosphate, albumin, pre-transplant diabetes, vascular comorbidity, time in 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 on October 18, 2017. The results in the Cox model remained unchanged whether vitamin D was treated as a continuous or a categorical variable.

ANOVA and paired samples *t* tests were used to compare creatinine levels at 10 weeks post-transplant and at 1 year (423).

Paper 3

Area under the curve (AUC) was calculated for each biomarker at different time points during 1-year follow-up, and correlations were measured cumulatively. ANOVA was used to test for differences between the variables measured at multiple time points, with Bonferroni post-hoc testing where appropriate. Univariate correlations were performed using non-parametric methods (Spearman). Non-parametric tests (Mann-Whitney U, Kruskal-Wallis) were used to test for differences between groups.

Paper 4

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

ANOVA and paired samples t-tests were used to compare variables measured at 8 weeks post-transplant and in the end of 2020.

Survival analysis was performed using Kaplan-Meier estimate and multivariable Cox proportional hazard analysis including iFGF23, sKlotho, vitamin D, age, BMI, eGFR, HbA1c and haemoglobine measured at 8 weeks as independent variables. The selection of variables was based on clinical knowledge and previous publications. The proportionality assumption was checked using a log-minus-log plot. Two different Cox regression models were performed using uncensored graft loss (including death and death censored graft loss) and death as dependent variables.

To compare variables measured at 8 weeks and 1 year post-transplant, a 2 sided p-value was estimated using paired sample t-test in normally distributed variables and Wilcoxin Signed Ranks test in not-normally distributed variables (426).

## Ethical considerations

### Paper 1

All participants drew blood and delivered an extra urine sample to be included in the study. The donors, controls and patients were not informed about their biomarker levels, but controls with deviating standard laboratory levels were informed. One of the controls had albuminuria, and he was informed about this via telephone. He was asked to contact his primary phycisian for control and blood pressure measurement.

### Paper 2

The kidney recipients were examined at 10 weeks as a part of their standardized post-transplant follow-up. They agreed to draw a few extra tubes of blood at the same time as standard blood testing. They were not subjected to extra examinations or reporting besides the normal follow-up post kidney transplantation.

### Paper 3

The patients with MI were given standard treatment in the acute fase of the MI. They were given the opportunity to be followed with extra CMRs during the next year. Many patients feel important and extra safe when being included into a study, and may be extra careful with self-care and adherence to doctor's advice/ intake of medication, others may have felt that the follow-ups were close in time and a hassle to do. The patients were given repeated doses of a contrast-agent that is taken off the marked after the study was completed, but no reports on averse events have been filed.

### Paper 4

The kidney recipients were examined at 8 weeks and 1 year as a part of their standardized post-transplantant follow-up. They agreed to draw a few extra tubes of blood at the same time as standard blood testing. They were not subjected to extra examinations or reporting besides the normal follow-up post kidney transplantation.

# 4. Results

Paper 1

Long-term kidney donors with eGFR >60 ml/min/1.73m$^2$ had significantly increased levels of NGAL when comparing with controls 5-38 years (median 15) after kidney donation. As expected, we found increasing levels of log NGAL with declining kidney function, figure 19. In the donors, there was no association between NGAL levels and time since nephrectomy, NGAL levels and age, or NGAL levels and 1st-degree relationship between donors and recipients.

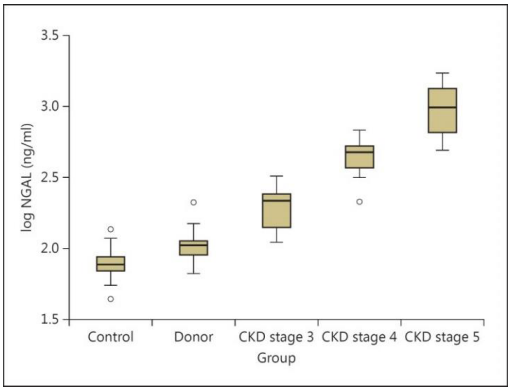



**Figure 19.** Boxplt showing the distribution of NGAL in the different groups. *Reprinted with permission from Karger (422).*

In a multiple regression analysis using log NGAL as the dependent variable and age, sex, 25(OH) vitamin D, phosphate, iPTH, albumin-corrected calcium, log sKlotho, log FGF23, and creatinine as independent variables, only creatinine remained in the model using a backward selection model (422).

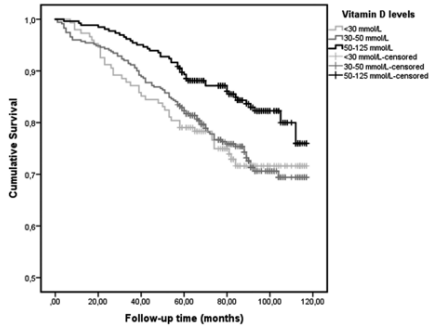
log iFGF23 levels were nonsignificantly higher in donors than in controls. As expected log iFGF23 levels increased significantly with declining kidney function. There was no difference in log sKlotho levels between controls and kidney donors. log sKlotho levels declined with declining kidney function, and the log sKlotho levels were significantly lower in patients with CKD stages 4 and 5 than in controls (422).

Paper 2

In the 762 kidney 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 >50 nmol/L (vitamin D sufficiency) 10 weeks post-transplant. None had 25(OH)D levels >125 nmol/L.

During a median follow-up of 82 months after the 10-week post-transplant control, 118 (15%) recipients died, and 172 (23%) patients lost their grafts. Patient and graft survival were significantly better in patients with vitamin D sufficiency at 10 weeks post-transplant compared with patients with vitamin D insufficiency or deficiency. Eighty-six percent of the transplant recipients with sufficient vitamin D levels were alive with a well-functioning graft after 5 years using Kaplan-Meier survival estimates, compared with 79% and 76% of the patients with vitamin D deficiency and insufficiency, respectively (P = 0.006) (423).

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, figure 20.




Number at risk:

| Vitamin D | 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 20.** Overall survival in patients with vitamin D insufficiency, deficiency and sufficiency. *Reprinted with permission from Wiley* (423).

In multivariable analysis, the patients with vitamin D deficiency and insufficiency had a higher risk of graft failure or death when comparing with patients with sufficient vitamin D levels. Using graft failure as the main end-point, censoring for death with functioning graft, the results show the same tendency with increased risk of graft failure with vitamin D insufficiency and deficiency. 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. Vitamin D level at 10 weeks post-transplant was associated with graft failure and death in all three Cox models (423).

NGAL was not significantly different between the groups of patients with vitamin D insufficiency, deficiency, and sufficiency. Median aPWV at 10 weeks showed no significant difference between the vitamin D groups of patients. Mean $T_{50}$ was significantly higher in the vitamin D sufficient group of patients compared with the patients with vitamin D deficiency and insufficiency (423).

One-year post-transplant follow-up laboratory samples were analyzed in 710 of the 762 transplant recipients. In the group of patients with vitamin D deficiency (137 patients) and insufficiency (322 patients), the creatinine levels increased insignificantly. In contrast, patients with vitamin D sufficiency (251 patients) had a significant decrease in creatinine. Creatinine increased ≥20% from week 10 to 1 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).

In the 1-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, that is, 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) (423).

Several significant patterns were revealed comparing the pattern during follow-up in MI patients. First, iFGF23 was significantly lower day 0 and day 2 compared with the levels measured in healthy adults, with a significant increase in concentrations during follow-up reaching levels comparable/ higher than healthy adults on day 7 with the highest concentrations at 1 year, figure 21.

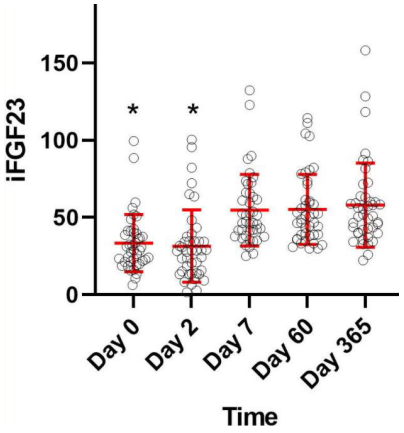



**Figure 21.** FGF23measurements at different timepoints. Mean ± SD in red. *Reprinted with permission from Elsevier (424).*

Second, CITP increased significantly from day 0 to day 2, and remained elevated throughout the observation period indicating a prolonged increased collagen type I degradation following MI. Third, in contrast to this pattern, PINP showed a late, significant increase from day 0 reaching the highest level at 1 year, suggesting a slower increase in collagen type I synthesis. Fourth, PIIINP showed a rapid increase during the first week, with persistent elevation for the rest of the observation period, suggesting a rapid increase in type III collagen synthesis. Finally, there was no significant changes during follow-up in 25(OH) vitamin D, CTGF, TGF-β, creatinine, calcium, phosphate and iPTH levels. There was no correlation between iFGF23 levels and the other biomarkers at any time-points. However, there was a strong correlation

between the AUC of FGF23 and CTGF during the 1-year follow-up ($r = 0.53$, $p < 0.01$).

In the whole group of patients there was a significant reduction in infarct size and myocardial mass from day 1 to 1 year post MI. LVEF increased from 47.2% at baseline to 52.7% at one year follow-up. LVESVI and LVEDVI did not change significantly. In the group as a whole, there was no correlation between iFGF23 levels and the CMR findings at any time-points.

The studied cohort was divided according to the presence or absence of LV dysfunction or heart failure during the acute course of the MI. A total of 8 patients had LVEF <40% and signs of acute heart failure. These patients had larger infarct sizes, lower LVEF and increased LVEDVI and LVESVI on CMR, figure 22.

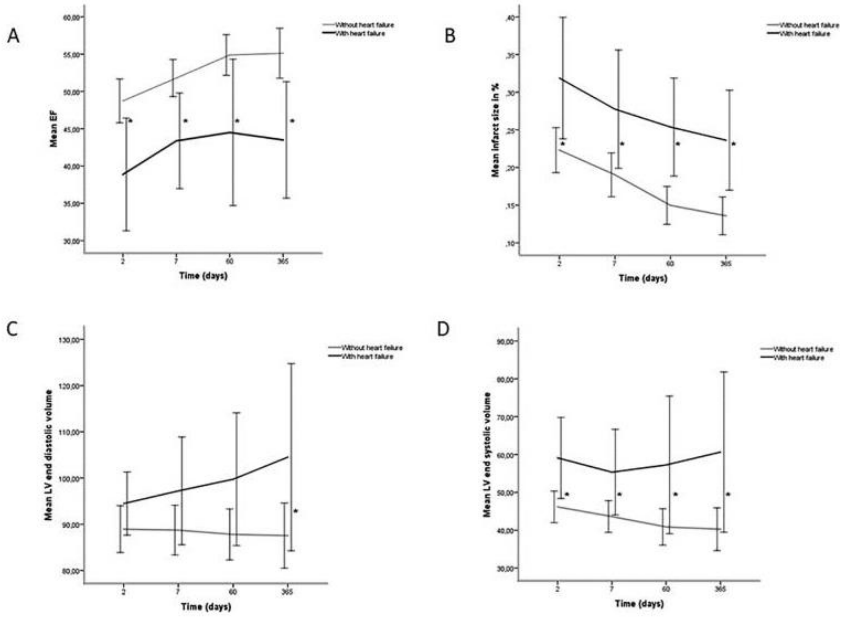



**Figure 22.** (A) LVEF, (B) infarct size, (C) LVEDVI and (D) LVESVI in patients with and without heart failure. *Reprinted with permission from Elsevier (424)*

The 8 patients with heart failure were treated with aldosterone antagonists following revascularization. iFGF23 levels were higher at all time-points in this group, figure 23. In the patients with acute heart failure, there was a significant correlation between the AUC of the iFGF23 concentrations 7 days post MI and CMR findings on day 7, with LVEF, infarcted mass and infarct size in percent of LV mass (424).

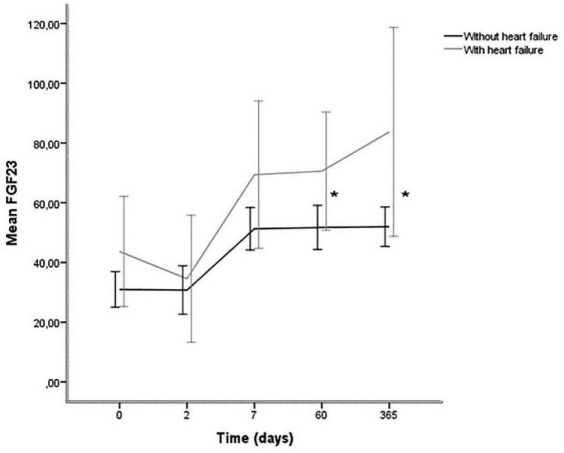



**Figure 23.** iFGF23 levels in patients without and with heart failure post MI.
*Reprinted with permission from Elsevier (424)*

Paper 4

iFGF23, sKlotho and vitamin D status 8 weeks post-transplant

Of the 132 selected kidney transplant recipients, 49 (37%) had 25(OH)D levels <30 nmol/L, 83 (63%) had 25(OH)D levels ≥30 nmol/L. Mean age was 51.1 years, 58% were male. There were no significant differences in iFGF23 levels (p=0.17) or sKlotho levels (p=0.37) between the vitamin D groups at 8 weeks. Median follow-up time was 7.4 (1.2-8.1) years (426).

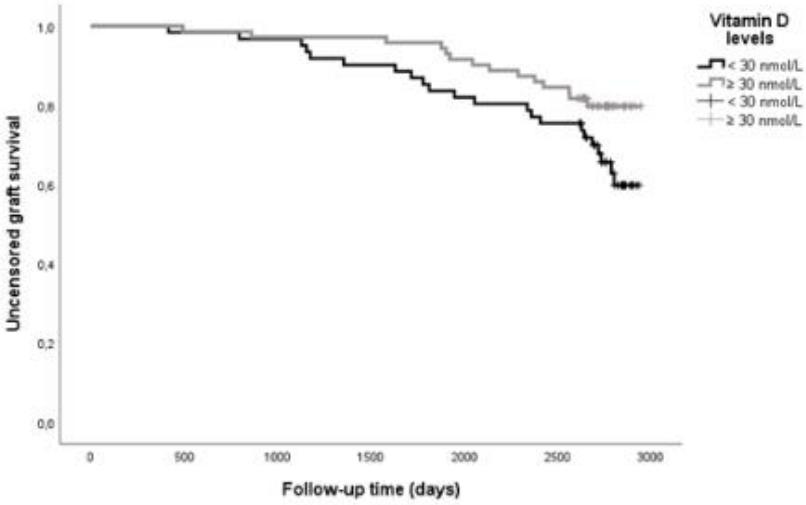
FGF23, Klotho and vitamin D status 1 year post-transplant

In total 128 of the 132 patients attended the 1 year follow-up at the transplant center. In 4 of the 128 attendees, vitamin D was missing, all other measurements were registered. In the cohort as a whole, vitamin D increased from mean 43±20 nmol/L 8 weeks post-transplant to 60±26 nmol/L (p<0.001) 1 year post-transplant. Thirteen patients (11%) had 25(OH)D levels <30 nmol/L, and 111 patients (87%) had 25(OH)D levels ≥30 nmol/L 1 year post-transplant. In the whole group, iFGF23 levels decreased from median 110 (15-1990) pg/mL to 89 (32-727) pg/mL, (p<0.001) from 8 weeks to 1 year post-transplant. In the same period, sKlotho leves increased from mean 417±129 pg/mL to 637±207 pg/mL, (p<0.001), eGFR increased from mean 62±19 mL/min/1.73m$^2$ to 73±25 mL/min/1.73m$^2$, (p<0.001), and iPTH levels decreased from median 10.5 (2.3-132.2) µmol/L to 8.9 (2.3-72.5) µmol/L, (p<0.001) (426).

Graft survival and overall survival

During the follow-up period, a total of 36 grafts were lost, 27 (75%) of these were due to death with a functioning graft. Overall, graft survival was significantly different between the two vitamin D groups. Twenty (41%) of the patients with vitamin D deficiency (<30 nmol/L) at 8 weeks post-transplant suffered graft loss or death compared with 16 (19%) in the non-deficient vitamin D group (≥30 nmol/L), p= 0.007 (426).

Crude Kaplan Meier estimated 5 year uncesored graft survival was 84% ± 5% (SE) in the patients with deficient vitamin D levels, compared with 94% ± 3% in the patients with non-deficient vitamin D levels, (p= 0.014). After 7 years follow-up, the uncensored graft survival was 74% ± 6% and 84% ± 4%, respectively (p=0.007), figure 24 (426).




Number at risk:

| | 0 | 500 | 1000 | 1500 | 2000 | 2500 (days) |
|---|---|---|---|---|---|---|
| <30 nmol/L | 49 | 48 | 47 | 43 | 40 | 36 |
| ≥30 nmol/L | 83 | 82 | 81 | 81 | 75 | 70 |

(vitamin D levels/ days of follow-up)

**Figure 24:** Kaplan Meier plot showing overall survival in patients with vitamin D deficiency, insuffiency and sufficiency. *Reprinted with permission from Wolters Kluwer Health Inc. (426)*

In the multivariable Cox regression models with uncensored graft loss as the dependent variable, high age, high HbA1c, low hemoglobin and low vitamin D were associated with uncensored graft loss. In the Cox regression model with patient survival as the dependent variable, high age, high HbA1c and low vitamin D were associated with death. sKlotho, iFGF23 and eGFR at 8 weeks were included as variables in both models, but had no impact on the dependent variables. Haemoglobine did not influence the model with death as dependent variable (426).

In the adjusted model, the patients with vitamin D levels ≥30 nmol/L nmol/L, had a hazard ratio for uncensored graft loss of 0.46 (0.23-0.95) compared with patients with vitamin D levels <30 nmol/L. Using death as the main end-point, the patients with vitamin D ≥30 nmol/L had a risk of death of 0.40 (0.17-0.92), compared with patients with vitamin D levels <30 nmol/L (426).

7 year patient follow-up

Ninety-six (73%) of the 132 patients still had a functioning graft 7 years post-transplant, 29 (59%) of the 49 patients with deficient vitamin D, and 67 ((81%) of the 83 patients with non-deficient vitamin D levels post-transplant, (p=0.007). Mean eGFR was 64 mL/min/1.73m$^2$, with no significant difference between the two vitamin D groups. iPTH remained elevated above the normal reference range in both groups with a higher mean level in the whole group of patients 7 years compared with 8 weeks post-transplant, median (range) 10.5 µmol/L (2.3 µmol/L-132.3 µmol/L) at 8 weeks vs 11.0 µmol/L (2.7 µmol/L-44.7 µmol/L) after 7 years (426).

# 5. Discussion

**Methods:**

The groups of patients included in these 4 studies do not overlap. They include different groups of patients representing a large portion of the patients followed in a nephrology department, as well as patients with acute MI without kidney disease and healthy controls. This gives us a broad overview of the selected biomarkers in many different patient categories.

**Biomarkers**

The quality of commercially available immunoassays to measure biomarkers is no guarantee, and all assays should be evaluated carefully. When using differing kits and different labs (technique), each laboratory has to establish a reference range, and results in published studies may not be automatically comparable.

The same person in the laboratory of biochemistry at SUS performed the biomarker analyses done locally in all 4 studies.

*Klotho:*

When study 1 was performed, three different Klotho kits were available for purchase. The IBL kit used in study 1 was most widely used at the time, and it showed a within-run variation of 4%. There is a vast increase in available kits, but we chose to use the same IBL kit in study 4 as well. In study 1 we analysed sKlotho in serum, and in study 4 we analysed sKlotho in EDTA-plasma due to sample availability. A good agreement between serum and EDTA plasma in 3 different Klotho assays have been established in one study ($R^2$= 99; n=20) (434).

*FGF23:*

Intact FGF23 (iFGF23) is enzymatically cleaved into inactive c-terminal and n-terminal fragments. Different types of assays exist for FGF23 testing, those targeting the iFGF23 and those targeting terminal fragments (cFGF23). The cFGF23 kits measure both cleaved and iFGF23. The best way of analyzing FGF23 is a matter of debate. iFGF23 appears to be the active form of the hormone. The two assays show poor agreement, especially in healty subjects (435-437). As kidney function is reduced, the agreement between the assays improves (437). Some studies have shown a steeper cFGF23 slope as kidney function is reduced, supporing an accumulation of c-terminal fragments or reduced renal elimination (435, 438, 439), However, this is not consistent with other reports which claim that during disease progression, iFGF23 increases as cFGF23 decreases, suggesting a reduced cleavage of the intact form, or an increased production of iFGF23 by bone in advanced CKD (108, 438).

