INTRODUCTION
Chronic kidney disease (CKD) is a major health problem, affecting 8-16% of the global population.\(^1\) Diabetes and hypertension, \(i.e.\) chronic lifestyle-related diseases, are the leading causes of CKD in all developed and many developing countries.\(^1\) Progressive renal function loss ultimately results in end-stage renal disease (ESRD) requiring renal replacement therapy, \(i.e.\) dialysis or transplantation. Albuminuria and blood pressure are the main factors contributing to progressive renal function loss. Therefore, both are considered key targets for intervention in chronic kidney disease. Pharmacological blockade of the renin-angiotensin-aldosterone system (RAAS) targets both blood pressure and albuminuria, and has been the cornerstone of CKD treatment for the last two decades. In many patients however, RAAS-blockade is insufficient to normalize blood pressure and albuminuria. The residual albuminuria, in particular, is a major risk factor for adverse renal and cardiovascular outcomes in CKD. Adjunct therapies are therefore urgently needed to further reduce albuminuria.

Of note, CKD is characterized by a strikingly elevated risk for cardiovascular complications, including heart failure, stroke, coronary heart disease and peripheral artery disease. The excess cardiovascular risk is proportional to the decrease in renal function, and further aggravated in patient with albuminuria. In patients with eGFR 30-59 mL/min (CKD stage 3) cardiovascular mortality is twice as high as in individuals with normal kidney function, and when eGFR is 15-29 mL/min (CKD stage 4) the risk triples compared to individuals with normal kidney function.\(^2\) Among dialysis patients the cardiovascular mortality risk is even 10 to 30 times higher than the general population.\(^3\)

For patients with ESRD, kidney transplantation is the preferred mode of renal replacement therapy, resulting in substantial improvements in quality of life and life expectancy compared to dialysis.\(^4\) In 2014 approximately 1,000 patients received a kidney transplant in the Netherlands, of whom 53% from a living donor.\(^5\) Over the past decades, the short-term patient and graft survival have improved markedly thanks to improved immunosuppressive therapy, surgical techniques, and treatment of infectious complications. For instance, the incidence of acute rejection during the first year has declined from around 50% in 1996 to approximately 10% in 2006.\(^6\) At variance with the substantial improvements in short-term outcome, improving long-term graft and patient survival remains a major challenge.\(^7\) The proportion of patients with graft failure within the first decade after transplantation remains over 30% (26% for living donor transplant and 36% for deceased donor transplant).\(^6\) Moreover, in spite of restoration of kidney function, mortality remains substantially higher than in the general population with a 10-year mortality rate of 24% for recipients of a living donor kidney and 38% for recipients of a deceased donor kidney.\(^6\) Cardiovascular disease is the leading cause of death,\(^8\) being nearly twice as common as infection or malignancy.\(^6\) Strikingly, three years after transplantation, nearly 40% of the patients have experienced a cardiovascular event.\(^6\)

Thus, improving outcome in kidney patients requires not only protection of kidney function, but also protection against cardiovascular disease. This requires proper treatment of traditional risk factors such as blood pressure and albuminuria, as well as exploration of other pathways of cardiorenal damage, such as abnormalities in mineral metabolism. The latter are frequent in patients with kidney disease and were traditionally linked to bone disease in particular. More
recent evidence, however, highlights the progressive vascular calcification that occurs in many renal patients and strongly predicts cardiovascular disease and all-cause mortality. Of note, vascular calcification may progress substantially even in stable renal transplant recipients.

Interestingly, there is evidence for interaction between the RAAS, as a main pathway for the traditional risk factors, and derangement of mineral metabolism in CKD. Abnormalities in vitamin D, fibroblast growth factor 23 (FGF23) and klotho are common, and interlinked in CKD patients. In 2011 our group proposed the concept of cross-talk between the RAAS and the vitamin D-FGF23-klotho axis, and its impact cardiorenal outcomes. In this thesis, we will study clinical implications of deregulated cross-talk between the RAAS and the vitamin D-FGF23-klotho axis.

PART I – CLINICAL IMPACT OF TARGETING CROSS-TALK: ALBUMINURIA AND RENO-PROTECTIVE TREATMENT

**Albuminuria**

Albuminuria is a hallmark of CKD, and a predictor of renal function loss and cardiovascular complications. Reduction of albuminuria, either spontaneously or by intervention is associated with a beneficial effect on long term renal function loss, delay of the onset of ESRD, and protection against cardiovascular complications. The underlying mechanism of its effect on renal prognosis seems to be related to tubulotoxic effects of albumin leaking through the diseased glomerular filter. The consistent association of albuminuria with cardiovascular morbidity and mortality across CKD populations might be explained by the Steno-hypothesis that states that albuminuria reflects a generalized vascular endothelial dysfunction. Inflammation and thrombogenic factors have also been implicated to underlie the relationship between albuminuria and cardiovascular disease.

**Renin-angiotensin-aldosterone system**

The RAAS is considered a major pathway underlying hypertension and progressive renal damage in CKD, by its effects on blood pressure and volume regulation, as well as by direct profibrotic and pro-inflammatory effects in the kidney. RAAS-blockade is the pharmacological cornerstone of treatment in CKD, most commonly by angiotensin converting enzyme inhibition (ACEi) or angiotensin receptor blockade (ARB). RAAS-blockade reduces albuminuria and blood pressure, retards progression of renal function loss, and lowers the risk of cardiovascular morbidity and mortality. However, despite optimally dosed RAAS-blockade residual proteinuria (or albuminuria) persists in a substantial number of CKD patients, which is strongly associated with both long-term renal and cardiovascular adverse outcomes. This is, at least partly, attributed to limitations inherent to RAAS-blockade as such. Pharmacological blockade with ACEi or ARB elicits a reactive increase of renin, upstream in the RAAS. This rise may limit the efficacy of RAAS-blockade and also has adverse effects in itself. Several add-on therapies have been investigated, including dual RAAS-blockade, add-on by other drugs and dietary measures, to potentiate the cardiorenal protective efficacy of RAAS-blockade. A consistent line of evidence shows that sodium overload annihilates the antihypertensive
and antiproteinuric effects of RAAS blockers. Post-hoc studies demonstrated that renal and cardiovascular protective effects of RAAS blockers are blunted with high sodium intake. Conversely, combining RAAS-blockade with a moderate sodium restriction synergistically increases the maximum therapeutic gain of single-agent RAAS-blockade in both diabetic and non-diabetic CKD patients. Furthermore CKD patients generally have a salt-sensitive blood pressure, related to inappropriate activity of sodium-retaining pathways, and possibly also diminished non-osmotic sodium storage capacity.

**Vitamin D**

Vitamin D₃ is synthesized in the skin under the influence of UV-B radiation or is obtained from dietary sources, such as fish and dairy products, by absorption through the intestine. In the liver, vitamin D₃ is converted to 25(OH)D by 25-hydroxylase. Afterwards 25(OH)D is converted to 1,25(OH)₂D in the kidney by the enzyme 1α-hydroxylase. 1α-hydroxylase is regulated by several mechanisms, including PTH and FGF23.

![Vitamin D-FGF23-PTH axis](image)

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PTH regulate each other in a negative feedback loop, where PTH stimulates FGF23 production and FGF23 in turn suppresses PTH synthesis (Figure 1).

In CKD, vitamin D deficiency is common, generally attributed to insufficient renal conversion of 25(OH)D. Vitamin D deficiency not only leads to secondary hyperparathyroidism and mineral bone disorder (MBD), but has also been implicated in extraskeletal conditions including hypertension, insulin resistance, a more rapid decline in renal function, increased albuminuria and eventually an increased risk of cardiovascular disease and mortality (as reviewed by Doorenbos et al and De Borst et al). The Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines advise vitamin D supplementation in CKD stage 3-4 if serum 25(OH)D is below 75 nmol/L to treat secondary hyperparathyroidism, to increase bone mineral density and to reduce the risk of hip fracture. The increasing evidence on the renoprotective effects of vitamin D analogues (as reviewed by Mirkovic et al) could provide an additional reason for early initiation of vitamin D treatment in CKD.

FGF23 and klotho
FGF23 is a hormone involved in the regulation of phosphate metabolism by inducing phosphaturia. Klotho is an essential co-factor for the FGF23 receptor, FGFR1, in the kidney and parathyroid gland. Of interest, both FGF23 and klotho have been implicated in the cardiorenal complications of CKD. Klotho is a single-pass transmembrane protein with a large extracellular domain and is predominantly expressed in the kidney. The extracellular domain of klotho is cleaved and secreted (shedded) into extracellular fluids, including blood, urine, and cerebrospinal fluid. The secreted and membrane-bound klotho have distinct functions. The shedded part of klotho retains enzymatic activity, which affects distant channels including transient receptor potential vanilloid type 5 (TRPV5), renal outer medullary K+ channel (ROMK1) and type 2a sodium-dependent phosphate co-transporters (NaPi-2a) which increases calcium reabsorption, potassium excretion and phosphate reabsorption, respectively (reviewed by John et al). Reduction of klotho gene expression in mice results in a syndrome resembling human ageing, characterized by a short life span, osteoporosis, renal fibrosis, arteriosclerosis and calcifications. CKD patients remarkably share phenotypic properties of accelerated ageing, including the tendency to develop soft tissue calcification, bone disease and reduced life expectancy. Furthermore, in CKD both membrane-bound and secreted klotho are decreased. Injection of recombinant soluble klotho and transgenic overexpression of klotho dramatically improve kidney function and morphological lesions in several experimental CKD models. Restoring soluble klotho levels might be a target for cardiorenal protective therapy in CKD, but whether soluble klotho levels are affected by the current standard renoprotective therapy is largely unknown.