A comparison between the two assays is a challenge, since the assays lack standardization between them, and they measure two different forms of FGF23. The results are given in different units, cFGF23 is expressed as RU/mL, and iFGF23 as pg/mL. The assays display different analytical performance (440). There are also more than one available cFGF23 and iFGF23 kits on the market, and the analytical agreement is variable at best, and the performance of the assays vary in precision, range, detection limits and sample matrices (441). There is need for standardization and the development of an international standard, if such kits are to be imported into clinical practice (442). Thus, FGF23 concentrations reported in different studies are not necessarily comparable because there are two different tests, the assays are not standardized and there are differences between laboratories because of the manual nature of the assays (443). We decided to use the concentration of the biologically active intact FGF23 with a half-life of about 1 hour.

In study 1 iFGF23 was analyzed using the commercially available ELISA kit from Kainos Laboratories Inc (Tokyo, Japan). The level of iFGF23, measured with Kainos ELISA kit, in 104 healthy adults from Japan was reported to be in the range of 8.2-

54.3 ng/l (ng/L=pg/ml) (444), and a reference range of 10-50 ng/l has been suggested based on these results (445). In our 35 healthy controls FGF23 was 51.5 pg/ml (±15.4 pg/ml) (422). In studies 3 and 4 we chose a different supplier for iFGF23, partly because it had a much lower prise, and the reproducibility was reported as high, and variation as low. iFGF23 was analyzed in one session per study, using ELISA kits from Immunotopics Int. (San Clemente, CA, USA). The reference interval for iFGF23 in this kit was 43.9 pg/ml (±19.0 pg/ml) based on samples from 8 healthy adults measured in the same laboratory (424).

The iFGF23 kits from both Kainos and Immunotopics are sandwich ELISAs that measure antigens between two layers of antibodies (capture and detection antibody). The kits measures active, full length FGF23. The Kainos assay has been used in many studies, and has been used for a long time. The ELISA kits are not approved for clinical use, and are marked for research only (444, 446).

The interest of this biomarker has stimulated the development of automated methods as a way to overcome the limitations of the ELISA kits. An automated chemiluminescent iFGF23 assay has been developed more recently. The advantages are shorter run times, smaller sample volumes and a wider analytical range (108). Some European countries, including Norway, have approved the use of such a chemiluminescence immunoassay for measuring iFGF23 (Liaison FGF23), which is superior to the manual assays when it comes to performance and accuracy (443, 447). In Norway, plasma can be sent to OUS, hormonlaboratoriet, for FGF23 analysis. The development and implementation of automated assays like this is a step towards standardization, where measuring FGF23 can be included in routine clinical work if indicated. If therapies aimed at FGF23 reduction prove useful, routine measurement of this hormone might be useful. However, this warrents that the different pre-analytical and analytical issues are addressed. Reference intervals and clinical decision limits are necessary before inserting this into standardized care. In patients with X-linked hyperphosphatemia treated with burosumab (Crysvita) this could be very beneficial (447). In CKD, FGF23 research has changed the understading of the physiological mechanisms of CKD-MBD, but it has not been implemented into

clinical use in CKD patients, despite its promise in research. FGF23 measurement is expensive and the therapeutic strategies are still limited. With time, this might change (440).

*NGAL:*

Serum NGAL was measured in studies 1 and 2. In study 1 the analysis was done at SUS, and in study 2 the analysis was done at OUS, RH. The chosen kits are not the same, and results may not be completely comparable. Kits are chosen based on price, availability and experience at the lab doing the analysis.

In study 1 NGAL concentration in EDTA-plasma was measured by a commercially available ELISA kit, with microwells pre-coated with a monoclonal antibody raised against human NGAL (KIT 036, Bio-Porto Diagnostics, Gentofte, DK) (448). In study 2 NGAL was analyzed by enzyme immunoassay using antibodies from R&D systems (Stillwater, Minnesota, USA) (423).

There are several ELISA kits to measure serum NGAL on the market, and the results may not be comparable between different kits and different labs. It is possible to measure serum NGAL and urinary NGAL, and both measurements appear to be valuable biomarkers for AKI. NGAL is produced in many organs, and increase as a part of inflammation. A two-compartment model has been suggested for NGAL in the kidneys, where uNGAL is produced from in the distal part of the nephron within hours of an injury. Proximal tubule NGAL come from systemic NGAL which often comes from extrarenal sources (449). Many studies report changes in NGAL with AKI or kidney surgery, and they often report urinary NGAL changes (or both urinary and serum NGAL) (450). Urinary NGAL may be the biomarker that performs best in the very acute setting, but serum NGAL performs equally well/ better after only a few hours. In the very acute setting, obtaining a urine sample may be a challenge (451, 452). NGALs role in more chronic disease remains unclear, but it's potential as a prediction tool for AKI is shown in many studies (449). Our patients were not admitted in an acute setting, and we chose to measure NGAL in serum and not in

urine. At the time of our studies, urinary NGAL testing was considered to be more unstable and less informative than serum NGAL.

A Danish company, Bioporto, has recently developed an NGAL urinary dipstick quick test similar to a streptest or pregnancy test, it is approved for clinical use in Europe, and is hoping for FDA approval for use in the US. In a newly published study on Covid 19 patients, there was an independent relationship between elevated urinary NGAL and severe clinical outcomes. The dipstick correlated with ELISA-based measurements, and could be a helpful tool in emergency rooms in the future (453). It has not yet been approved in Europe.

*Vitamin D:*

Vitamin D was measured in the laboratory of biochemistry at SUS in studies 1, 2 and 3, and at OUS for study 4.

At SUS vitamin D was measured en bloc for each of the studies. 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 analysis by liquid chromatography coupled with tandem mass spectrometry detection. The analysis is incorporated in the routine laboratory workflow and is available for routine measurement (423, 427).

At OUS, 25(OH) D was measured in fresh serum samples continuosly as patients came to their 8-week evaluations. 25-OH Vitamin D2 and 25-OH Vitamin D3 were analysed separately, but the total 25(OH) D was used in the analyses. 25-OH Vitamin D2 and 25-OH Vitamin D3 are separated from possible interferences by reversed-phase liquid chromatography coupled with tandem mass spectrometry detection (426).

**Seasonal variation in vitamin D levels**

The major source of vitamin D is cutaneous production by exposure to UVB-irradiation of 7-dehydrocholesterol in the skin. Inadequate exposure to UVB is a major cause of vitamin D deficiency (454). The production of vitamin D in the skin is proportional to sunlight-exposure, and it varies with season and geographic location (latitude) (455). In Norway, solar angle and weather conditions are most favorable for vitamin D synthesis between May and September (455). In addition to sunlight, diet, skin pigmentation and lifestyle factors such as sunseeking behavior influence vitamin D levels (455).

Vitamin D deficiency is an increasingly recognized condition worldwide, and transplant recipients are at particularly high risk (194, 456). Transplant recipients are instructed to avoid sunlight exposure to minimize the risk of developing skin cancer due to immonusuppressive medication (457).

A Polish study, on 71 long-term transplant recipients, found vitamin D deficiency in 96% during the winter, and in 89% during the summer. None of the participants took vitamin D supplements (272). 105 long-term kidney transplant recipients were included in a study from the dermatology department at OUS, Norway. Vitamin D levels were measured in February and August 2013. Low vitamin D levels were frequent, but less frequent than reported in earlier studies from other countries. Seasonal variations were very modest, and not evident in patients that did not travel to southern latitudes in the last three months before blood sampling. When excluding the “travelers” there was no significant seasonal variation detected (271). 75% of 103 Israeli kidney transplant recipients had vitamin D deficiency (<30nmol/L) in a cross-sectional study, with no difference between testing in summer- and wintertime (458). Kidney transplant recipients living closer to the Equator are exposed to more UVB radiation than patients living in Norway and northern Europe, but a Danish study found similar results as the Egyptian study (459).

In study 2 patients were included 10 weeks post transplant, and vitamin D levels were measured at this “set” time, without consideration of season. In a subanalysis, we

divided the patients into 4 groups based on which season it was when they measured vitamin D. We did not find any significant differences when repeating the Kaplan Meier and Cox analyses in the four different seasonal subgroups.

We then divided the year into “summer” and “winter” according to sun exposure, and we did not find a significant difference between the two seasons, figure 25. The patients included during the fall-months are fewer compared with the other seasons, most likely because one does not plan for LD-transplantation during the summer holiday.

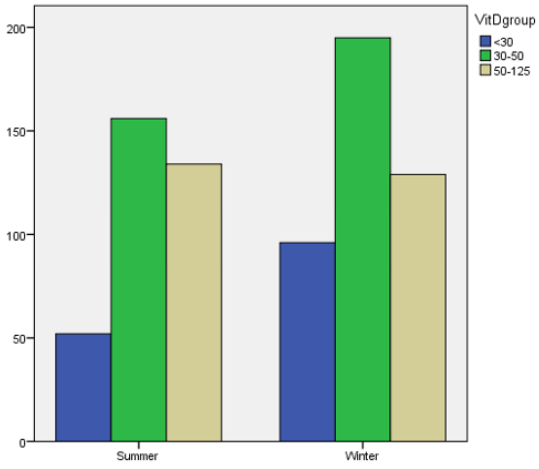



**Figure 25.** Vitamin D measurements (divided by 3 main groups) measured summer and winter. Created from data from study 2. Not previously published.

**Discussion of the results:**

**Kidney donation:**

For patients with ESRD, a successful kidney transplantation is the best treatment choise. A transplantation offers improved survival and improved quality of life, compared to treatment with dialysis (52). With an increasing number of patients on the organ donation waiting list, the use of living donor donations is essential to keep the waiting list stable. The key advantages are that the recipients may be transplanted preemptively (before dialysis) or the time in dialysis is reduced. Reports on better results with living donors compared with deceased donors supports an effort for the recruitement of living donors (460). Some new studies show less difference between graft survival after living vs deceased donor transplantations than reported earlier (461, 462), this could be due to advances in immunosuppression regimes and changes in handling of deceased donor organs. Changes in donor criteria, with the acceptance of donors with well-treated hypertension and donors with a history of kidney stones may result in poorer graft survival (461, 463, 464). In Norway, the results after living donor transplantation is still significantly better than after deceased donor transplantations, figure 26 (5).

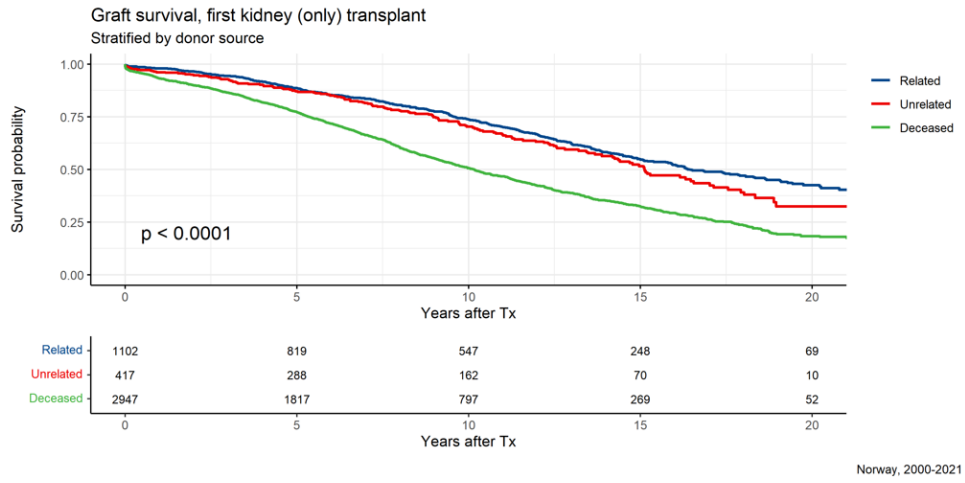



**Figure 26.** Graft survival in norwegian first-time kidney transplant recipients
*Reprinted with permission from the NRR (5).*

As nephrologists we promote living kidney donations, but with reports of increased risk of CKD developement and a shorter life-expectancy, a careful selection of the donor is important. The donors need to be well informed prior to transplantation, and this new prognostic information is implemented in the information from OUS which is mandatory for all potential donors.

In the general population, reduced GFR is associated with CVD and death (12), but the association between reduced GFR and adverse events could be due to other cardiovascular risk factors that the reduced GFR in itself. Kidney donors are ideal subjects to study the relationship between GFR and CVD, since they are thoroughly screened before kidney donation, and all other potential risk factors are investigated. A few prospective studies have investigated the cardiovascular effects of kidney donation. Altmann et al studied kidney donors with CMR before kidney donation and after 1 year, they found increased LV mass, reduction in diastolic function and elevated procollagen type III, a biomarker of cardiac fibrosis (465). Moody et al found increased LV mass, increased aortic stiffness, elevated iPTH, CRP, cardiac troponin and microalbuminuria 1 year post kidney donation, but after 5 year there was no significant difference in LV mass or blood pressure in kidney donors vs controls. Serum urate and FGF23 remained elevated after 5 years (289). Kasiske et al also found no effect of kidney donation on peripheral blood pressure or PWV, but small artery elasticity was significantly reduced in kidney donors. They also found elevated iPTH, homocystein and uric acid 9 years after donation. These factors have been associated with adverse cardiovascular outcomes and may themselves have direct adverse effects on LV geometry (466). In a Norwegian study, Haugen et al found significantly increased risk of ischaemic heart disease after kidney donation, but no increased risk of cancer or cerebrovascular events. The long follow-up is a strength of the study, and the controls were matched, and persons with comorbidities were excluded (87). The above mentioned studies provide insight into the potential mechanisms by which adverse cardiovascular effects of kidney donation may be mediated. Other studies do not suppert these findings, Price et al did not find an association between kidney donation and change in cardiovascular structure and

function after 5 years of follow-up based on CMR and PWV measurements in donors and controls (467).

In our first study, we found that long-term kidney donors have significantly higher levels of NGAL than healthy controls. The donors had eGFR >60 ml/min/1.73m$^2$, and were included 5-38 years after donation (median 15 years) (422). The elevated NGAL levels could be associated with the renal hyperfiltration seen in the remaining kidney after kidney donation. It is possible that these changes are present many years post-transplant. The elevated NGAL levels may also reflect the partial loss of renal function seen post kidney-donation. There is not much data on NGAL and kidney donors, at least not long-term studies. One study found that high serum NGAL 7 days after donor nephrectomy was predictive of lower eGFR 6 months after kidney donation (119). In 76 healthy living kidney donors, urinary NGAL (uNGAL) and serum NGAL (sNGAL) were measured preoperatively and regularly over the next 6 months. uNGAL was highest on postoperative day 1, and became stable on day 3. sNGAL was highest on day 7, and stabilized 1 month postoperatively. There was no association between uNGAL and postoperative creatinine levels (450). NGAL is a potential biomarker for predicting donors at risk of developing CKD and premature mortality postoperatively, and this should be tested prospectively in a much larger cohort. In accordance with many publications, we found that iFGF23 levels have an inverse relationship to eGFR, and the level of sKlotho declines when eGFR declines. However, we found no significant difference in sKlotho and iFGF23 levels between long-term kidney donors and controls.

With increasing evidence suggesting that donors are subjected to a long-term health risk when compared with matched controls, a special focus in the donor selection process is necessary. Studies on the long-term risks are coming, and they must continue. The ideal study includes donors and controls from the time of donation, with the control group being subjected to the same screening procedures as the donors. A long observation period would be necessary to obtain a sufficient number of events and adequate power, since most donors are relatively young (on average 48 years old the last decade in Norway), and they are healthy at time of donation.

**Vitamin D and kidney transplant recipients:**

In study 2, we found that vitamin D sufficiency (25(OH)D >50 nmol/L) 10 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 (423). In study 4 we found that patients with vitamin D deficiency (<30 nmol/L) at 8 weeks post-transplant had significantly reduced graft- and patient survival compared with patients with vitamin D levels >30 nmol/L. sKlotho and iFGF23 at 8 weeks showed no significant association with graft- or overall survival (426).

25(OH)D deficiency is common in patients referred for kidney transplantation. Prevalence varies across cohorts, reflecting different supplementation policies, sunshine exposure, and comorbidity. After a successful transplantation and restored kidney function, many transplant recipients remain 25(OH)D insufficient. This may, in part, be related to avoidance of sunexposure post-transplant to avoid skin cancer (271, 423, 468), as well as insufficient vitamin D supplentation before and after transplantation, increased vitamin D catabolism induced by immunosuppressive drugs (270, 458, 469). In study 2, serum creatinine decreased during the first year post-transplant, and there were fewer patients with significant fibrosis in the 1-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, supporting our findings (423, 470). Others have found an association between low 25(OH)D levels at time of transplantation and/ or a few months post-transplant, and a higher risk of DGF (269), rejection (471, 472), increased interstitial fibrosis (268), increased proteinuria (473, 474) and lower eGFR (258, 268, 471, 475, 476). Low 25(OH)D measured on average 6 years post-transplant was in another study independently associated with increased mortality and a higher annual decline in eGFR (477). There are conflicting reports on the link

between vitamin D deficiency and acute rejections (269, 471, 478, 479), study 2 is in accordance with Ban and Falkiewicz, with higher incidence of acute rejection in the group with vitamin D deficiency (269, 423, 479). Mehotra et al recently found a link between vitamin D deficiency and low VDR-activity before kidney transplantation, and the development of donor specific antibodies as well as decreased eGFR 12 months post-transplant (480). Vitamin D affects the adaptive immune system, with suppression of T-lymphocyt proliferation and modulation of cytokine production and differentiation, and weakening of the antigen presenting cells (481, 482). Vitamin D also influence the innate immune system by enhancing the antimicrobial activities of monocytes, macrophages and natural killer cells (483). These effects may explain why vitamin D deficiency may increase the risk of rejection and opportunistic infections (484). There are reports on vitamin D deficiency and increased risk of cytomegalo- and BK virus infections in transplant recipients (485, 486). We did not have data on opportunistic infections in our studies. Severe vitamin D deficiency may also be associated with post-transplant diabetes mellitus (487). It is a matter of debate if vitamin D is just a marker of poor health in general in these patients, making them predisposed for increased mortality and graft failure, or if low vitamin D triggers a cascade of events leading to the actual findings. Due to the design of studies 2 and 4, it is unfortunately not possible to investigate a cause-effect relationship.

Given the high prevalence of vitamin D deficiency in kidney transplant recipients, many studies have tried to clarify if correction of this deficiency can contribute to better bone health, improved graft survival, reduced cardiovascular risk, fewer infections and lower mortality. The interventional studies on vitamin D supplementation in kidney transplant recipients have given conflicting results. This may be attributed to differences in patient selection, use of vitamin D supplementation, time since transplantation and more. A direct comparison of the results is difficult. On one hand, vitamin D supplementation post-transplant was associated with decreased iPTH levels (488-490), reduced rejection rates (491, 492), better graft function (493) and improved graft survival (492, 494, 495). And yet another study did not find improved renal function or better protection against

rejection in kidney transplant recipients receiving vitamin D supplementation the first year post-transplant (496). The above mentioned trials were observational studies.

The VITA-D study randomized 200 adult kidney transplant recipients with vitamin D deficiency (<50 nmol/L) to treatment with oral vitamin D3 or placebo (497). With 1-year follow-up, they did not find improved short-term outcome. The results have not been published, but they were presented in the EDTA-ERA conference in 2015 (498). Another intervention study with paricalcitol, a synthetic vitamin D analogue, post-transplant found a reduction in iPTH but not in vascular health, or influence on allograft gene expression (499). Among kidney transplant recipients, studies have not showed a consistent association between vitamin D deficiency and CVD or malignancies (500-502), but a small study found that paricalcitol reduced proteinuria in kidney transplant recipients (503). This is supported in a more recent study who also found that therapy with paricalcitol, as an add-on to RAAS inhibition, reduced proteinuria in kidney transplant recipients with moderate CKD and mild proteinuria (504). A brief intervention study with paricalcitol 2 to 4 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, IL-4, and IL-10 in the aneurysm wall samples (505). These findings support that paricalcitol as a VDR 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. Larger, randomized studies are needed to confirm if paricalcitol has potential nefroprotective effects in kidney transplant recipients.

The VITALE study is a prospective randomized study that will evaluate the effect of high and low dose vitamin D supplementation in 640 kidney transplant recipients, with focus on fractures, diabetes mellitus, CV events, cancer and mortality (506). The results are not published yet, but in an abstract presented at the 2019 EDTA-ERA congress as preliminary results were presented. They found that the currently recommended doses of vitamin D supplementation are not sufficient to protect

against fractures post-transplant. They found no difference between high and low dose in the risk of developing diabetes mellitus, CV events cancer and mortality. High dose treatment was well tolerated, and there was not increased risk of vascular calcification or elevated phosphate or calcium levels (507).

The CANDLE-KIT study was a two-by-two, open-label, randomized trial that recruited 153 kidney transplant recipients at least 12 months post transplant, examining the effects of anemia correction and vitamin D supplementation. Inclusion criteria did not include vitamin levels, but hemoglobin <10.5 g/dL and eGFR 16-60 ml/min/1.73m$^2$. 2-year decline in eGFR was smaller in the high hemoglobin group, but did not differ in the cholecalciferol vs control group, suggesting aggressive anemia correction as a potential target to preserve kidney grafts (508). A randomized clinical trial in 180 Japanese kidney transplant recipients receiving cholecalciferol or placebo from 1 month to 1 year post-transplant did not find beneficial effects of cholecalciferol on change in eGFR, in histology or on urinary biomarkers related to kidney damage 1-year post-transplant. There was no long-term follow-up. The treatment was efficient in achieving sufficient vitamin D levels, and the treatment was well-tolerated (509).

From prior studies, it is 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 CV death, and low Klotho with aging and mortality (138). In study 4 we explored the the relationship between Klotho and FGF23 in transplant recipients with different levels of vitamin D. Patients with vitamin D deficiency (<30 nmol/L) had reduced graft- and patient survival compared with the group that had vitamin D levels ≥30 nmol/L in the early post-transplant phase. sKlotho and iFGF23 at 8 weeks showed no significant association with survival. High age, high HbA1c, low hemoglobin and low vitamin D levels were associated with reduced graft survival, and high age, high HbA1c and low vitamin D levels were associated with reduced patient survival (426). Overall, the patients with functioning kidney grafts at the end of follow-up, had higher eGFR at both 8 weeks and 1 year post transplant

than the patients who died or lost their kidney. The “survivors” were almost 10 years younger at the time of transplantation (426).

In our study the median iFGF23 levels declined from 8 weeks to 1 year post-transplant, but were still higher than in normal controls at 1 year, suggesting a significant difference between kidney transplant recipients and healthy individuals. The mean sKlotho levels increased from 8 weeks to 1 year, but were still lower than in normal controls at 1 year. The kidney rescipients in this study trended towards the sKlotho and iFGF23 levels measured in healty controls as time passed, which is in accordance with an increase in eGFR in the same period of time (426).

Vitamin D is a hot topic in research and in everyday life, in patients with and without kidney disease, and in the general population. There is overwhelming evidence on the negative effects of vitamin D deficiency. Low vitamin D is connected to demineralization of bones which in turn leads to increased risk of fractures (510). Low vitamin D is associated with hypertension in animal studies, and a direct effect on the renin-angiotensine-aldosterone axis has been found (192). Human trials, on the other hand, have not found an association between vitamin D supplementation and reduced blood pressure, and vitamin D is not recommended as a part of the antihypertensive treatment (511). Vitamin D is important for brain function, and vitamin D deficiency is also associated with several neurological diseases such as multiple sclerosis, stroke, Parkinson’s disease and Alzheimer’s disease (512). Low vitamin D levels is associated with increased risk of cancer and a decreased survival rate (513). Vitamin D has antiproliferative, antiinflammatory and antifibrotic properties, which could be beneficial in the treatment of certain types of cancers (513, 514). Vitamin D is important for the immune system, and activate immune cells to fight viral and bacterial infections (515).

There is ongoing debate as to wether vitamin D should be considered a supplement, a prophylactic or as treatment for multiple disorders. Many clinical trials have showed positive effects of vitamin D supplements on overall health, and is has been suggested as a possible treatment for several diseases, including cancer (513, 514) and CVD

(516). However, this has not been documented in RCTs to date (372, 517, 518). The results are inconclusive, and could be explained by too short follow-up time and small study-sizes with low statistical power (510).

When looking at systematic reviews and metanalysis on vitamin D in kidney transplant recipients the conclusion seems to be that vitamin D deficiency is associated with advese outcomes following transplantation, with higher mortality, lower graft function, more proteinuria and more rejection episodes (205, 484, 519). If vitamin D therapy ameliorates these outcomes is a matter of debate, but vitamin D supplementation is inexpensive, easy to use, and it does not seem to harm a kidney transplant recipient with low levels of vitamin D. If calcium levels are monitored to avoid hypercalcemia, vitamin D supplementation is safe in kidney transplant recipients (520). Patients with ESRD waiting for a transplant should also have their vitamin D levels assessed, and supplementation should be considered if vitamin D deficiency is discovered. Normal vitamin D status at time of transplantation can reduce the chance of acute rejection, and should be relatively easy to achieve (519). Vitamin D levels should be measured regularly after transplantation, yearly monitoring should be adequate (205).

The debate on vitamin D also entails a discussion on wether there is a causality between low vitamin D and disease or if it just an association. Observational studies do not meet the criteria to establish a cause-and effect relationship. Low levels of vitamin D may reflect chronic illness or poor health in general. Having in mind the multiple effects of vitamin D, serum levels have to be evaluated in clinical practice, and deficiency should be treated, to improve general health and support our fight against diseases (515). Large, well-designed RCTs will most likely shed more light on the vitamin D debate in the future, and at this time we lack compelling evidence to recommend mandatory vitamin D supplementation to all kidney transplant recipients. But overall, the opinion is that vitamin D deficiency should be supplemented (190).

**FGF23 and acute MI**

In study 3, we found that iFGF23 levels were significantly decreased prior to, and at 2 days following primary PCI in subjects with a first-time STEMI successfully revascularized. iFGF23 levels normalized on day 7 in patients without heart failure. There was a gradual increase in iFGF23 levels, above normal levels, during one-year follow-up in patients with signs and/or symptoms of heart failure during the acute phase of the MI despite the use of aldosterone antagonist. We found no direct relationship between iFGF23 levels and infarct size, myocardial volumes- or function measured by CMR, or with systemic markers of MI, inflammation, collagen turnover or LV remodeling. Taken together, the present findings suggest that the early suppression of systemic iFGF23 levels in the acute phase of MI is not directly related to the size of myocardial injury or myocardial reperfusion. Interestingly, the largest reduction in iFGF23 levels occurred in the group of patients without evidence of acute LV dysfunction, potentially suggesting that the iFGF23 reduction in the acute phase of MI may represent a physiological response to counteract myocardial failure (424).

Our findings are in accordance with clinical data from Takahashi et al who found lower levels of FGF23 in MI patients compared with controls, looking at 44 patients with acute MI who underwent PCI. They followed 22 patients with echocardiography 6 months post MI and found an association between high FGF23 and low EF at 6 months (521). Our study extends these observations to a larger population and a longer duration of one year of follow-up, using cardiac MRI to quantify the size of MI and function; also including a large number of biomarkers of collagen turnover (424).

Increased FGF23 levels in subjects with acute heart failure or LV dysfunction following acute MI has also have been reported by others. Fuernau et al. measured FGF23 in patients with acute MI complicated by cardiogenic shock. They found that non-survivors had significantly higher FGF23 levels at 1, 2 and 3 days post MI. The negative prognostic association between FGF23 and adverse outcomes was only significant in patients with creatinine levels above median at admittance (522). Poss

et al. found significantly higher levels of FGF23 in patients with cardiogenic shock, compared with patients with uncomplicated MI. Elevated FGF23 was associated with poor outcome (523). Reindl et al. looked at 88 consecutive patients with acute MI who underwent PCI. They used CMR at 2 days and 4 months post MI, and found elevated FGF23 levels in the 13% who developed LV remodeling (524). A recent study on patients with left coronary artery disease undergoing coronary artery bypass surgery found that the preoperative FGF23 level was correlated with cumulative rate major adverse cardiac and cerebrovascular events. The authors suggested FGF23 as a potential prognostic biomarker in patients with coronary artery disease (525).

Our findings are in line with these previous reports, demonstrating higher systemic FGF23 levels in subjects with the clinically worst prognosis. However, our subjects were healthier than many of the included individuals in the above mentioned studies. Our study adds to the findings described above, demonstrating that there is no direct relationship between systemic levels of FGF23 in the acute phase of an MI and the infarct size, LV mass, LV volumes or LVEF assessed by CMR. Our findings show a reduction in FGF23 levels immediately following an acute MI, which may be a normal, physiological compensatory respons to MI. Elevated FGF23 levels may represent increased aldosterone levels which is a poor prognostic sign, and elevated aldosterone leads to increased FGF23 (353, 424). Our findings are in apparent contrast to the increasing number of publications suggesting that iFGF23 have direct myocardial actions that may be relevant in the acute MI (424).

In a mouse model using transverse aortic constriction to increase FGF23 levels, Slavic et al found that the aldosterone receptor blocker spironolactone normalized serum FGF23 levels by recurring bony FGF23 transcription (352). In patients with non-ischemic heart failure without CKD, both FGF23 levels and serum aldosterone levels correlated with degree of systolic dysfunction (526). FGF23 expression is up-regulated by aldosterone in vitro as well as in mice treated with DOCA leading to hypertension and end-organ damage (353). These findings indicate that aldosterone stimulates bone-mediated FGF23 synthesis. This is consistent with our findings where the group of patients with heart failure, treated with an aldosterone antagonist, had higher FGF23 compared with patients without heart failure. The level of FGF23

might have been even higher in these patients without treatment with an aldosterone antagonist (424).

In a recently published study, a mouse model with intracardiac FGF23 synthesis was used to investigate how cardiac FGF23 affects cardiac remodeling and function in healthy mice. They did not find increased LVH in this mouse model, suggesting that FGF23 excess alone does not affect the heart (527). These results differ from other studies that suggest a direct link between FGF23 and LVH (160), but the methods differ between the two studies, and the last word on FGF23 effects in the heart has not been spoken.

It has been established that high FGF23 levels increase the risk of MI after adjustment for established CV risk factors (528). But is has yet not been determined if a reduction of FGF23 levels in patients with high CV risk levels could influence the risk of MI (440).

**Clinical implications / Prognostic value:**

Several therapies exist to target abnormalities seen in CKD-MBD, some with better documentation than others. The different treatment strategies also influence FGF23 and Klotho levels. There are several points for possible interventions. Standardized and more experimental treatment strategies are reviewed below.

*Phosphate binders*

Circulating FGF23 levels are associated with dietary phosphate load (529). Phosphate is reduced in dialysis, and phosphate levels can be influenced by reduced dietary intake, which is suggested as a first intervention in the KDIGO guidelines (184, 530). Oral phosphate binders represent the principal treatment strategy to treat hyperphosphatemia, they work by reducing the absorption of dietary phosphate in the gastrointestinal tractus (531). In the early years of dialysis, aluminum salts were used as an efficient phosphatebinder (532). It was discovered that patients receiving aluminum containing substances showed elevated aluminum levels in plasma, and some of them presented with symptoms of dialysis encephalopathy, osteomalacia and anaemia, which is related to the toxic effects of aluminum (532, 533).

Aluminum containing phosphatebinders were then replaced by calcium-based phosphatebinders in the 1980s (532). Calcium carbonate reduces phosphate levels, and for many years, calcium-based phosphate binders were widely used to control hyperphosphatemia and hyperparathyroidism in patients with ESRD (534, 535). In 2002, a comparison between calcium-based phosphate binders to the newly-developed polymer sevelamer was published. Calcium-based phosphate binders and non-calcium containing phosphate binders provided equal phosphate control in HD patients, but the use of calcium salts to correct hyperphosphatemia led to hypercalcemia and progressive coronary artery and aortic calcification, while treatment with the non-calcium containing sevelamer did not show similar calcification (536). These results changed the standardized treatment in Norway, and in 2020, 1480 patients included in the NRR were treated with non calcium-based phosphate binders, 82 received treatment with calcium-based phosphate binders, and

69 were treated with a combination of both types of phosphate binders.
Non-calcium based phosphatebinders can be used in all ESRD patients with hyperphosphatemia. KDIGO guidelines recommend avoiding hypercalcemia, they also suggest restricting the dose of calcium-based phosphatebinders (184). Phosphatebinders are taken with every meal (537), and it adds a lot of pills to the patients' already very high pill burden. For some patients, this is very challenging. When used properly, the use of calcium-based phosphate binders increase FGF23 levels (538), while non-calcium phosphatebinders are associated with improved control of SHPT and a modest reduction in FGF23 even in patients without hyperphosphatemia (539).

Recent studies indicate beneficial effects of treatment with ferric citrate as a phosphate binder, with a reduction in both phosphate and FGF23 levels (540), but ferric citrate is not commonly used in Norway. The phosphate transporter nicotinamide (niacin, vitamin B3) has also shown beneficial effects on phosphate levels (541) and reduction in FGF23 levels in mice (542). In CKD patients, nicotinamide monotherapy (543) or in combination with a phosphate binder (544) did not influence phosphate levels and other markers of mineral metabolism significantly. Magnesium has also been shown to reduce phosphate in CKD patients with elevated phosphate levels, but less efficiently than the standard treatments. Many patients experience diarrhea as a side effect (532, 545). Another approach to control phosphate levels is to target the phosphate absorption and reabsorption in the renal tubules directly using a phosphate transporter inhibitor (546), and the promotion of increased phosphate excretion might be beneficial to combine with "standard" medication inhibiting intestinal absorption (547).

In a large metanalysis from 2018, the effects of phosphatebinders on biochemical, musculoskeletal and cardiovasvular morbidity as well as death were reviewed. The conclusion in patients with CKD stage 5 was that sevelamer might lower the incidence of death (all causes) when compared to calcium-based binders, but there was no clinically significant benefit on CV death, MI, stroke or coronary artery

calcification. In CKD stages 2-5 the effects on mortality and morbidity were inconclusive, and the adverse effects were dominating (548).

*Vitamin D*

In observational studies vitamin D therapy has been linked with improved survival in patients with CKD and ESRD, despite the increased levels of calcium and phosphate following vitamin D therapy (549, 550). In an RCT on patients with CKD stages 4 and 5, paricalcitol (activated vitamin D2 analogue) did not reduce LV mass after 48 weeks of treatment (551).

Calcitriol, the synthetic analogue of vitamin D3, blocks FGF23-induced activation of FGFR4 and the growth of cardiomyocytes in cell cultures. Calcitriol has also been shown to reduce FGF23 expression, FGFR4-induced PLCɣ/ calcineurin/ NFAT signaling and LVH in rats (552). Vitamin D increases Klotho expression and reduces calcification in mice with CKD (553), suggesting that vitamin D treatment could have a positive effect on the Klotho deficiency present in patients with CKD. Taken together, a combination of VDR activation and FGF23/ FGFR4 blocade could be a future treatment strategy.

*PTH*

Calcimimetics are approved for the treatment of hyperparathyroidism in patients on dialysis. Calcimimetics activate the CaSR and inhibit PTH secretion. They are termed "calcimimetics" because they mimic or potentiate the effects of extracellular calcium on the parathyroid glands (554), which leads to reduced levels of PTH and sequentially reduced levels of FGF23 (555, 556). In secondary analysis of the EVOLVE trial, an >30% reduction of FGF23 in patients randomized to cinecalcet was associated with reduced CV mortality (557). The two available calcimimtics are cinacalcet with a p.o. administration and etecalcid with an i.v. administration. Their efficiency have been compared in an RCT, and both significantly reduced PTH in patients in dialysis. Patient preference (i.v. vs. p.o. treatment) and price should be taken into concideration when chosing strategy (558). In pre-dialysis CKD a

significant reduction in FGF23 levels is reported, but poor effect on PTH-levels and high portion of patients developing hypocalcemia and hyperphosphatemia is reported. Calcimimetics are not lisenced for use in pre-dialysis CKD patients (559, 560).

*Old strategies to influence Klotho and FGF23:*

Intensified HD treatment (561), pre-emptive transplantation (562), reduced inflammation (563, 564) and treatment of iron deficiency (565, 566) are possible targets for reducing FGF23 in HD patients. To increase Klotho one may consider exercise (567), angiotensin-receptor antagonists (568) and statins (569). The medical indications for these approaches are well established, however, they are not considered to be novel, direct targets for FGF23 and Klotho.

*New ways to taget FGF23 and Klotho:*

There is no human equivalent to a Klotho-knockout animal model, but there are examples of alterations in Klotho expression. For example, the missense mutation H193R leads to a point mutation in the Klotho gene, causing decreased Klotho, inducing hyperphosphatemia, hypercalcemia, elevated PTH, FGF23 and vitamin D levels. The mutation results in skeletal osteopenia and tumoral calcinosis with ectopic and vascular calcification (570). On the other hand, overexpression of the Klotho-gene resulted in hyperparathyroidism and hypophosphataemic rickets, with high levels of FGF23 (153). One study examined Klotho gene variations in large cohorts, and found that certain klotho genotypes are associated with decreased cognitive ability throughout life (571). There are not many examples of Klotho dysregulation in humans, but it seems like minor alterations may increase the risk of disease (572).

The development of drugs acting via Klotho is theoretically a promising new strategy for treating CKD patients, but it is still at an early stage.

Mice with αKlotho deficiency display features similar to mice with CKD, such as hyperphosphatemia, high FGF23 levels, and high CV morbidity and mortality (247). Exogenous Klotho-supplementation may be a novel therapy to delay or inhibit CKD progression, but so far this has only been tested successfully in animal models (573).

In animal models, the administration of a Klotho-gene (an adeno-associated virus containing mouse full-length cDNA) reversed many of the traits seen in Klotho-deficient mice, with a longer life-span and weight gain (574), improved kidney function after AKI (575) prevented the progression of hypertension and kidney damage (576) and protected against uremic cardiomyopathy (577) and cardiac fibrosis (578). Although gene therapy is effective in animal studies, its safety in humans has not been established, and to my knowledge, human, clinical trials are not in the pipeline. There seems to be a rationale to explore the therapeutic benefits of exogenous Klotho supplementation in the future.

Both ageing and CKD are associated with high levels of FGF23 and low levels of Klotho (123, 577, 579). An age-related physiological decline may, in part, be secondary to increased levels of FGF23 and decreased levels of Klotho (580). If this is the case, one could assume that sKlotho acts as an inhibitor of Klotho-independent actions of FGF23 by inhibiting FGF23/FGFR4 signalling by binding to the FGFR4-receptor, or by interfering with FGF23 as a decoy receptor (580). This means that the development of a Klotho-mimetic is a possible therapeutic option in the treatment of CKD (310). However, it remains unclear if Klotho is protective for the heart and kidneys in the absence of increased FGF23, or if Klotho administration could be used as an anti-ageing therapy in all individuals, independently of FGF23 levels.

The discovery of the structure of Klotho:FGF23:FGFR1 in a 1:1:1 relationship has provided new insight into the dynamics between the factors. This might reveal new therapeutic options for patients with CKD with drugs targeting the Klotho:FGF23:FGFR1 complex directly (581). Small molecules that enhance Klotho expression have been identified, with Klotho transcription activators showing the most promise, demonstrating an effect on FGF23 signalling. These compunds can serve as a starting point for research into pharmacological tools to modulate Klotho in vivo (572).

The use of anti FGF23 monoclonal antibodies such as burosumab (approved to treat x-linked hypophosphatemia) cause severe side-effects in patients with CKD by

decreasing phosphaturia (582). FGF23 neutralization with monoclonal antibodies has been shown to increase hyperphoshatemia and increase vascular calcification and mortality in rat models (583, 584).

Several studies have shown that repetitive administration of FGF23 in mice induces cardiac hypertrophy within days (160, 351, 585). FGFR-blocade is efficient in reversing LVH in rodents (160, 586), but results in hyperphosphatemia, ectopic calcium deposition and cardiac toxicity (587). These studies reflect that some systemic FGF23 effects are necessary, such as avoidance of hyperphosphatemia.