A consequence of klotho downregulation is a disrupted FGF23 signaling, resulting in (excessively) high FGF23 levels. Higher FGF23 levels suppress the activation of vitamin D and are associated with CKD progression, left ventricular hypertrophy, vascular calcification and increased risk of cardiovascular events and all-cause mortality in CKD patients. Serum levels...
of FGF23 increase gradually as kidney function decreases. Moreover, higher FGF23 is associated with proteinuria in patients with moderate to severe CKD.60

**Cross-talk between RAAS and vitamin D-FGF23-klotho axis**

The RAAS and the vitamin D-FGF23-klotho axis interact at several levels under physiological conditions, and this cross-talk appears to be disturbed in CKD, as proposed by *De Borst et al.* Several preclinical studies have shown negative regulation of renin production by vitamin D. Vitamin D receptor (VDR) knock-out mice have increased renin expression, angiotensin II (Ang II) synthesis and subsequently develop hypertension and myocardial hypertrophy.61 Mechanistically, the VDR can directly downregulate prorenin gene expression through interaction with its promoter region.62 Conversely, Ang II negatively regulates renal klotho expression.63,64 This subsequently causes FGF23 resistance and rising FGF23 levels, leading to suppression of 1α-hydroxylase and reduced production of 1,25(OH)2D. These lower vitamin D levels then in turn stimulate the RAAS, causing a vicious cycle (Figure 2). In light of these findings, it is plausible that vitamin D deficiency contributes to the inappropriate RAAS activation in the initiation and progression of chronic proteinuric nephropathies.

![Figure 2. Cross-talk between renin-angiotensin-aldosterone system and vitamin D-FGF23-klotho axis](image)

The clinical implications of this cross-talk are not yet clear. Up until now deregulations of the RAAS or vitamin D-FGF23-klotho axis are largely considered separately, regarding the natural course of kidney disease as well as regarding intervention treatment. The cross-talk concept elicits the hypothesis that derangements in both pathways interact in the development of cardiorenal
damage. Moreover, for intervention treatment the cross-talk concept suggest that the vicious cycle of deregulation of the RAAS and the vitamin D-FGF23-klotho axis can best be interrupted by simultaneous modulation of both pathways. It is hypothesized that treatment with a vitamin D receptor activator (VDRA), in addition to RAAS-blockade, could provide additional cardiorenal protection by suppressing renin. Indeed, several preclinical studies confirm that renoprotective effects of VDRA are mediated – at least in part – by suppression of renin. Other possible mechanisms explaining the renoprotective effects of VDRA include anti-inflammatory and anti-fibrotic effects. Vitamin D suppresses NF-κB activity in macrophages, resulting in inhibition of inflammatory genes. Furthermore, VDRA administration blocks Wnt/β-catenin signaling, a pathway involved in the development and progression of renal fibrosis, and subsequently improves podocyte function, proteinuria and kidney injury. In several clinical trials among different CKD populations, VDRA treatment reduced proteinuria. Furthermore, a recent meta-analysis confirmed that vitamin D analogues reduced proteinuria by 16% in CKD patients. The next step would be to investigate whether VDRA treatment in combination with optimized RAAS-blockade, by means of dietary sodium restriction, would provide further reduction of albuminuria in CKD patients.

PART II – CLINICAL IMPACT OF CROSS-TALK: NOVEL CLINICAL READOUTS AND DOWNSTREAM FACTORS

To provide more insight in the clinical implications of cross-talk between the RAAS and vitamin D-FGF23-klotho axis, it is important to consider the best clinical readouts. In the second part of this thesis we study the clinical impact of novel clinical readouts and factors downstream of the cross-talk in the renal transplantation population. Novel clinical readouts are needed to investigate how deregulations of the cross-talk are related to worse cardiorenal outcomes and to guide treatment to improve cardiorenal outcomes. Identifying novel readouts and downstream factors of the cross-talk might also provide insights in novel pathways, extending the original cross-talk paradigm.