Selectively blocking the cardiac FGFR4 sounds like an ideal target from a pharmacological perspective, with a drug blocking the FGFR responsible for the adverse cardiac effects on FGF23, not FGFR1 which is critical for maintaining normal phosphate levels (310). FGFR4-antibodies have been shown to inhibit FGF23 induced hypertrophia in rat cardiomyocytes, and it decreased LVH in a nephrectomy rat model (154). Little is known whether FGFR4 blocking prevents or reverses cardiac fibrosis (588), but development of these agents could be a real possibility in the future of nephrology. FGF signaling is dysregulated in many cancer types, and FGFR activation is relevant in carcinogenesis (589). Several FGFR-inhibitors and monoclonal antibodies are currently being developed for use in oncology, and evidence suggest that FGFR-inhibitors may present antitumor activity (589, 590). In CKD a partial FGFR inhibition might be a potential mechanism to reduce the effects of very high levels of FGF23, but a full inhibition will most likely cause severe adverse effects. The fact that these drugs are being developed for use in oncology, opens the door for similar drugs being developed for use in CKD (310).

Another possibility is to target FGF23 synthesis in bone, and recently it has been suggested that the sodium-phosphate cotransporter PiT2 found in bone is a possible regulator of FGF23 synthesis. Targeting PiT2 could potentially reduce FGF23 synthesis in hyperphosphatemic disorders like CKD (591).

Recent studies show that FGF23 can activate cardiac fibroblasts, but the mechanism and its role in the development of fibrosis is not completely understood (143, 588,

592). As cardiac fibroblasts do not express Klotho, the alternative pathway through FGFR4 and the PLCɤ/ calcineurin/ NFAT alternate pathway is likely (160, 161, 552).

Oxidative stress is a major cause of MI, and MI cause local and systemic oxidative stress (593). Oxygen free radicals may damage myocardial cells and induce apoptosis and necrosis (171). Antioxidants inhihit post MI oxidative stress and improve the disease progression (593). In a rat model of MI, the Klotho-gene's antioxidative properties were explored. The Klotho-gene was injected in rats with MI to explore the effects of Klotho. They found that overexpression of the Klotho gene can repair some of the damage to myocardial tissue caused by post MI oxidative stress (594), thus finding a method to increase Klotho-levels post MI could be a possible new method to treat MI.

The osteocyte protein dentin matrix protein 1 (DMP1) has been suggested as a new target to control FGF23. In animal models DMP1 reduced FGF23, prevented LVH, corrected bone mass reduction and improved survival. But, as an adverse effect, DMP1 significantly increased phosphate levels (595). DMP1 combined with phosphate lowering therapy could be a potential new therapeutic approach.

Patients with CKD or ESRD are at increased risk for cardiac arrhythmias, due to the structural changes in their hearts. Studies with implanted loop recorders suggest that bradyarytmias are the main causes of sudden cardiac deaths in these patients, and not tachyarrythmias as one might expect (596). The precise causes of these deaths are yet to be established. Klotho has been found in the sinoatrial node of mice, and klotho-deficient mice have sinoatrial dysfunction and a higher rate of bradyarrythmias (597), suggesting that Klotho deficiency may play a role in the bradyarrythmias of patients with ESRD. FGF23 interrupts intracellular calcium cycling in the cardiomyocyte, increasing the risk for arrhythmias (347). In a rat model, the administration of FGF23 to rat cardiomyocytes caused rhythm alterations and contractile dysfunction, but administration of klotho or a FGFR-blocker reduced the pro-arrythmic activity (598). One study found that the genetic deletion of FGFR1 in mice lead to hypertension and LVH. This suggests that FGFR1 in the renal tubules play a physiological role in

regulating systemic blood pressure, and that these receptors could be a new potential target for treating hypertension (599).

*Calcification inhibitors:*

In homeostatic conditions, pro-calcifying stimuli are counterbalanced by several inhibitors. If these inhibitors are deficient or inactive, the result may be faster and more severe calcification (323). Matrix Gla Protein (MGP) is produced in VSMCs, and has a high affinity for hydroxyapatite crystals and calcium. MGP also inhibits VSMC differentiation (600). MGP is activated via a vitamin K dependent carboxylation, and MGP levels are associated with vitamin K status. MGP is consideres a predictor of calcification in CKD patients (601). Vitamin K deficiency is common in CKD patients and in kidney transplant recipients. These patients usually have low levels of MGP, which is associated with increased mortality (602). Anticoagulation with the vitamin K antagonist warfarin leads to reduced MGP activation and accelerated calcification in CKD patients and in transplant recipients (600, 603). Vitamin K supplementation in HD patients significantly reduced MGP levels, suggesting a possible effect on vascular calcification (604). Treatment with vitamin K could be beneficial in patients with both CKD and transplant recipients, but so far the results are inconclusive, and some studies are ongoing (605-608).

Magnesium is another potential calcification inhibitor in patients with CKD. Magnesium preventes VSMC differentiation and calcification in vitro (609, 610). Magnesium supplementation reduced vascular calcification in rats (611), and coronary artery calcification in patients with high CVD-risk. The study was terminated prematurely due to efficacy (612). These promising results could be relevant in kidney transplant recipients, but trials are needed before a conclusion can be made. Magnesium supplementation could be an easy, inexpensive and safe preventive approach (323).

The ongoing research on FGF23, Klotho and other possible interventions is a constantly evolving field, and more questions must be answered before therapeutic intervensions are ready for clinical use.

**Strenghts and limitations**

Paper 1

Restriction and matching are the main methods of preventing confounding in an observational cohort study. Restriction is used in this study by having selected kidney donors with an eGFR ≥60 ml/min/1.73 $m^2$ to reduce confounding by reduced kidney function. We did not perform any matching. To further examine the results, we compared the 15 controls with the lowest eGFR (mean 87 and range 73-96 ml/min/1.73 $m^2$) to the 15 donors with the best eGFR (mean 88 and range 81-103 ml/min/1.73 $m^2$). The NGAL levels were nonsignificantly higher in donors than in controls (86 vs. 105 ng/ml), showing a trend toward higher NGAL levels in a small sample. This finding supports - but does not confirm - that the increased level of NGAL in kidney donors is associated with the donation, and is not only the result of reduced postoperative kidney function. If, however, the increase in NGAL in this population of donors is the result of reduced kidney function, NGAL levels may still be important as a biomarker for people at risk of increased morbidity and mortality after donation. The small number of patients included in this study is a limitation, and prospective studies are needed to evaluate these biomarkers as prognostic markers in kidney donors (422).

Paper 2

The investigated cohort of transplanted patients represents 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 10 weeks and 1 year post-transplant (OUS, RH), and all the renal biopsies were 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 1-year investigation. We found a significant association between vitamin D levels 10 weeks post-transplant and death, and vitamin D levels and graft loss showed a trend toward 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, DGF 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 (423).

Paper 3

The study explores a relatively small sample size, which makes it susceptible to the impact of biological heterogeneity, but the trial was designed with strict selection criteria to reduce the number of confounders. The cohort represents a unique and homogenous patient sample. When dividing into two groups based on the presence or absence of acute heart failure, we faced a power-problem since the group with the additional treatment contained only 8 patients, meaning that the data provide trends rather than statistically significant data (424).

Paper 4

The median follow-up period of 89 months is longer than in most other studies. The reporting to the NRR is close to 100%, which gives complete data on the patients with a functioning graft after 7 years. The patients were all included at the same time after transplantation which is a strength in the comparability of the patients and the results. The sample size is a limitation, and it is difficult to draw definite conclusions due to a relatively low power, and the results only show trends. Vitamin D, iFGF23 and sKlotho only measured twice, and a longitudinal profile may have added additional information. The number of events (graft loss or death) are low, and we pooled the two events in the survival analyses (426).

# 6. Conclusions and future prospects

Biomarkers in CKD can help us assess disease severity, predict progression and outcome, determine CVD risk and metabolic abnormalities in CKD patients. The pathophysiology in CKD is complex, and involves many processes during its progression. Comorbidity is present in almost all patients with CKD, and underlying renal disease must also be taken into consideration. A single biomarker is probably insufficient to meet all of those expectations, but maybe a panel of biomarkers could be useful in the future.

Improved understanding in all aspects of CKD development is likely to enable the development of novel therapeutic interventions capable of reducing the risks associated with CKD/ ESRD, and to optimize the treatment for the individual patient.

NGAL is a biomarker that has been widely studied in different clinical settings, and NGAL plasma has been suggested as a candidate biomarker of AKI in patients hospitalized in intensive care units if the analysis is done shortly after admittance (613). Since there is a relationship between elevated urinary NGAL and severe clinical outcomes, the dipstick developed for emergency rooms could also be used in intensive care settings. The relationship between plasma and urinary NGAL tests, as well as their clinical usefulness should be studied more in the future. NGAL as a possible predictive biomarker for kidney injury in potential kidney donors would also be interesting to explore further.

The discovery of FGF23 and Klotho and their effects on CKD-MBD is a good example of how even well-studied processes are susceptible to new contributions, which improve knowledge and possibly also develop new targeted therapies with a potential clinical benefit. FGF23 is a potential new CKD biomarker with high value because of its early increase that preceed the increase of both PTH and phosphate. This could benefit the patient, since FGF23 rises before symptom occur. Thus, lifestyle changes and medical treatment could be initiated earlier. FGF23 is also a prognostic biomarker of CKD progression, CV morbidity and all-cause mortality in patients with CKD (440). FGF23 is both a predictor and a major player in the

pathogenesis of CKD and CVD, and therapies aimed at FGF23 control could influence the future of nephrology. Klotho is an early predictor of renal injury, but studies suggest that Klotho ameliorates acute renal injury (575) as well as the progression of CKD in glomerulonephritis (122). Klotho could, theroretically, function as a renal- and cardiovascular- protective factor.

Vitamin D deficiency is associated with adverse short- and long-term outcomes after kidney transplantation. The clinical effects and the optimal supplementation is unknown, and a large, well-designed RCTs will most likely shed more light on the vitamin D debate in the future. At this time we lack compelling evidence to recommend mandatory vitamin D supplementation to all kidney transplant recipient.

Taken together, a combination of Klotho supplementation, VDR activation and FGF23/ FGFR4 blocade could be a future treatment strategy. The development of SGLT2 inhibitors showed us that medications might have other effects than the intended one. Instead of a revolution for patients with diabetes mellitus type 2, it proved itself as a promising drug class for patients with heart failure and patients with proteinuria. Other new therapies might also surprise us in the future.

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**Original Paper**

# Neutrophil Gelatinase-Associated Lipocalin, Fibroblast Growth Factor 23, and Soluble Klotho in Long-Term Kidney Donors

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**Key Words**
Kidney donors · Biomarkers · Neutrophil gelatinase-associated lipocalin · Klotho · Fibroblast growth factor 23 · Vitamin D · Kidney transplantation

**Abstract**
***Background:*** The best treatment for end-stage renal disease (ESRD) is kidney transplantation. Twenty-seven percent of transplantations in Norway are from living donors. Recent studies have shown an increased risk of ESRD and increased mortality in donors. The aim of this study was to determine if the levels of the new biomarkers neutrophil gelatinase-associated lipocalin (NGAL), soluble Klotho (sKlotho), and fibroblast growth factor 23 (FGF23) are changed in kidney donors with normal kidney function defined as an estimated glomerular filtration rate (eGFR) >60 ml/min/1.73 $m^2$ compared to patients with chronic kidney disease (CKD) stages 3–5 and healthy controls. ***Methods:*** This is a cross-sectional, observational, single-center study including 35 kidney donors with an eGFR ≥60 ml/min/1.73 $m^2$ 5 years after donation, 22 patients with CKD stage 3 (eGFR 30–59 ml/min/1.73 $m^2$), 18 patients with CKD stage 4 (eGFR 15–29 ml/min/1.73 $m^2$), 20 patients with CKD stage 5 (eGFR <15 ml/min/1.73 $m^2$), and 35 controls comparing levels of biomarkers in long-term kidney donors with those in CKD patients and healthy controls. ***Results:*** The level of log NGAL was significantly higher in donors than in healthy controls (2.02 ± 0.10 vs. 1.89 ± 0.10 ng/ml; $p < 0.001$), and the level increased with declining kidney function. The log FGF23 level was nonsignificantly higher in donors than in controls, but it significantly increased with declining kidney function. The log sKlotho levels were significantly lower in patients with CKD stages 4 and 5 than in controls, but no difference was revealed between controls and donors. ***Conclusion:*** Kidney donors have significantly higher levels of NGAL than healthy controls after a median of 15 years (range 5–38). NGAL could be a valuable diagnostic marker in the future. FGF23 and sKlotho were not significantly different between donors and controls.



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## Introduction

In Norway the prevalence of chronic kidney disease (CKD) is 10.2% according to the second Health Survey of Nord-Trøndelag [1]. The same prevalence is reported from the USA [2]. CKD is characterized by progressive destruction of the renal parenchyma and loss of functional nephrons [3]. Only a group of patients with CKD stages 3–4 develop end-stage renal disease (ESRD) with the need for dialysis or transplantation [4].

The best treatment for ESRD is kidney transplantation. In 2013, 246 kidney transplantations were performed in Norway, of which 68 (27.6%) were from a living donor [5]. The short- and long-term effects of unilateral nephrectomy on living donors have been an important issue for more than 60 years. A living donor must undergo a medically unnecessary procedure, and safety concerns have always been the focus of attention. The short-term risk is well established with a 0.03% risk of mortality and <1% risk of major morbidity [6]. The loss of renal mass from unilateral nephrectomy in living kidney donors is associated with compensatory changes in the remaining kidney [7], and a rapid compensatory increase in glomerular filtration rate (GFR) in the remaining kidney is well documented [8].

Earlier studies claimed that living kidney donors neither have an increase in all-cause mortality nor an increased risk of ESRD compared to the general population. However, donors are selected from a group of very healthy individuals thoroughly screened for conditions such as hypertension and kidney and coronary diseases, and they do not reflect the general population. New studies comparing donors with equally healthy controls indicate an increased risk of metabolic derangements particularly involving calcium homeostasis, kidney failure, and mortality [9, 10].

The function of neutrophil gelatinase-associated lipocalin (NGAL) is largely unknown, but NGAL is associated with cellular stress, acute kidney injury, and CKD [11, 12]. Fibroblast growth factor 23 (FGF23) has an important role in calcium-phosphate metabolism, and FGF23 levels rise quickly as kidney function declines [13]. Soluble Klotho (sKlotho) is linked to aging and CKD progression. These biomarkers have a known association with CKD progression, but they are not adequately explored in long-term kidney donors [14]. The aim of this study was to determine if the levels of the new biomarkers NGAL, sKlotho, and FGF23 are changed in kidney donors with normal kidney function defined as an estimated GFR (eGFR) >60 ml/min/1.73 $m^2$ compared to healthy controls and patients with CKD.

## Subjects and Methods

The study is a cross-sectional, observational, single-center trial. Donors and patients with CKD stages 3 and 4 were identified in a local database containing all patients visiting the outpatient clinic at the renal unit of Stavanger University Hospital. Patients were included in connection with planned appointments and after giving informed consent. Patients with CKD stage 5 were all recruited from the hemodialysis unit. Patients with severe comorbidity and a limited life expectancy, as well as previously parathyroidectomized patients, were excluded. We included donors with an eGFR ≥60 ml/min/1.73 $m^2$ to avoid confounding with regard to the levels of the new biomarkers from reduced eGFR, and only patients with a follow-up of >5 years after donor nephrectomy. The time since nephrectomy varied from 5 to 38 years (median 15). We included 35 kidney donors with an eGFR ≥60 ml/min/1.73 $m^2$, 22 patients with CKD stage 3 (eGFR 30–59 ml/min/1.73 $m^2$), 18 patients with CKD stage 4 (eGFR 15–29 ml/min/1.73 $m^2$), and 20 patients with CKD stage 5 (eGFR <15 ml/min/1.73 $m^2$).

Thirty-five healthy controls were recruited from among colleagues and friends who regarded themselves as healthy. All patients and controls were older than 18 years. Written

informed consent was obtained from all patients, donors, and controls prior to their inclusion. The study adhered to the Declaration of Helsinki and was approved by the Regional Medical and Health Research Ethics Committee Western Norway.

Routine hematological and biochemical analyses, including hemoglobin, creatinine, calcium, phosphate, intact parathyroid hormone (iPTH), albumin, and electrolytes, were performed at the hospital's analytical laboratory. Urine samples were analyzed for albumin, creatinine, calcium, and phosphate in all participants except ESRD patients.

NGAL was analyzed in EDTA plasma, whereas 25(OH) vitamin D, sKlotho, and FGF23 were analyzed in serum. Serum and EDTA plasma were separated from blood cells within 1 h after collection by centrifugation for 15 min at 2,500 *g* at 4°C, and stored in aliquots at –76°C until analysis.

The eGFR was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [15]. Serum phosphate (reference range: females 0.85–1.44 mmol/l; males 13–49 years 0.78–1.52 mmol/l, ≥50 years 0.69–1.31 mmol/l), total serum calcium (reference range 2.15–2.55 mmol/l), and albumin (reference range: 0–39 years 36–48 g/l, 40–69 years 36–45 g/l, ≥70 years 34–45 g/l) were analyzed using Architect c16000TM (Abbott Diagnostics, Abbott Park, Ill., USA). Albumin-corrected serum calcium (reference range: females <54 years 2.17–2.52 mmol/l, ≥55 years 2.20–2.62 mmol/l; males 2.17–2.56 mmol/l) was calculated as follows: serum calcium + 0.02 × (41.3 – serum albumin). iPTH (reference range: 1.6–8.5 pmol/l) was determined using an intact PTH assay from Abbott Diagnostics, analyzed on an Architect i2000SR (Abbott Diagnostics).

Serum iFGF23 and sKlotho were measured by commercially available ELISA kits from Kainos Laboratories Inc. (Tokyo, Japan) and IBL (Immuno-Biological Laboratories GmbH, Hamburg, Germany), respectively. Freshly thawed samples were measured in duplicate, and the reproducibility of the methods was monitored by analyzing 3–5 aliquots of a serum control with each assay. The intra- and interassay coefficients of variation for sKlotho were <15%, and for iFGF23 they were <7 and <11%, respectively. The level of iFGF23, measured with the Kainos ELISA kit, in 104 healthy adults from Japan was reported to be in the range of 8.2–54.3 ng/l [16], and a reference range of 10–50 ng/l has been suggested based on these results [17]. sKlotho, measured by the IBL ELISA kit in serum from 142 healthy subjects, was reported to range from 239 to 1,266 pg/ml, with a mean of 562 ± 146 pg/ml, in the original publication on the method [18]. Reference ranges for males of 252–652 pg/ml and for females of 177–804 pg/ml in a healthy Danish population were recently published using the same ELISA kit [19]. Serum 25(OH) vitamin 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 analysis by liquid chromatography coupled with tandem mass spectrometry detection [20]. Levels of serum 25(OH) vitamin D are deficient below 50 nmol/l, insufficient between 50 and 75 nmol/l, and sufficient above 75 nmol/l [21]. The NGAL concentration in EDTA plasma was measured with a commercially available ELISA kit, with microwells precoated with a monoclonal antibody raised against human NGAL (KIT 036; Bio-Porto Diagnostics, Gentofte, Denmark). The analyses were performed in accordance with the manufacturer's protocol. Freshly thawed samples were measured in duplicate, and the reproducibility of the method was monitored by analyzing 4 duplicates of a plasma control with each assay. The intra- and interassay coefficients of variation were <10 and <8%, respectively [22].

The urine samples were analyzed for concentrations of creatinine (reference range: 4.5–20.0 mmol/l), albumin, calcium (reference range: 2.0–9.0 mmol/l), and phosphate. The fractional excretion of phosphate ($FePO_4$) was calculated as follows: (urine phosphate × serum creatinine) × 100/(serum phosphate × urine creatinine). The reference range for $FePO_4$ in healthy subjects is reported to be 0–20% [23]. The fractional excretion of calcium ($FeCa_3$) was calculated as follows: (urine calcium × serum creatinine) × 100/(serum calcium × urine creatinine).

**Table 1.** Baseline characteristics

| Group | Control (n = 35) | Donor (n = 35) | CKD stage 3 (n = 22) | CKD stage 4 (n = 18) | CKD stage 5 (HD) (n = 20) | p value |
|---|---|---|---|---|---|---|
| Age, years | 43.6 ± 13.1 | 56.5 ± 9.4 | 62.7 ± 16.7 | 61.7 ± 17.1 | 70.9 ± 16.6 | <0.001 |
| Sex – male/female, n | 16/19 | 21/14 | 15/7 | 11/7 | 12/8 | 0.102 |
| NGAL, ng/ml | 97.6 ± 18.3 | 110.6 ± 31.7 | 209.2 ± 67.5 | 459.5 ± 111.3 | 1,012.6 ± 374.1 | <0.001 |
| log NGAL, ng/ml | 1.89 ± 0.10 | 2.02 ± 0.10 | 2.3 ± 0.15 | 2.65 ± 0.12 | 2.98 ± 0.17 | <0.001 |
| FGF23, pg/ml | 51.8 (25.9–90) | 62.6 (6.6–112) | 97.5 (44–308) | 337.0 (139–11,000) | 806.0 (121–16,100) | <0.001 |
| sKlotho, pg/ml | 725.4 (458–1,222) | 669.3 (409–1,161) | 597.8 (449–979) | 460.8 (288–790) | 377.6 (223–784) | <0.001 |
| 25(OH) vitamin D, nmol/l | 58.67 ± 25.2 | 76.4 ± 17.0 | 44.01 ± 19.4 | 53.55 ± 22.5 | 59.65 ± 29.5 | <0.001 |
| eGFR, ml/min/1.73 $m^2$ | 99.0 ± 13.1 | 75.8 ± 12.3 | 43.7 ± 9.8 | 19.1 ± 5.8 | 7.3 ± 2.6 | <0.001 |
| Creatinine, µmol/l | 73.1 ± 12.6 | 90.3 ± 16.3 | 141.0 ± 28.1 | 280.1 ± 84.2 | 621.2 ± 203.6 | <0.001 |
| Albumin-corrected calcium, µmol/l | 2.4 ± 0.07 | 2.41 ± 0.06 | 2.46 ± 0.13 | 2.45 ± 0.13 | 2.5 ± 0.18 | 0.006 |
| iPTH, pmol/l | 5.82 ± 2.47 | 7.1 ± 2.29 | 13.63 ± 13.19 | 32.73 ± 24.34 | 47.43 ± 46.86 | <0.001 |
| Phosphate, mmol/l | 1.12 ± 0.17 | 1.06 ± 0.19 | 1.11 ± 0.16 | 1.35 ± 0.27 | 1.46 ± 0.49 | <0.001 |
| Urine phosphate, mmol/l | 16.4 (2.4–49.9) | 25.8 (2.6–50.7) | 17.2 (4.1–31.8) | 13.7 (6.5–27.8) | nn | 0.001 |
| Urine calcium, mmol/l | 2.84 ± 2.06 | 1.96 ± 1.09 | 1.53 ± 1.11 | 0.77 ± 0.12 | nn | <0.001 |
| Urine albumin/creatinine, mg/mmol | 0.61 (0.18–30.8) | 2.3 (0.1–19.9) | 21.5 (2.0–2,000.0) | 198 (3.3–1,753.0) | nn | <0.001 |
| $FePO_4$ | 12.0 (4.0–22.9) | 22.7 (3.1–41.9) | 30.5 (19.9–56.9) | 47.9 (31.6–69.1) | nn | <0.001 |
| $FeCa_3$ | 0.84 (0.20–2.5) | 0.78 (0.16–1.73) | 0.79 (0.35–3.03) | 1.13 (0.43–3.15) | nn | 0.025 |

Laboratory results are given as means ± SD of normally distributed data, and as medians (ranges) of non-normally distributed data. p values display differences between groups, using one-way ANOVA for numeric values and the $\chi^2$ test for the categorical value 'sex'. Urine values in CKD stage 5 are marked as 'nn', because the included patients were on HD and had very limited urine production, which is why no urine samples were obtained. HD = Hemodialysis.

*Statistical Analysis*

All variables normally distributed are expressed as means ± standard deviations. Other distributed data are expressed as medians and ranges. NGAL, sKlotho, and FGF23 were logarithmically transformed to obtain normal distributions.

The variables were compared by one-way ANOVA. The Mann-Whitney U test and Kruskal-Wallis test gave similar results. We used a multiple regression model with the biomarkers NGAL, FGF23, and sKlotho as dependent variables to explore the possible effects of the independent variables age, sex, 25(OH) vitamin D, phosphate, iPTH, albumin-corrected calcium, creatinine, and the other 2 biomarkers on the dependent variables. NGAL, FGF23, and sKlotho were used as dependent variables in 3 different models. We excluded nonsignificant variables using a backward selection model. We performed the analysis on the complete study population to ensure an adequate sample size.

The level of statistical significance was defined as $p < 0.05$. All statistical analyses were conducted using IBM SPSS Statistics version 22.0.

## Results

The baseline characteristics are given in table 1. The mean age of the controls was significantly lower than that of the other groups, and the mean age of the patients with CKD stage 5 was significantly higher.

The levels of log NGAL were significantly different between all groups, showing increasing levels with declining kidney function (fig. 1). Post hoc testing showed a significantly higher level of log NGAL in donors than in healthy controls ($p < 0.01$). Post hoc testing also showed differences between controls and patients with CKD stages 3–5 and between each group of patients with CKD stages 3, 4, and 5. There was no association between NGAL levels and time since nephrectomy, NGAL levels and age, or NGAL levels and 1st-degree relationship between donors and recipients. In a multiple regression analysis using log NGAL as the dependent variable and age, sex, 25(OH) vitamin D, phosphate, iPTH, albumin-corrected calcium, log sKlotho, log FGF23, and creatinine as independent variables, only creatinine remained in the model using a backward selection model.

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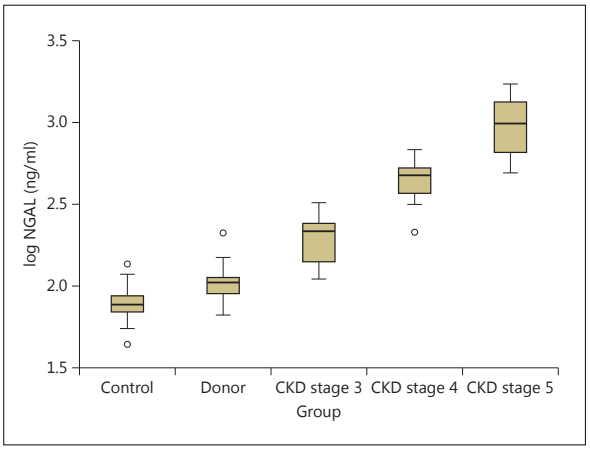


**Fig. 1.** Boxplot showing the distribution of plasma log NGAL in the different groups. The levels of log NGAL are significantly different between the groups. CKD = Chronic kidney disease.

log FGF23 levels were nonsignificantly higher in donors than in controls – and, as expected, log FGF23 levels increased significantly with declining kidney function. In a multiple regression analysis using log FGF23 as the dependent variable and age, sex, 25(OH) vitamin D, creatinine, phosphate, albumin-corrected calcium, iPTH, log sKlotho, and log NGAL as independent variables, only serum phosphate, albumin-corrected calcium, and iPTH remained in the model using a backward selection model.

There was no difference in log sKlotho levels between controls and kidney donors. log sKlotho levels declined with declining kidney function, and the log sKlotho levels were significantly lower in patients with CKD stages 4 and 5 than in controls. There was no difference in log sKlotho levels between donors and controls. In a multiple regression analysis, there was no association between log sKlotho levels and age, sex, 25(OH) vitamin D, phosphate, iPTH, albumin-corrected calcium, log NGAL, or log FGF23. Creatinine was the only variable remaining in a backward selection model.

The kidney donors had an eGFR ≥60 ml/min/1.73 $m^2$, but the eGFR was significantly lower and creatinine significantly higher than in the control group. In comparison to healthy controls, living kidney donors had significantly higher levels of iPTH ($p = 0.03$), significantly higher fractional urinary excretion of phosphate ($p < 0.001$), nonsignificantly lower serum phosphate ($p = 0.23$), and similar serum calcium levels. The donors also had significantly higher 25(OH) vitamin D levels than all the other groups. 25(OH) vitamin D levels were significantly lower in patients with CKD stage 3, with 73% of the patients having serum levels <50 nmol/l.

## Discussion

Long-term kidney donors with an eGFR >60 ml/min/1.73 $m^2$ have significantly increased levels of NGAL compared to controls as well as to patients with CKD stages 3–5 after 5–38 years (median 15). Levels of NGAL and FGF23 have an inverse association with eGFR, and the level of sKlotho declines when the eGFR declines.

NGAL was originally isolated from the supernatant of activated neutrophilic granulocytes [24, 25]. NGAL is also produced in other types of tissue, such as the kidneys. The function

of NGAL is largely unknown, but NGAL is increased in cells under 'stress' – for instance, during infections, inflammation, degeneration, or neoplastic transformation [11]. NGAL is associated with reduced kidney function in patients with CKD [12]. It is also associated with reduced kidney function in kidney transplant patients, and it is increased in stable transplant patients with subclinical tubulitis [26]. Studies of delayed kidney graft function have shown an association between delayed kidney graft function and urinary levels of NGAL and interleukin-18 on the first day after transplantation [27, 28]. Interleukin-18 is a proinflammatory cytokine and a marker of oxidative stress [29]. The increased levels of NGAL in our kidney donors may reflect a state of increased cellular 'stress' which can induce cardiovascular disease (CVD) and kidney failure.

In a prospective observational cohort study on patients with CKD stage 3 or 4, urinary NGAL was associated with an independent higher risk of death and initiation of renal replacement therapy [30]. In another study, plasma and urinary levels of NGAL were independent predictors of CKD progression even after adjusting for eGFR [31].

The hypothesis called the 'forest fire theory' claims that the increase in NGAL in CKD patients is the result of a sustained production of 'inflamed' but vital tubular cells, whereas reduced kidney function is a general loss of functional cells and nephrons. NGAL may therefore represent a real-time biomarker for ongoing kidney damage [32].

Immediately following a unilateral nephrectomy, renal blood flow increases by approximately 40%. This is associated with glomerular hypertrophy and an increase in renocortical volume. Adaptive hyperfiltration by the remaining kidney is maintained at a constant level for at least 6–8 years after donation [33]. Renal hyperfiltration is associated with an increased risk of developing hypertension and CVD, and there is an association between renal hyperfiltration and decline in eGFR [34]. This may in part explain the increased risk of cardiovascular morbidity and mortality among living donors.

In a recent study by Yoon et al. [35], NGAL levels 1 week after donor nephrectomy, but not preoperative NGAL levels, were associated with an eGFR <60 ml/min/1.73 $m^2$ 6 months postoperatively. This supports our hypothesis that increased NGAL levels in kidney donors may be a biomarker for an increased risk of development of CKD.

FGF23 is a phosphaturic hormone with an important role in calcium-phosphate metabolism. FGF23 increases phosphate excretion, and FGF23 levels rise quickly as kidney function declines [13].

Increased levels of FGF23 are associated with increased mortality in hemodialysis patients [36], as well as in patients with CKD stages 2–4 [37] and kidney transplant patients [38]. Increased FGF23 levels combined with a reduced GFR predicts rapid progression toward ESRD [39, 40]. Observational studies reported independent associations of elevated serum phosphate and FGF23 levels with risk of ESRD, CVD, and death. Excessive phosphate induces arterial calcification, and increased FGF23 probably reflects the phosphate load of the body [41]. Donor Nephrectomy Outcomes Research (DONOR) Network investigators found increased FGF23 levels 6 months after donation in an unselected group of donors [42]. In our study, the FGF23 levels were nonsignificantly increased in donors compared to controls, probably reflecting the subgroup of donors with well-preserved kidney function or a power problem.

There was no significant difference in levels of sKlotho between donors and controls, probably reflecting a near-normal eGFR in the donors. There was, however, a significant decrease in serum sKlotho in patients with CKD stages 4 and 5 compared to donors. Ever since its discovery about 15 years ago, sKlotho has been linked to reduced kidney function. sKlotho was originally identified as an antiaging protein, but at a later stage it has been shown to possess a number of biological functions [14]. sKlotho is expressed in different organs, but mostly in kidney tissue, especially in the distal tubular cells [43]. sKlotho was reported to be

closely associated with CKD progression [44–46], but another study found stable sKlotho levels in patients with CKD stages 2–4 [47]. Our results support the work of Pavik et al. [45], with a significant decrease in sKlotho in CKD stages 4 and 5, but it is still unclear if the serum levels of sKlotho reflect tissue Klotho.

*Strengths and Limitations*

The commercially available methods for the measurement of sKlotho differ in quality; thus, results may be difficult to compare between studies. The IBL kit used in this study showed a within-run variation of 4%, with good agreement between serum and EDTA plasma in a study of the 3 different Klotho assays available [48].

Restriction and matching are the main methods of preventing confounding in an observational cohort study. Restriction is used in this study by having selected kidney donors with an eGFR ≥60 ml/min/1.73 $m^2$ to reduce confounding by reduced kidney function. We did not perform any matching. To further examine the results, we compared the 15 controls with the lowest eGFR (mean 87 and range 73–96 ml/min/1.73 $m^2$) to the 15 donors with the best eGFR (mean 88 and range 81–103 ml/min/1.73 $m^2$). The NGAL level was nonsignificantly higher in donors than in controls (86 vs. 105 ng/ml), showing a trend toward higher NGAL levels in a small sample. This finding supports – but does not confirm – that the increased level of NGAL in kidney donors is associated with the donation, and is not only the result of reduced postoperative kidney function. If, however, the increase in NGAL in this population of donors is the result of reduced kidney function, NGAL levels may still be important as a biomarker for people at risk of increased morbidity and mortality after donation.

The small number of patients included in this study is a limitation, and prospective studies are needed to evaluate these biomarkers as prognostic markers in kidney donors.

## Conclusion

Long-term kidney donors have significantly higher levels of NGAL than healthy controls. This may reflect a partial loss of renal function in kidney donors as compared with a healthy control group. Renal hyperfiltration as a consequence of nephrectomy may induce cellular stress. NGAL is a potential biomarker for predicting donors at increased risk of developing CKD and premature mortality postoperatively. FGF23 levels have an inverse relationship to eGFR, and the level of sKlotho declines when eGFR declines; however, there is no significant difference in sKlotho and FGF23 levels between long-term kidney donors and controls. The hypothesis using NGAL as a prognostic marker for kidney donors should be tested prospectively in a larger cohort.

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## Authors' Contributions

- I.S. Thorsen: study design, recruiting patients, data collection, data analysis, and preparing the manuscript
- I.H. Bleskestad: data analysis and revision of the manuscript
- G. Jonsson: collecting and analyzing laboratory samples and revision of the manuscript
- Ø. Skadberg: analyzing laboratory samples and revision of the manuscript
- L.G. Gøransson: study design, data collection, data analysis, and preparing the manuscript
- All authors read and approved the final manuscript

## Disclosure Statement

The authors declare no conflicts of interests.

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# The relationship between Fibroblast Growth Factor 23 (FGF23) and cardiac MRI findings following primary PCI in patients with acute first time STEMI

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ABSTRACT

*Background:* Fibroblast growth factor 23 (FGF23) is a regulator of mineral metabolism, that has been linked to myocardial remodeling including development of left ventricular (LV) hypertrophy and myocardial fibrosis. The aim of this study was to investigate the relationship between intact FGF23 (iFGF23), myocardial infarct size and LV remodeling following a first acute ST-elevation myocardial infarction (STEMI).

*Methods and results:* Forty-two consecutive patients with first-time STEMI, single vessel disease, successfully treated with primary percutaneous coronary intervention were included. Cardiac magnetic resonance (CMR) imaging was performed at day 2, 1 week, 2 months and 1 year post MI, and blood samples were drawn at admittance and at the same time points as the CMRs. The cohort was divided according to the presence or not of heart failure post MI. In the total cohort, iFGF23 (mean ± SD) was significantly lower at day 0 (33.7 ± 20.6 pg/ml) and day 2 (31.5 ± 23.4 pg/ml) compared with a reference interval based on 8 healthy adults (43.9 pg/ml ± 19.0 pg/ml). iFGF23 increased to normal levels (55.8 ± 23.4 pg/ml) seven days post MI. In the subset of patients with signs of acute heart failure, FGF23 was higher at all measured timepoints, reaching significantly higher FGF23 levels at 2 months and 1 year following revascularization.

*Conclusion:* There was a reduction in iFGF23 levels during the acute phase of MI, with a normalization at seven days following revascularization. During one-year follow-up, there was a gradual increase in iFGF23 levels in patients with heart failure.



## 1. Introduction

Fibroblast growth factor 23 (FGF23) is a circulating phosphaturic molecule secreted mainly from osteocytes in bone, acting on the kidney and parathyroid glands to regulate the phosphate-homeostasis and vitamin D metabolism. FGF23 is also implicated in the sodium-phosphate cotransport in the proximal tubule in the kidney. Circulating FGF23 increases early in the development of chronic kidney disease (CKD), and FGF23 has emerged a powerful predictor of adverse outcomes in patients with CKD, and a potential target for medical therapy in these disorders [1–3].

Several studies have also demonstrated an association between high levels of FGF23, cardiovascular events and all-cause mortality irrespective of the presence of CKD [3–6]. To this end, the pathophysiological role of FGF23 in cardiac disease has not been established. A recent study has suggested that cardiac myelofibroblasts are the main source of FGF23 within the myocardium [7], and persistent elevation of FGF23 has been associated with

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[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation

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myocardial remodeling including the induction of left ventricular (LV) hypertrophy and myocardial fibrosis [8].

In contrast to chronic FGF23 elevation, the role of FGF23 in the setting of acute myocardial damage, such as myocardial infarction (MI) is less clear. FGF23 may induce positive ionotropic effects through alterations in intracellular calcium handling in cardiomyocytes [9]. This may potentially contribute to a compensatory enhancement of contractility in non-infarcted myocardium following acute MI. In animal models, FGF23 is upregulated in myofibroblasts following MI, suggesting a potential physiologic role of FGF23 in the acute phase of a MI [7]. Simultaneously, transforming growth factor-β (TGF-β) was found to be the strongest inhibitor of FGF23 expression, indicating the presence of counter-regulatory mechanisms attenuating the effects of FGF23. Moreover, activated macrophages and dendritic cells express high levels of FGF23, linking this mediator to inflammation. Hence, current data suggest that FGF23 reflects different aspects of myocardial injury and healing at different time-points following acute MI. However, although elevated FGF23 was found to be negatively associated with adverse outcomes and heart failure after MI [5,10], the impact of FGF23 on post-MI remodeling is still unclear. In particular, few studies have addressed the dynamic relationship between myocardial remodeling and FGF23 following acute MI.

The aim of this study was therefore to investigate the time-dependent changes in FGF23 and the relationship between circulating FGF23 levels and MI size, LV remodeling and systemic biomarkers of inflammation and collagen turnover following MI.

## 2. Materials and methods

### 2.1. Study population and study design

This is a single center observational study including patients with a first-time ST-elevation MI (STEMI) due to an occlusion of a single, large coronary artery, successfully revascularized with primary percutaneous coronary intervention (pPCI), assessed by cardiac magnetic resonance (CMR) at 4 time-points during the first year following infarction: at 2 days, 1 week, 2 months and 1 year. The CMR examinations were used to assess infarct size and left ventricular (LV) volumes and ejection fraction (LVEF). Blood samples were drawn at admittance (before revascularization), and at the same time points as the CMRs.

Inclusion and exclusion criteria have previously been described [11]. In brief 42 patients with their first-time STEMI (typical chest pain and ST-elevations on ECG) were recruited if they had acute proximal/ mid-occluded single vessel disease and went through a successful revascularization (pPCI) defined as TIMI 3 flow, and no significant residual stenosis. Patients were excluded if they had evidence of previous MI based on history, ECG or angiography, or if they had more than single vessel disease on angiography or CMR. Patients were excluded if they had co-morbidities such as chronic atrial fibrillation, malignancies, autoimmune disorders or infectious diseases, as well as reinfarction during the first week or contraindications against CMR. All patients were treated according to standard percutaneous coronary intervention (PCI) procedure, with aspirin, clopidogrel, heparin and statins. Post-MI patients were treated according to current guidelines with ACE inhibitor/angiotensin receptor blockers, diuretics and beta-blockers. They were treated with an aldosterone antagonist if indicated, in accordance with the criteria used in the EPHESUS study. Patients with left ventricular dysfunction as documented by a left ventricular ejection fraction of 40 percent or lower, and heart failure as documented by the presence of pulmonary rales, chest radiography showing pulmonary venous congestion. In patients with diabetes who met the criteria for left ventricular dysfunction after acute myocardial infarction, symptoms of heart failure did not have to be demonstrated, since such patients have an increased risk of cardiovascular events similar to that of nondiabetic patients with symptoms of heart failure.[12]. None of our 8 patients receiving aldosterone antagonist, were started because of diabetes. They all had EF < 40% and clinical signs of heart failure. We divided the cohort according to the presence or absence of heart failure post MI, using +/- indication for aldosterone antagonist to divide the group.

The Regional Ethics Committee at the University of Bergen approved the study, and all the patients gave their written informed consent prior to inclusion. The investigation was performed according to the principles in the Declaration of Helsinki.

### 2.2. Cardiac magnetic resonance imaging

The CMR images were ECG-gated and obtained during breath holding. Patients were scanned in a supine position by a 1.5 T whole body scanner (Intera™ R10, Philips Medical Systems, Best, The Netherlands) using a dedicated cardiac coil. Resting LV function was determined with cine images using a steady-state free precession technique. For first-pass perfusion, a turbo field echo sequence with three short-axis slices per heartbeat (prospective triggering) and a selective saturation recovery prepulse was used. Gadolinium-based contrast agent (Omniscan™, Amersham Health, Little Chalfont, UK) was given at a dose of 0.075 mmol/kg. Immediately following the completion of the first-pass imaging, another 0.175 mmol/kg of contrast agent was infused, and 10–15 min following the latter infusion-delayed hyper enhancement images were acquired. All post- processing was performed on the View Forum™ Software (Philips Medical Systems, Best, The Netherlands) in a random, blinded fashion. Assessment of body surface indexed end-diastolic (LVEDVi), end-systolic (LVEDVSi), and EF was done by short-axis volumetry. Infarct size was assessed manually with planimetry on each short-axis slice by delineating the hyper enhanced area from the non-enhanced myocardium [13].

### 2.3. Blood sampling protocol

Venous blood samples were collected on admission to the hospital, immediately prior to PCI, and 2 days, 7 days, 2 months and 1 year following MI. The pyregen-free blood collection tubes were immediately immersed in melting ice (ethylenediaminetetraacetic acid-containing tubes, plasma) or placed in room temperature (tubes without any additives, serum) and centrifuged within 20 min at 2500*g* for 20 min to obtain platelet-poor plasma, or centrifuged at 1000*g* for 10 min after coagulation (serum). All samples were stored at −50 °C and thawed only once. Biomarkers were analyzed in one run, after the study was completed.

### 2.4. Biomarkers

Plasma **iFGF23** was measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits from Immutopics International, San Clemente, CA, USA. Freshly thawed samples were measured in duplicates, and the reproducibility of the method monitored by analyzing 4 aliquots of a control with each assay. Intra- and inter-assay coefficient of variation for iFGF23 was <8%. The reference interval for iFGF23 based on 8 healthy adults measured in the same laboratory was on average 43.9 pg/ml (±19.0 pg/ml). Serum levels of connective tissue growth factor (s-**CTGF**) were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) [14]. The concentration of transforming growth factor b1 (**TGF-b1**) was measured by EIA (R&D Systems) [15]. Serum **25(OH) vitamin D** were 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 analysis by liquid chromatography coupled with tandem mass spectrometry detection [16]. In this study, levels of serum 25 (OH) vitamin D are termed deficient < 30 nmol/l (<12 ng/mL), insufficient at 30–50 nmol/L (12–20 ng/mL), sufficient at >50 nmol/L (>20 ng/mL) [17].

Amino-terminal propeptide of procollagen type I (PINP), amino-terminal propeptide of procollagen type III (PIINP) and carboxyterminal type I telopeptid (CITP) as markers of extracellular matrix (ECM) remodelling were analysed using a radioimmunoassay from Orion Diagnostica (Finland). The lower detection limit was 2.0 ng/mL for PINP, 0.20 ng/mL for PIIINP and 0.50 ng/mL for CITP respectively.

C-reactive protein (**CRP**) concentrations were measured by a particle enhanced immunoturbidimetric method with the use of Roche ModularP automated clinical chemistry analyser (Roche Diagnostics, Basel, Switzerland) and reagents of Tina-quant C-reactive protein (latex) assay (Roche Diagnostics), reference range 0–7 mg/L. **Creatinine** was analysed on Roche Modular P, enzymatic colorimetric assay (CREA plus), (ref.range 60–105 μmol/L for men and 45–90 μmol/L for women), **calcium** was analyzed on Roche Modular P using colorimetric assay (ref.range 2.15–2.55 mmol/L), inorganic phosphorus (**phosphate**) was analyzed on Roche modular P (ref.range 0.85–1.44 mmol/L). Troponin T (**TnT**) concentrations were measured on Roche Elecsys 2010 (Roche Diagnostics), with the immunoassay Troponin T (Roche Diagnostics), using biotinylated monoclonal troponin T-specific antibody and a monoclonal troponin T-specific antibody labelled with ruthenium forming a sandwich complex, reference range $\leq$ 14 μg/L. N-terminal pro B-type natriuretic peptide (**NT-proBNP**) was measured with a Roche Diagnostics NT-proBNP assay on an Elecsys 2010 analyser, reference range 0–100 pg/mL. Plasma intact parathyroid hormone (**iPTH**) was determined using an intact PTH assay from Abbot Diagnostics, analyzed on Architect i2000SR (Abbott Diagnostics), reference range 1.6–8.5 pmol/L.

### 2.5. Statistical analysis

Baseline characteristics were assessed by standard descriptive statistics. Data are expressed as mean ± SD for normally distributed data or median and range for non-normally distributed data. Area under the curve (AUC) was calculated for each biomarker at different time points during 1-year follow-up, and correlations were measured cumulatively. ANOVA was used to test for differences between the variables measured at multiple time points, with Bonferroni post-hoc testing where appropriate. Univariate correlations were performed using non-parametric methods (Spearman). Non-parametric tests (Mann-Whitney U, Kruskal-Wallis) were used to test for differences between groups. A two-tailed p-value of <0.05 was considered significant. SPSS version 24.0 was used for statistical analysis.

## 3. Results

### 3.1. iFGF and markers of extracellular matrix turn over following MI

The mean (SD) age was 58 (12) years, 34 males and 8 females, Mean (SD) BMI was 27.2 (3.4), mean (SD) blood pressure was systolic 140 (27) mmHg, and diastolic 84 (20) mmHg. 10 patients were previously treatment for hypertension, 3 were diabetic, 20 were current smokers. Mean (SD) creatinine levels were 74.9 (17.4) μmol/L.

Several significant patterns were revealed comparing the pattern during follow-up in MI patients. First, iFGF23 was significantly lower day 0 and day 2 compared with the levels measured in healthy adults, with a significant increase in concentrations during follow-up reaching levels comparable/higher than healthy adults on day 7 with the highest concentrations at 1 year, Fig. 1.

Second, type I collagen degradation marker (CITP) increased significantly from day 0 to day 2, and remained elevated throughout the observation period indicating a prolonged increased collagen type I degradation following MI. Third, in contrast to this pattern, type I collagen production marker (PINP) showed a late, significant increase from day 0 reaching the highest level at 1 year, suggesting a slower increase in collagen type I synthesis. Fourth, collagen type III production marker (PIIINP) showed a rapid increase during the first week, with persistent elevation for the rest of the observation period, suggesting a rapid increase in type III collagen synthesis. Finally, there was no significant changes during follow-up in 25 (OH) vitamin D, CTGF, TGF-β, creatinine, calcium, phosphate and PTH levels, Table 1. There was no correlation between iFGF23 levels and the other biomarkers at any time-points. However, there was a strong correlation between the AUC of FGF23 and CTGF during the 1-year follow-up ($r = 0.53$, $p < 0.01$).

### 3.2. Cardiac magnetic resonance imaging following MI

CMR findings are listed in Table 1. In the whole group of patients there was a significant reduction in infarct size from 16.1 to 9.8 g/m$^2$ ($p < 0.001$) and myocardial mass from 66.0 g/m$^2$ to 58.3 g/m$^2$ ($p = 0.002$) from day 1 to 1 year post MI. LV ejection fraction (EF) increased from 47.2% at baseline to 52.7% at one year follow-up ($p < 0.001$). Left ventricular end systolic volume (LVESVi) and left ventricular end diastolic volume (LVEDVi) did not change significantly, Table 1. In the group as a whole, there was no correlation between iFGF23 levels and the CMR findings at any time-points.

### 3.3. FGF23 levels in patients with impaired post-MI myocardial function in the acute phase

As previously described in methods, the studied cohort was divided according to the presence or absence of LV dysfunction or heart failure during the acute course of the MI. A total of 8 patients had LVEF < 40% and signs of acute heart failure. These patients had larger infarct sizes, lower EF and increased end diastolic- and end systolic volume on MRI, Fig. 2.

All the patients with heart failure (n = 8) were treated with aldosterone antagonists following revascularization. iFGF23 levels were higher at all time-points in this group compared with the other patients (n = 34), and significantly higher at day 60 (70.6 ± 23.7 pg/ml vs 51.7 ± 21.2 pg/ml, $p = 0.025$) and 1 year (83.7 ± 41.8 pg/ml vs 52.0 ± 18.9 pg/ml, $p = 0.019$) following MI, Fig. 3. Absolute change in iFGF23 levels from baseline to 1 year follow up was 40.0 pg/ml in the heart failure group, and 21.0 pg/ml in the no heart failure group.

In the patients with acute heart failure, there was a significant correlation between the AUC of the iFGF23 concentrations 7 days post MI and CMR findings on day 7, with LVEF ($r = -0.76$, $p < 0.05$), infarcted mass ($r = 0.75$, $p < 0.05$) and infarct size in percent of LV mass ($r = 0.71$, $0 < 0.05$).

## 4. Discussion

In the present study we show a significant reduction in systemic iFGF23 levels in the very early phase of acute MI followed by a gradual increase during follow-up reaching the highest levels after one year. Notably, patients with heart failure during the acute phase following MI had a significantly higher iFGF23 concentration from day 7 post MI. In contrast, no direct relationship was found

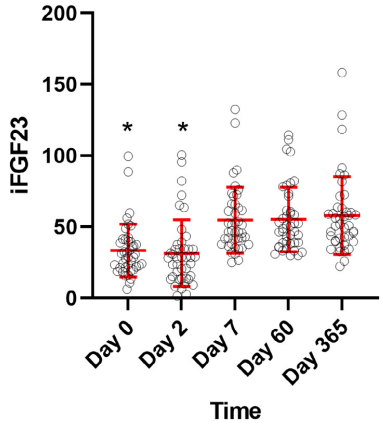


**Fig. 1.** iFGF23 measurements at the different timepoints, Mean ± SD in red. Y-axis: iFGF23, X-axis: time, *p < 0.05, compared with reference interval of 43.9 pg/ml (±19.0 pg/ml). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between iFGF23 levels and infarct size, myocardial volumes- or function measured by cardiac MRI. There was no correlation with other systemic markers of MI, inflammation, collagen turnover or LV remodeling. Taken together, the present findings suggest that the early suppression of systemic iFGF23 levels in the acute phase of MI is not directly related to the size of myocardial injury or myocardial reperfusion. Interestingly, the largest reduction in iFGF23 levels occurred in the group of patients without evidence of acute LV dysfunction, potentially suggesting that the iFGF23 reduction in the acute phase of MI may represent a physiological response to counteract myocardial failure.

Our findings are in accordance with clinical data from Takahashi et al who found lower levels of FGF23 in MI patients compared with controls, looking at 44 patients with acute MI who underwent PCI. They followed 22 patients with echocardiography 6 months post MI and found an association between high FGF23 and low EF at 6 months [18]. Our study extends these observations to a larger population and a longer duration of one year follow-up, using cardiac MRI to quantify the size of MI and function; also including a large number of biomarkers of collagen turnover.

We found increased FGF 23 levels in subjects with acute heart failure or LV dysfunction following acute MI as also have been reported by others. Fuernau et al. measured FGF23 in patients with acute MI complicated by cardiogenic shock [19]. They found non-survivors to have significantly higher FGF23 levels at 1, 2 and 3 days post MI. However, the negative prognostic association of FGF23 with adverse outcomes was only significant in patients with creatinine levels above median at admittance [19]. Poss et al. found significantly increased levels of FGF23 in patients with cardiogenic shock, compared with patients with uncomplicated MI [8]. Reindl et al. looked at 88 patients with acute MI who also underwent PCI. They found higher FGF23 levels in patients who developed LV remodeling (13%) on MRI, examined 2 days and 4 months post MI [20]. Our findings are in line with these previous reports, demonstrating higher systemic FGF23 levels in subjects with the clinically worst prognosis. However, our study adds to these findings demonstrating that there is no direct relationship between systemic levels of FGF23 in the acute phase of an MI and the infarct size, LV mass, LV volumes or LVEF assessed by CMR.

Our findings are in apparent contrast to the increasing number of publications suggesting that iFGF23 have direct myocardial actions that may be relevant in the acute MI [7,21]. FGF23 has been shown to induce LV hypertrophy independently of the most common FGF23 receptor complex consisting of Klotho/ FGF23 receptor, by activating FGF receptor 4 (FGFR4) and subsequently the calcineurin/ nuclear factor of activated T cells (NFAT) signaling in vivo and in vitro [22,23]. In situations with decreased FGF23 post MI, calcitriol levels increase leading to a blockade of the NFAT signaling pathway. This is possibly a protective mechanism attenuating the development of LV hypertrophy and myocardial fibrosis. It may be speculated that in large MIs, this downregulation is overturned into upregulation of FGF23 and enhanced development of fibrosis post-MI with increased levels of local produced FGF23 and an upregulation of FGFR4 in the myocardium secondary to inflammation [24].

In vitro data also indicate that FGF23 stimulate proliferation and migration of fibroblasts essential to development of replacement fibrosis necessary for normal scar healing and the prevention of wound rupture [25]. In the acute phase a downregulation of FGF23 might protect the heart from developing scarring and fibro-

**Table 1**
Biomarkers and MRI results.

| Laboratory parameters | Day 0 | Day 2 | Day 7 | Day 60 | Day 365 | p-value (ANOVA) |
|---|---|---|---|---|---|---|
| iFGF23 (pg/ml) | 33.4 ± 18.5 | 31.5 ± 23.4 | 54.7 ± 23.1 | 55.3 ± 22.7 | 58.0 ± 27.3 | <0.001 |
| 25 (OH)D (nmol/l) | 51.9 ± 19.7 | 48.6 ± 18.1 | 51.3 ± 19.1 | 51.7 ± 18.8 | 54.4 ± 19.6 | 0.641 |
| CITP (ng/mL) | 3.0 ± 1.4 | 4.1 ± 1.6 | 4.0 ± 1.8 | 4.4 ± 3.1 | 4.6 ± 2.5 | 0.006 |
| PINP (ng/mL) | 37.9 ± 17.0 | 37.9 ± 16.4 | 37.7 ± 16.0 | 46.0 ± 19.3 | 57.1 ± 46.0 | 0.002 |
| PIIINP (ng/mL) | 4.1 ± 1.3 | 4.9 ± 1.7 | 4.9 ± 1.6 | 4.6 ± 1.3 | 4.9 ± 1.5 | 0.083 |
| CRP (mg/L) | 5.5 ± 7.5 | 46.1 ± 45.7 | 17.3 ± 26.2 | 2.2 ± 1.8 | 1.8 ± 2.1 | <0.001 |
| TnT (μg/mL) | 0.16 ± 0.24 | 7.14 ± 5.18 | 4.25 ± 3.25 | 3.46 ± 2.83 | 3.13 ± 2.47 | <0.001 |
| TGF-β (ng/mL) | 19.7 ± 7.2 | 21.1 ± 6.6 | 23.3 ± 7.1 | 21.1 ± 6.4 | 22.0 ± 5.3 | 0.163 |
| CTGF (ng/mL) | 134.3 ± 66.3 | 140.8 ± 81.4 | 158.5 ± 183.3 | 163.7 ± 209.9 | 130.9 ± 71.6 | 0.749 |
| NT-proBNP | 12.9 (2.5–345.7) | 157.0 (24.7–1556.0) | 55.4 (14.4–886.4) | 34.9 (7.7–446.0) | – | <0.001 |
| **CMR** | | | | | | |
| Myocardial mass (g/m$^2$) | – | 66.0 ± 10.1 | 65.2 ± 10.2 | 59.8 ± 10.9 | 58.3 ± 12.5 | 0.002 |
| Infarcted myocardium (g/m$^2$) | – | 16.1 ± 7.4 | 13.7 ± 6.9 | 10.5 ± 6.5 | 9.8 ± 6.6 | <0.001 |
| Infarct size (%) | – | 24 ± 9 | 21 ± 9 | 17 ± 8 | 16 ± 8 | <0.001 |
| LVEF | – | 47.2 ± 9.0 | 50.2 ± 7.9 | 52.9 ± 9.5 | 52.7 ± 10.6 | 0.020 |
| LVEDVI (mL/m$^2$) | – | 90.0 ± 13.6 | 90.1 ± 15.5 | 89.6 ± 16.5 | 91.1 ± 21.9 | 0.996 |
| LVESVI (mL/m$^2$) | – | 48.5 ± 12.8 | 45.9 ± 12.8 | 45.6 ± 16.8 | 44.9 ± 19.9 | 0.614 |

*Normally distributed values are expressed as mean ± SD.*
iFGF23 = intact fibroblast growth factor 23, 25 (OH)D = 25-hydroxyvitamin D, CITP = C-terminal telopeptide of type I collagen, PINP = Amino terminal propeptide of type I procollagen, PIINP = amino terminal propeptide of type III procollagen, CRP = C-reactive protein, TnT = troponine T, TGF-β = transforming growth factor-β, CTGF = connective tissue growth factor, NT-proBNP = N-terminal pro B-type natriuretic peptide, EF = ejection fraction, LVEDVI = left ventricular end diastolic volume per m$^2$, LVESVI = left ventricular end systolic volume per m$^2$.

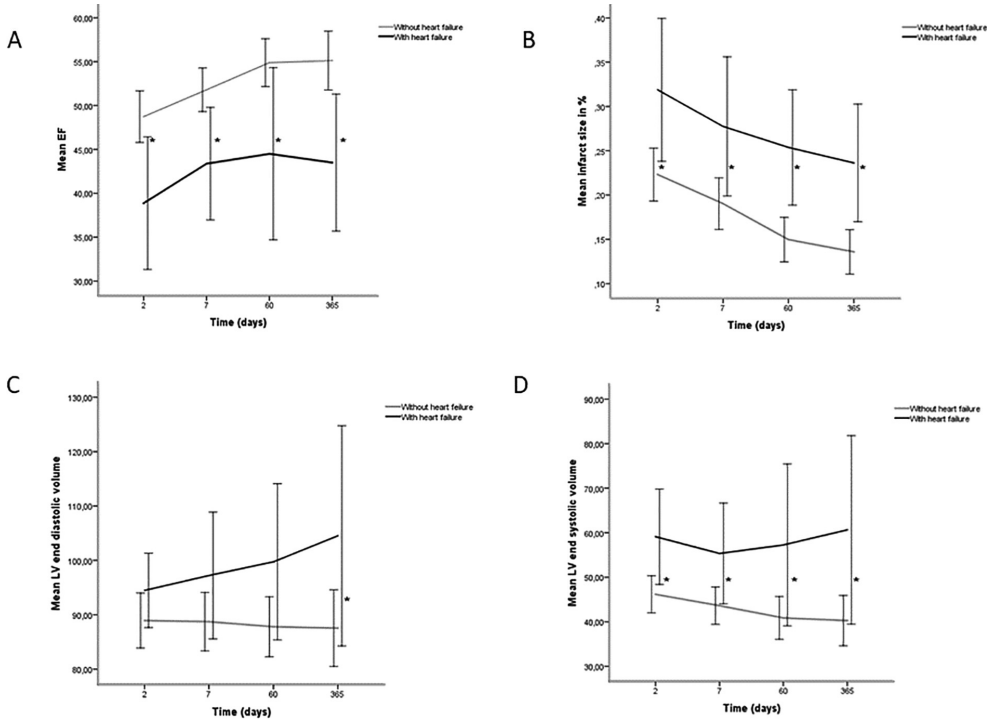


**Fig. 2.** (A) Ejection fraction, (B) infarct size, (C) left ventricular end diastolic volume per $m^2$, (D) left ventricular end systolic volume per $m^2$ in patients without heart failure and with heart failure on treatment with an aldosterone antagonist post MI expressed as mean (95% CI), *p < 0.05.

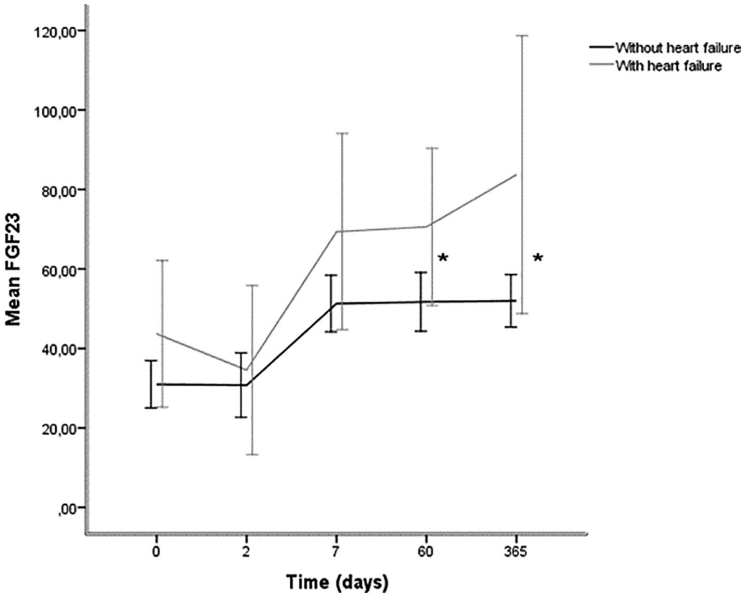


**Fig. 3.** FGF23 levels in patients without heart failure and with heart failure on treatment with an aldosterone antagonist post MI, mean and 95% CI. Y-axis: iFGF23 concentration, X-axis: time, *p < 0.05.

sis by increasing the level of calcitriol and thereby blocking the PCγ/C/NFAT signaling pathway for FGF23 in the myocardium [9]. Inflammation might be a mechanism linking FGF23 to cardiovascular disease [26]. It has been shown that FGF23 in cardiac fibroblasts, is upregulated in systemic inflammation [24]. Herein we found a significantly increased CRP ($p < 0.001$) at day 2 and day 7 post-MI, Table 1, but there was no significant difference between CRP levels in the 2 groups of patients in our study, questioning the link between FGF23 and inflammation in clinical MI.

Slavic et al have identified aldosterone as an important stimulator of bony FGF23 transcription, where blockade with spironolactone normalized serum FGF23 levels and bone transcription [27]. Serum aldosterone levels correlated with FGF23 in patients with non-ischemic heart failure [28]. Aldosterone increased FGF23 levels in vitro as well as in mice treated with deoxycorticosterone acetat (DOCA) leading to hypertension and end-organ damage [29]. These findings indicate that aldosterone stimulates bone-mediated FGF23 synthesis. This is consistent with our findings where the group of patients with heart failure, treated with an aldosterone antagonist, had higher FGF23 compared with patients without heart failure. The level of FGF23 might have been even higher in these patients without treatment with an aldosterone antagonist.

## 5. Strengths and limitations

The study explores a relatively small sample size, which makes it susceptible to the impact of biological heterogeneity, but the trial was designed with strict selection criteria to reduce the number of confounders. The cohort represents a unique and homogenous patient sample. When dividing into two groups based on the presence or absence of acute heart failure, we faced a power-problem since the group with the additional treatment contained only 8 patients, meaning that the data provide trends more than statistically significant data.

## 6. Conclusion

iFGF23 levels were significantly decreased prior to, and at 2 days following pPCI in subjects with a first-time STEMI successfully revascularized. iFGF23 levels normalized on day 7 in patients without heart failure. There was a gradual increase in iFGF23 levels, above normal levels, during one-year follow-up in patients with signs and/or symptoms of heart failure during the acute phase of the MI despite the use of aldosterone antagonist.

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## Declaration of Competing Interest

The authors report no relationships that could be construed as a conflict of interest.

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# Klotho and Fibroblast Growth Factor 23 are independent of vitamin D, and unlike vitamin D, are not associated with graft- and patient survival after kidney transplantion

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**Authorship page:**

Inga S Thorsen participated in the design of the study, data analysis, writing and reviewing the manuscript. Inger H Bleskestad participated in critically reviewing the article. Anders Åsberg participated in the design of the study, supplying data from the Norwegian Renal Registry, data analysis and critically reviewing the article. Grete Jonsson and Øyvind Skadberg participated in the analysis of Klotho and FGF23, and critically reviewing the article. Kristian Heldal participated in writing and critically reviewing the article. Lasse G Gøransson participated in making the study design, data analysis, writing and critically reviewing the manuscript.

All authors have read and approved the final manuscript.

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**Abbreviations:**

| | |
|---|---|
| BMI | body mass index |
| BP | blood pressure |
| CKD | chronic kidney disease |
| CKD-MBD | chronic kidney disease-mineral bone disorder |
| CVD | cardiovascular disease |
| eGFR | estimated glomerular filtration rate |
| ESRD | end stage renal disease |
| FGF23 | Fibroblast Growth Factor 23 |
| Hba1c | glycosylated hemoglobin |
| iFGF23 | intact Fibroblast Growth Factor 23 |
| iPTH | intact parathyroid hormone |
| MR | mineralcorticoid receptor |
| NRR | Norwegian Renal Registry |
| OUS-RH | Oslo University Hospital, Rikshospitalet |
| PWV | pulse wave velocity |
| SD | standard deviation |
| sKlotho | soluble α-Klotho |
| SUS | Stavanger University Hospital |
| Tx | transplantation |

**Abstract**

Introduction:

Short-term survival after kidney transplantation is excellent, but long-term survival remains suboptimal. The aim of the study was to explore the relationship between soluble α-Klotho (sKlotho) and intact Fibroblast Growth Factor 23 (iFGF23) measured 8 weeks and 1 year post-transplant with long-term graft- and patient survival in a cohort of kidney transplant recipients with deficient and non-deficient vitamin D (25(OH)D) levels.

Subjects and methods:

Vitamin D, sKlotho and iFGF23 were measured 8 weeks and 1 year post-transplant in 132 recipients transplanted between November 2012 and October 2013.

Results:

Of the 132 kidney transplant recipients, 49 had deficient vitamin D levels (<30 nmol/L), 83 had non-deficient vitamin D levels (≥30 nmol/L) at eight weeks post-transplant. Mean age was 51 years and median follow-up was 7.4 years. At 1 year post-transplant, vitamin D increased significantly. There were no significant difference in sKlotho- or iFGF23 levels between the two vitamin D groups neither at 8 weeks nor 1 year. sKlotho increased significantly and iFGF23 decreased significantly in the whole cohort. During follow-up, there were 36 graft losses (27%) and 27 deaths (20%). 94% of the transplant recipients with non-deficient vitamin D levels were alive with a well-functioning graft after 5 years using Kaplan-Meier survival estimates, compared with 84% of the patients with deficient vitamin D levels (p=0.014). Klotho and FGF23 levels did not influence graft- and patient survival.

Conclusion:

In this nation-wide cohort of kidney transplant recipients, long-term graft- and patient survival were significantly better in patients with vitamin D ≥ 30 nmol/L 8 weeks post-transplant compared with those with vitamin D <30 nmol/L. sKlotho levels increased and iFGF23 levels decreased from 8 weeks to 1 year post-transplant. Klotho and FGF23 levels were not associated with graft- and patient survival.

## Introduction

Kidney transplantation is the preferred renal replacement therapy for most patients with end stage renal disease (ESRD) [1,2]. Observational studies show that a successful kidney transplantion is associated with a substantial reduction in the risk of mortality and cardiovascular events, as well as a relevant improvent in quality of life, compared with patients remaining in dialysis [1,2]. Short-term results after kidney transplantation have become excellent during recent decades, but long-term prognosis has not improved substantially since the 1990s [3]. The mortality risk in transplant recipients is still very high compared with the general population, and five-year survival after transplantation is actually comparable to the prognosis associated with many invasive malignancies [4]. Cardiovascular disease (CVD) is the number one cause of death, followed by malignant diseases and infections [5].

Chronic kidney disease-mineral bone disorder (CKD-MBD) is a term that describes a broad clinical syndrome that develops as a systemic disorder of mineral and bone due to CKD. The syndrome manifests in bone and mineral abnormalities, histological bone disease, calcification of vasculature and soft tissues, and increased cardiovascular morbidity and mortality [6].

Vitamin D is a steroid hormone with many effects in the human body. One of the most important roles of vitamin D is to maintain skeletal calcium homeostasis. 25(OH)D is the form of vitamin D measured in serum, and reflects body stores [7]. Increased PTH secretion induce calcitriol formation. Calcitriol, on the other hand, inhibits the synthesis and secretion of PTH, providing negative feedback regulation of calcitriol [8]. The levels of vitamin D are hypothesized to affect the immune system and may have protective effects against cancer [9], CVD [10], infection [11] and mortality [9].

Vitamin D deficiency can result in reduced bone density, osteoporosis and increased risk of fractures [12].

Arterial stiffness kan be estimated by measuring pulse wave velocity (PWV), which is the velocity at which the blood pressure pulse propagates through the circulatory system. PWV is the gold standard for a non-invasive evaluation of arterial stiffness [13]. Increased PWV predicts future cardiovascular events and all-cause mortality independently of conventional risk factors[14]. Calcification of central arterial vessels contributes to increased PWV. PWV is recognized as an indicator of target organ damage and a useful additional test in the investigation of hypertension [14].

Fibroblast growth factor 23 (FGF23) is a circulating phosphaturic hormone secreted mainly from osteocytes in bone, acting on the kidneys and parathyroid glands to regulate the phosphate-metabolism and vitamin D metabolism [15]. Circulating FGF23 increases early in the development of CKD to increase phosphate excretion [15]. At a certain point, this mechanism is overloaded, and in dialysis patients, FGF23 levels are increased up to 1000 times the normal range [16]. FGF23 is synthesized as an inactive form, and is activated when a signal peptide is cleaved off, and the active form, the intact FGF23 (iFGF23) peptide is formed [17]. FGF23 inhibits 1-α-hydroxylase in the kidney which leads to reduced vitamin D levels [18]. Elevated FGF23 levels are also associated with CVD [19], mortality [19] and reduced graft-function post-transplant [20]. FGF23 can be measured with different immunoassays, where some measure iFGF23, and others measure C-terminal FGF23 [21]. In this study we measured iFGF23.

Klotho was discovered in 1997 as an anti-ageing membrane protein in a mouse model of human ageing. In animal studies Klotho has kidney protective characteristics, participates in the calcium-phosphate homeostasis, in addition to anti-

ageing and cardiovascular protective properties [22]. The Klotho gene encodes a transmembrane protein called α-Klotho that contains a short intracellular domain and an extracellular domain. Klotho protein exists in several forms including the membrane bound full-length form, and a soluble circulating form (sKlotho) [23]. The membrane bound Klotho acts as a co-receptor for FGF23, and the tissue specific Klotho expression influence the organ specific effects of FGF23[22,23]. sKlotho is present in the blood, urine and cerebrospinal fluid where it performs a multitude of functions, but the details are not fully understood [23]. sKlotho was measured in the present study. sKlotho is stimulated by vitamin D receptor activators [24] and by inhibition of the renin-angiotensin-aldosterone system [25]. sKlotho levels are down-regulated in the presence of albuminuria, inflammation and acute kidney injury [26]. sKlotho levels are reduced when kidney function declines [27], and low levels of sKlotho are associated with increased risk of cardiovascular events and mortality in patients with CKD and ESRD [28].

In a previous study [29], we found better long-term graft- and patient survival in 762 first-time kidney transplant recipients with vitamin D sufficiency compared with patients with vitamin D insufficiency and deficiency. The aim of this study was to explore the relationship between sKlotho and iFGF23 measured 8 weeks and 1 year post-transplant with long-term graft and patient survival in two groups of kidney transplant recipients with deficient and non-deficient vitamin D levels 8 weeks post-transplant.

**Patients and methods**

This is a prospective, observational cohort study including 132 kidney transplant recipients transplanted between November 19, 2012 and October 31, 2013. In Norway, solid organ transplantation is centralized to one center, Oslo University

Hospital, Rikshospitalet (OUS-RH). A total of 236 patients received a single kidney transplant in the same period, 170 from a deceased donor and 66 from a living donor. Thirtyfour of the 236 were re-transplantations. Eight weeks post-transplant all patients are subjected to an in-depth investigation at the laboratory for Renal Physiology at OUS-RH where vitamin D levels were analysed and blood samples biobanked. We randomly selected 132 transplant recipients based on vitamin D levels only without taking any other information into consideration. Patients with a functioning kidney graft 1 year post-transplant are invited to OUS-RH for clinical examination and laboratory follow-up. One hundred and twenty-eight of the 132 patients included (97%) attended the 1 year follow-up.

Standard immunosuppression regimen included basiliximab and methylprednisolone induction. The maintenance immunosuppression consisted of a calcineurin inhibitor (tacrolimus or cyclosporine) in combination with mycophenolate and prednisolone [30]. National guidelines regulate the immunosuppressive protocol, and the follow-up is done by the local nephrologists after the first 8 weeks post-transplant.

In the routine in-depth investigation 8 weeks and 1 year post-transplant, vital signs, blood pressure (BP) and blood samples for clinical chemistry were obtained. All patients were examined with aortic (carotid-femoral) PWV using SphygmoCore®. The blood samples were collected after an overnight of fasting (food, drugs and other beverages than water), and routine laboratory samples including 25(OH) D were analyzed in fresh samples at the Department of Medical Biochemistry at the transplant center. Blood samples were also biobanked at -70˚C in the Diagnostic and Treatment biobank “Nyrefysiologisk laboratorium” (Biobank nr 266-2005-142234) within one hour. Plasma samples for analysis of sKlotho and iFGF23 were obtained from the biobank and measured en bloc at Stavanger University Hospital (SUS). The

samples were only thawed once, and the biomarker results were not reported to the treating physicians.

Long-term outcomes from time of transplantation until graft failure, death or end of study March 01, 2022 were retrieved from the Norwegian Renal Registry (NRR) where annual data are collected on the entire Norwegian transplant population. The reporting to the NRR is closely monitored, and the coverage for individuals and annual data are >99.9% and 96-98%, respectively.

The study was approved by the Regional Medical and Health Research Comittee in South-East Norway (2014/455). Written and informed consent was obtained from all patients before any data or biological material was included in the NRR and the biobank at the transplant center.

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 estimated glomerular filtration rate (eGFR) was calculated using the original CKD Epidemiology Collaboration equation [31].

HbA1c results were given in % and converted to mmol/mol using the formula: IFCC HbA1c mmol/mol= (10.931*NGSP-HbA1c)-23.524 [32].

25(OH)D, including both 25-OH Vitamin D2 and 25-OH Vitamin D3 was measured in fresh serum samples by reversed-phase liquid chromatography coupled with tandem mass spectrometry detection. 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) [33] , and we pooled insufficiency 30-50 nmol/L (12-20 ng/mL) and sufficiency >50 nmol/L (>20 ng/mL as non-deficient vitamin D levels (≥30 nmol/L) to increase the clinical relevance and the statistical power.

iFGF23 and sKlotho were measured in EDTA-plasma. iFGF23 was measured with the commercially available Human Intact FGF23 ELISA kits from Immutopics International (San Clemente; CA, USA) and sKlotho was measured with the commercially available human soluable alpha klotho ELISA kit from IBL (Immuno-Biological Laboratoires GmbH, Japan). Freshly thawed samples were analyzed in duplicates, and the mean value reported. Coefficients of variation between duplicates were <17% for sKlotho and <16% for iFGF23. The intra- and inter-assay variability of the methods were <11% and <12% for iFGF23, respectively, and <13% and <12% for sKlotho, respectively (n=3-6). iFGF23 in EDTA-plasma in 8 healthy adults measured in the same local laboratory was mean 44 ±19pg/mL [34]. sKlotho, measured by IBL ELISA kit in serum from 142 healthy subjects, was reported to range from 239 to 1266 pg/mL, with a mean of 562 ± 146 pg/mL, in the original publication on the method [35]. In a group of 35 healthy controls measured at SUS, serum sKlotho levels ranged from 458 to 1222 pg/mmol, with a median of 525 pg/mmol [36]. We did not analyze sKlotho in plasma from healthy controls, but a review article on sKlotho and healthy middle-aged adults reported plasma sKlotho levels in 14 studies averaged between 600 and 700 pg/mmol [37]. Another article analyzed sKlotho in serum and plasma in 3 different ELISA kits, one of them the IBL kit. They found that measurements in serum and EDTA plasma were in agreement [38].

**Statistical analysis**

Normally distributed data are presented as mean ± standard deviation (SD) and skewed data as median and range.

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

samples t-tests were used to compare variables measured at 8 weeks post-transplant and in the end of 2020.

Survival analysis was performed using Kaplan-Meier estimate and multivariable Cox proportional hazard analysis including iFGF23, sKlotho, vitamin D, delayed graft function (DGF), age, BMI, eGFR, HbA1c and haemoglobine measured at 8 weeks as independent variables. The selection of variables was based on clinical knowledge and previous publications, and the number of variables had to be limited due to the low number of events. The proportionality assumption was checked using a log-minus-log plot. Two different Cox regression models were performed using uncensored graft loss (death and death censored graft loss) and death as dependent variables.

To compare variables measured at 8 weeks and 1 year post-transplant, a 2 sided p-value was estimated using paired sample t-test in normally distributed variables and Wilcoxon Signed Ranks test in not-normally distributed variables.

Statistical analysis was performed using IBM SPSS Statistics 26. P-value ≤ 0.05 was considered statistically significant.

## Results

iFGF23, sKlotho and vitamin D status 8 weeks post-transplant

Of the 132 selected kidney transplant recipients, 49 (37%) had 25(OH)D levels <30 nmol/L, 83 (63%) had 25(OH)D levels ≥30 nmol/L. Mean age was 51.1 years ±12.6, 58% were male. There were no significant differences in iFGF23 levels (p=0.17) or sKlotho levels (p=0.37) between the vitamin D groups at 8 weeks, table 1. Median follow-up time was 7.4 (1.2-8.1) years.

iFGF23, sKlotho and vitamin D status 1 year post-transplant

In total 128 of the 132 patients attended the 1 year follow-up at the transplant center. In 4 of the 128 attendees, vitamin D was missing, all other measurements were registered. In the cohort as a whole, vitamin D increased from mean 43±20 nmol/L 8 weeks post-transplant to 60±26 nmol/L (p<0.001) 1 year post-transplant. Thirteen patients (11%) had 25(OH)D levels <30 nmol/L, and 111 patients (87%) had 25(OH)D levels ≥30 nmol/L 1 year post-transplant. In the whole group, iFGF23 levels decreased from median 110 (15-1990) pg/mL to 89 (32-727) pg/mL, (p<0.001) from 8 weeks to 1 year post-transplant. In the same period, sKlotho leves increased from mean 417±129 pg/mL to 637±207 pg/mL, (p<0.001), eGFR increased from mean 62±19 mL/min/1.73m$^2$ to 73±25 mL/min/1.73m$^2$, (p<0.001), and intact parathyroid hormone (iPTH) levels decreased from median 10.5 (2.3-132.2) µmol/L to 8.9 (2.3-72.5) µmol/L, (p<0.001), figure 1, table 2.

Graft survival

During the follow-up period, a total of 36 grafts were lost, 27 (75%) of these were due to death with a functioning graft. Overall, graft survival was significantly different between the two vitamin D groups. Twenty (41%) of the patients with vitamin D

deficiency (<30 nmol/L) at 8 weeks post-transplant suffered graft loss or death compared with 16 (19%) in the non-deficient vitamin D group (≥30 nmol/L), p= 0.007, table 3. There were no significant difference in age (p=0.901), PWV (p=0.521), systolic/diastolic BP (p=0.117/ p=0.328) or cholesterol levels (p=0.571) between the patients in the 2 groups of vitamin D levels.

The proportion of preemptive transplantations and living donors was higher in the group of patients with non-deficient vitamin D levels (≥30 nmol/L), and the patients with deficient vitamin D levels (<30 nmol/L) had longer time in dialysis before transplantation, and more DGF post-transplant, table 1.

Crude Kaplan Meier estimated 5 year uncesored graft survival was 84% ± 5% (SE) in the patients with deficient vitamin D levels, compared with 94% ± 3% in the patients with non-deficient vitamin D levels, (p= 0.014). After 7 years follow-up, the uncensored graft survival was 74% ± 6% and 84% ± 4%, respectively (p=0.007), figure 2.

In the Cox regression models with uncensored graft loss as the dependent variable, high age (p=0.04), high HbA1c (p=0.03), low hemoglobin (p=0.03) and low vitamin D (p=0.04) were associated with uncensored graft loss. In the Cox regression model with patient survival as the dependent variable, high age (p<0.001), high HbA1c (p=0.01) and low vitamin D (p=0.03) were associated with death. sKlotho, iFGF23, DGF and eGFR at 8 weeks were included as variables in both models, but had no impact on the dependent variables. Haemoglobine did not influence the model with death as dependent variable, table 4.

In the adjusted model, the patients with vitamin D levels ≥30 nmol/L nmol/L, had a hazard ratio for uncensored graft loss of 0.46 (0.23-0.95) compared with patients with

vitamin D levels <30 nmol/L. Using death as the main end-point, the patients with vitamin D ≥30 nmol/L had a risk of death of 0.40 (0.17-0.92), compared with patients with vitamin D levels < 30 nmol/L, table 5.

At 8 weeks, PWV was median 9.1 (5.3-22.8) m/s. In the patients who died during follow up PWV was higher,11.4 (5.3-16.6) m/s, compared with 8.8 (5.6-22.8) m/s in the survivors, p=0.08. At 1 year the PWV was unchanged with a median of 9.2 (5.9-19) m/s.

7 year patient follow-up

Ninety-six (73%) of the 132 patients still had a functioning graft 7 years post-transplant, 29 (59%) of the 49 patients with deficient vitamin D, and 67 ((81%) of the 83 patients with non-deficient vitamin D levels post-transplant, (p=0.007). Mean eGFR was 64 mL/min/1.73m$^2$, with no significant difference between the two vitamin D groups. iPTH remained elevated above the normal reference range in both groups with a higher mean level in the whole group of patients 7 years compared with 8 weeks post-transplant, median (range) 10.5 µmol/L (2.3 µmol/L-132.3 µmol/L) at 8 weeks vs 11.0 µmol/L (2.7 µmol/L-44.7 µmol/L) after 7 years.

**Discussion**

The main finding in the present study was that patients with vitamin D deficiency (<30 nmol/L) 8 weeks post-transplant had reduced uncensored graft survival and reduced patient survival compared with the group that had vitamin D levels ≥30 nmol/L 8 weeks post-transplant. sKlotho and iFGF23 at 8 weeks showed no significant association with survival. High age, high HbA1c, low hemoglobin and low vitamin D levels were associated with reduced graft survival, and high age, high HbA1c and low vitamin D levels were associated with reduced patient survival.

Overall, the patients with functioning kidney grafts at the end of follow-up, had higher eGFR at both 8 weeks and 1 year post transplant than the patients who died or lost their kidney. The most plausible explanation to the finding of higher eGFR one year after transplantation is the toxicity due to mandatory co-trimoxazole treatment the first six months after transplantation. Somewhat higher calcineurin inhibitor levels in the early post-transplant phase may also contribute, at 8 weeks tacrolimus levels were 6.2µg/L ±1.7µg/L compared with 5.9 µg/L ±1.6 µg/L at 1 year after transplantation, (p=0.043). The "survivors" were almost 10 years younger at the time of transplantation.

The biochemical parameters of mineral metabolism often remain abnormal in transplant recipients for years after transplantation with persistent hyperparathyroidism, hypophosphatemia and hypercalcemia [39]. One study including 52 kidney transplant recipients found that FGF23 levels were elevated before transplantation, but decreased to normal levels within 3 months post-transplant as eGFR normalized [40]. Another study reported elevated FGF23 levels 3 months post-transplant [41], and others have showed persistently elevated FGF23 levels in transplant recipients 1 year post-transplant [42]. Even in kidney transplant recipients with a well-functioning graft >10 years post-transplant, the levels of FGF23 were increased compared with normal controls [43]. Elevated FGF23 post-transplant is associated with lower post-transplant eGFR [20], and increased risk of graft loss [44], cardiovascular [19] and all-cause mortality in long-term kidney transplant recipients [44].

Serum Klotho decrease in the initial post-operative phase after transplantation, but increase rapidly after a successful kidney transplantation, with the highest levels at 12 months post-transplant [42]. One study showed that Klotho levels 7 days and 1 month post-transplant were similar to Klotho levels before transplantation, but levels

started to rise approximately 4 months post-transplant [45]. Another study on long-term surviving kidney transplant recipients (>10 years after transplantation) showed a non-significant decline in Klotho levels [43], this might be as expected from 10 years of ageing.

In our study the median iFGF23 levels declined, but were still higher than in normal controls 1 year post-transplant [34], suggesting a significant difference between kidney transplant recipients and healthy individuals. The mean sKlotho levels increased, but were still lower than in normal controls at 1 year [36]. One year post-transplant, the sKlotho levels trend towards healthy controls, most likely as a result of increased eGFR.

Patients with CKD and kidney transplant recipients have the same traditional risk factors for CVD as the general population, and also to disturbed mineral metabolism, including elevated levels of FGF23 and decreased levels of Klotho [46]. Elevated FGF23 levels and reduced Klotho levels are both associated with left ventricular hypertrophy and CKD progression [47]. Klotho reduces hypertension, attenuates tissue fibrosis, CKD progression and vascular calcification [22,48]. In our study, PWV remained unchanged from 8 weeks to 1 year post-transplant, and higher PWV was associated with mortality. It is possible that the interval between PWV measurements is too short, and that the arterial stiffness associated with ESRD might resolve some after a longer period of follow-up post-transplant. Another possibility is that the arterial stiffness associated with CKD is irreversible, remaining as a continous risk factor for CVD in the post-transplant period.

The kidney is the main source of Klotho, and the Klotho levels are low in CKD patients, but still present [49]. Exogenous Klotho supplementation may be a new therapeutic approach to delay CKD progression and the development of CVD in both

CKD patients and transplant recipients. So far it has only been tested successfully in animal models [50]. The development of a Klotho-mimetic to maintain or elevate serum Klotho levels could be a future nephroprotective therapeutic option [51], but currently a well-functioning graft is the best prognostic marker for kidney transplant recipients.

Recently, mineralcorticoid receptor (MR) activation has emerged as a mediator for cardiovascular injury and CKD progression, in addition to Klotho deficiency and FGF23 excess. Mice exposed to mineralocorticoid deoxycorticosterone acetate displayed elevated FGF23 levels, hypertension and end-organ damage [52]. These changes were reversed by the administration of the MR- antagonists spironolactone and eplerenone [52]. In animal models, hyperphosphatemia was associated with upregulated aldosterone synthase, which is the terminal enzyme in the aldosterone biosynthesis pathway, leading to elevated aldosterone levels as a result of high phosphate levels [53]. Aldosterone inhibition using an aldosterone antagonist or an aldosterone synthase inhibitor may modify vascular calcification, and are possible candidates for improving cardiovascular outcomes in patients with CKD and kidney transplant recipients. There is an intricate interaction between increased iFGF23, reduced sKlotho, suboptimal vitamin D levels, increased aldosterone and several other factors interacting on the progression of CVD in kidney transplant recipients.

Limitations

The patients were all included at the same time after transplantation which is a strength in the comparability of the patients and the results. The sample size is a limitation, and it is difficult to draw definite conclusions due to a relatively low power, and the results only show trends. The number of events (graft loss or death) are low, and we pooled the two events in the survival analyses. Vitamin D, iFGF23 and

sKlotho were only measured twice, and a longitudinal profile may have added additional information.

## Conclusion

In this study patients with vitamin D deficiency (<30 nmol/L) at 8 weeks post-transplant showed reduced graft- and patient survival compared with the patients having higher vitamin D levels post-transplant. iFGF23 and sKlotho measured 8 weeks post-transplant were not associated with graft- and patient survival.

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## Visual abstract:

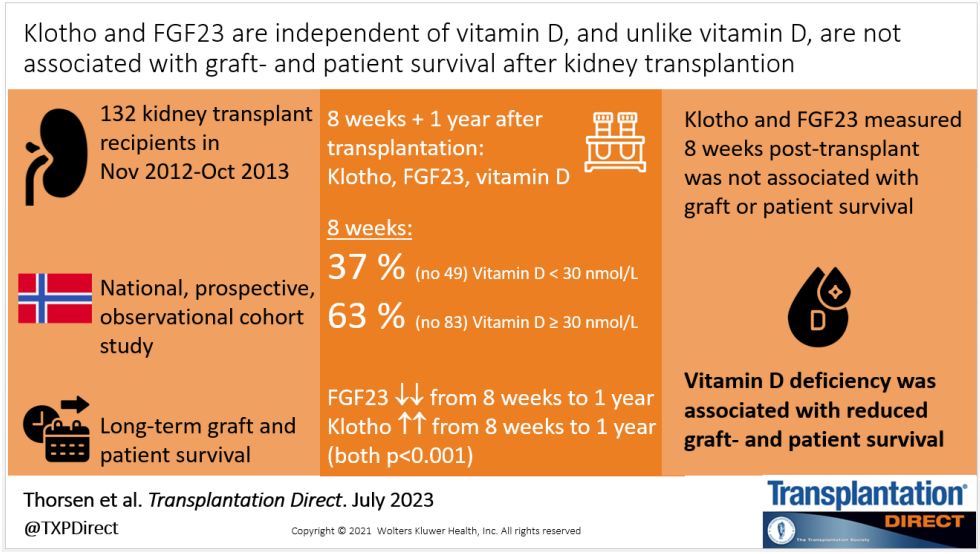


Table 1. Baseline characteristics 8 weeks post-transplant, for all patients as well as divided based on baseline plasma vitamin D levels (8 weeks post-transplant)

| Variables | All patients (n=132) | VitaminD <30 nmol/L (n=49) | Vitamin D ≥30 nmol/L (n=83) | p-value |
|---|---|---|---|---|
| Age, y | 51.1±12.6 | 50.7±12.1 | 51.3±12.9 | 0.901 |
| Sex m/f (% male) | 77/55 (58%) | 30/19 (61%) | 47/63 (57%) | 0.605 |
| BMI (kg/m$^2$) | 25.1±4.2 | 25.0±4.2 | 25.1±4.2 | 0.789 |
| Days in dialysis | 239 (0-3871) | 303 (0-3871) | 188 (0-11549 | 0.019* |
| Living donor, n (%) | 33 (25%) | 10 (20%) | 23 (28%) | 0.349 |
| First tx, n (%) | 110 (83%) | 39 (80%) | 71 (86%) | 0.309 |
| Preemptive tx, n (%) | 43 (33%) | 12 (24%) | 31 (37%) | 0.128 |
| DGF, n(%) | 16 (12%) | 11 (22%) | 5 (6%) | 0.005* |
| Vitamin D, nmol/L | 42.6±19.9 | 22.2±5.6 | 54.6±15.1 | <0.001* |
| sKlotho, pg/mL | 417±129 | 404±115 | 424±138 | 0.369 |
| iFGF23, pg/mL | 110 (31-1990) | 109 (49-1793) | 111 (31-1990) | 0.171 |
| eGFR, mL/min/1.73m$^2$ | 62±19 | 61±22 | 62±17 | 0.808 |
| Creatinine, µmol/L | 116±40 | 121±48 | 113±34 | 0.299 |
| Hemoglobin, g/dL | 12.0±1.3 | 11.9±1.2 | 12.0±1.4 | 0.651 |
| Calcium, mmol/L | 2.40±0.13 | 2.41±0.14 | 2.39±0.12 | 0.722 |
| iPTH, µmol/L | 10.5 (2.3-132.3) | 11.3 (2.5-132.3) | 9.9 (2.3-48.0) | 0.032* |
| Phosphate, mmol/L | 0.93±0.28 | 0.95±0.30 | 0.86±0.27 | 0.594 |
| Albumin, g/L | 41.6±2.9 | 41.0±3.3 | 41.8±2.6 | 0.096 |
| HbA1c, mmol/mol | 40.7±11.3 | 42.0±13.2 | 39.9±9.9 | 0.311 |

Median and range for dialysis duration, iFGF23 and iPTH, mean ± SD for other normally distributed variables. ANOVA was used on numeric variables and chi-square on categorical variables. *=significant difference between the groups (p≤0.05).

BMI, body mass index; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; iFGF23, intact fibroblast growth factor 23; iPTH, intact parathyroid hormone; Tx, transplantation.

Table 2: 8 weeks and 1 year data

| Variables | 8 weeks (n=132) | 1 year (n=128) | p-value |
|---|---|---|---|
| Age, y | 51.1±12.6 | 52.5±12.3 | - |
| Vitamin D, nmol/L | 42.6±19.9 | 59.8±26.0 | <0.001 |
| sKlotho, pg/mL | 417±129 | 637±207 | <0.001 |
| iFGF23, pg/mL | 110 (31-1990) | 89 (32-727) | <0.001 |
| eGFR, mL/min/1.73m$^2$ | 62±19 | 73±25 | <0.001 |
| Creatinine, µmol/L | 116±40 | 110±33 | 0.022 |
| Calcium, mmol/L | 2.40±0.13 | 2.43±0.19 | 0.093 |
| iPTH, µmol/L | 10.5 (2.3-132.3) | 8.9 (2.3-72.5) | <0.001 |
| HbA1c, % | 40.7±11.3 | 45.2±16.0 | <0.001 |
| BMI | 25.1±4.2 | 25.8±4.3 | <0.001 |

Median and range for iFGF23 and iPTH, mean ± SD for other normally distributed variables.

BMI, body mass index, eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; iFGF23, intact fibroblast growth factor 23; iPTH, intact parathyroid hormone

2 sided p-value is estimated using paired sample t-test in normally distributed variables and Wilcoxon Signed Ranks test in not-normally distributed variables

Table 3: Graft survival and overall survival

| | All patients (n=132) | VitaminD <30 nmol/L (n=49) | Vitamin D ≥ 30 nmol/L (n=83) | p-value |
|---|---|---|---|---|
| Death n (%) | 28 (21%) | 16 (33%) | 12 (14%) | 0.013* |
| Death cencored graft loss n (%) | 11 (8%) | 6 (12%) | 5 (6%) | 0.212 |
| Uncensored graft loss n (%) | 36 (27%) | 20 (41%) | 16 (19%) | 0.007* |

ANOVA was used to explore the differences between the 2 groups. *=significant difference between the groups (p≤0.05).

Table 4: Hazard ratios with 95% confidence intervals and p-values in the Cox regression models of graft and patient survival, respectively

| Variables | Uncensored graft loss HR (95% CI) | p-value | Death HR (95% CI) | p-value |
|---|---|---|---|---|
| sKlotho | 1.000 (0.997-1.003) | 0.847 | 0.999 (0.995-1.003) | 0.506 |
| iFGF 23 | 1.000 (0.999-1.001) | 0.989 | 1.001 (1.000-1.002) | 0.227 |
| eGFR | 1.005 (0.999-1.001) | 0.620 | 1.008 (0.985-1.031) | 0.499 |
| HbA1c | 1.029 (1.003-1.055) | 0.028 | 1.039 (1.008-1.071) | 0.013 |
| Vitamin D | 0.464 (0.228-0.947) | 0.035 | 0.379 (0.171-0.921) | 0.031 |
| Hb | 0.699 (0.505-0.967) | 0.030 | 0.977 (0.648-1.472) | 0.910 |
| Age | 1.036 (1.002-1.070) | 0.037 | 1.082 (1.040-1.136) | <0.001 |
| DGF | 1.377 (0.579-3.276) | 0.470 | 1.451 (0.542-3.890) | 0.506 |

Klotho, FGF23, eGFR, HbA1c, Hb, age and days in dialysis as continuous variables, DGF and vitamin D as categorical variables with no DGF and vitamin D deficiency as reference categories.

Table 5: Hazard ratios with 95% confidence intervals in the unadjusted and the adjusted Cox regression models

| Model | Uncensored graft loss HR (95% CI) | p-value | Death HR (95% CI) | p-value |
|---|---|---|---|---|
| Unadjusted | | | | |
| Vitamin D <30 nmol/L | reference | | reference | |
| Vitamin D ≥30 nmol/L) | 0.45 (0.23-0.86) | 0.02 | 0.42 (0.2-0.89) | 0.02 |
| Adjusted* | | | | |
| Vitamin D (<30 nmol/L) | reference | | reference | |
| Vitamin D ≥ 30 nmol/L) | 0.46 (0.23-0.95) | 0.04 | 0.40 (0.17-0.92) | 0.03 |

* Adjusted for the following variables: Age, eGFR, sKlotho, iFGF23, DGF, Hb and HbA1c. All adjustment variables were set to their empirical means when computing the adjusted survival curves. Vitamin D as a categorical variable with vitamin D deficiency (<30 nmol/L as reference category)

Figure 1. Spaghettiplots illustrating changes in Klotho, FGF23, Vitamin D and eGFR from 8 weeks to 1 year post-transplant

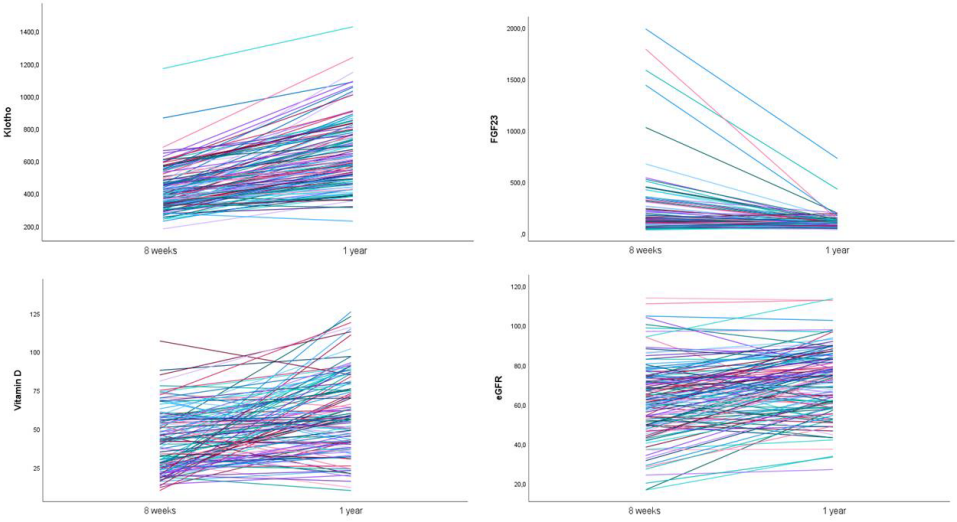
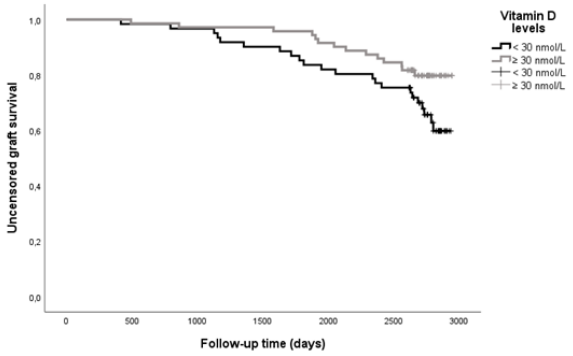


Figure 2. Kaplan Meier plot showing uncensored graft survival in patients with deficient and non-deficient vitamin D levels

Figure 2




Number at risk:

| | 0 | 500 | 1000 | 1500 | 2000 | 2500 (days) |
|---|---|---|---|---|---|---|
| <30 nmol/L | 49 | 48 | 47 | 43 | 40 | 36 |
| ≥30 nmol/L | 83 | 82 | 81 | 81 | 75 | 70 |

(vitamin D levels/ days of follow-up)

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