Vitamin D and Wnt/β-catenin pathway

25(OH)D deficiency is considered one of the main clinically relevant readouts of deregulations in the vitamin D-FGF23-klotho axis, and is associated with adverse cardiorenal outcomes. 25(OH)D deficiency is highly prevalent in CKD and after kidney transplantation. Many patients with CKD have 25(OH)D deficiency possibly due to reduced endogenous synthesis of vitamin D₃ in the skin and reduced activity of 1α-hydroxylase. In transplant recipients, the high prevalence of 25(OH)D deficiency is also due to avoidance of direct sunlight exposure to reduce the enhanced risk of non-melanoma skin cancer caused by immunosuppressive treatment. A recent study documented that low levels of 25(OH)D, but not the active metabolite 1,25(OH)₂D, measured 3 months after transplantation are associated with renal histological abnormalities and lower
renal function one year after kidney transplantation. However, vitamin D levels may vary considerably along with renal function, FGF23, and PTH during the first year after transplantation. Consequently, vitamin D status (both 25(OH)D and 1,25(OH)2D) early after transplantation may not represent vitamin D status during later stages post-transplantation. Therefore, the long-term clinical implications of vitamin D deficiency in the renal transplant population remain unclear.

Preclinical studies have demonstrated renoprotective effects of vitamin D analogues, including amelioration of renal fibrosis. Treatment with the VDRA paricalcitol significantly and dose-dependently reduced the fibrotic lesions in an aggressive renal fibrosis mouse model. These antifibrotic effects of vitamin D could at least in part be mediated through the Wnt/β-catenin pathway. Increasing evidence indicates that Wnt/β-catenin signaling, originally known as a major signaling cascade in bone development and bone homeostasis, plays an important role in renal tissue responses to injury. The Wnt/β-catenin pathway controls the expression of several pro-inflammatory and pro-fibrotic genes. Of interest, multiple RAAS genes are also downstream targets of Wnt/β-catenin signaling. Indeed, a previous preclinical study showed that paricalcitol improved podocyte function and reduced proteinuria and kidney injury, at least in part, by blocking Wnt/β-catenin-mediated gene transcription.

After kidney transplantation, renal interstitial fibrosis and tubular atrophy (IF/TA), previously included within the term chronic allograft nephropathy, is recognized as a final common pathway leading to graft failure. Pro-fibrotic processes drive the development of IF/TA and may determine an individual’s susceptibility to develop IF/TA. Aberrant regulation of the Wnt/β-catenin pathway has been observed in a preclinical model of chronic renal allograft damage. Inhibition of the aberrant Wnt/β-catenin pathway may reduce pro-fibrotic processes in the transplanted kidney. An endogenous extracellular antagonists of the Wnt signaling pathway is Dickkopf-1 (Dkk-1), which binds to the Wnt co-receptors low-density lipoprotein receptor-related protein 5 and 6 (LRP-5 and LRP-6) and subsequently prevents the formation of an active Wnt receptor complex. Indeed, ectopic expression of Dkk-1 resulted in suppression of Wnt/β-catenin target genes and attenuation of renal interstitial fibrosis. Considering the preclinical data Dkk-1 could, parallel to other bone-mineral markers, play an important role in the development and progression of renal damage after kidney transplantation.

**Vascular calcification**

Kidney transplantation restores renal function but incompletely mitigates cardiovascular risk, which remains strongly elevated compared to the general population. The long-term risk of redeveloping ESRD, i.e. returning to dialysis or retransplantation, has not improved over the last decades. The classical cardiovascular risk factors in the general population, i.e. tobacco use, diabetes, obesity, hypertension and dyslipidemia, are also considered risk factors for cardiovascular disease after kidney transplantation, according to current KDIGO guidelines. However, these classical risk factors underestimate the real risk of cardiovascular events in renal transplant recipients. After kidney transplantation, a combination of deregulated mineral metabolism, chronic inflammation and loss of calcification inhibitors give rise to an extracellular environment that triggers vascular mineralization. Of note, in the majority of stable renal
transplant recipients, coronary artery and aorta calcification may still progress substantially.\textsuperscript{10} Vascular calcification is determined by both inhibitors and promoters (Figure 3). Elevated serum phosphorus is a major promoter of vascular calcification\textsuperscript{98}. Furthermore, klotho loss also seems to be an intermediate factor in the pathogenesis of vascular calcification (as reviewed by Vervloet \textit{et al}\textsuperscript{99}). Matrix gla protein (MGP) is one of the strongest endogenous calcification inhibitors. Its suppression, by any cause, contributes importantly to vascular calcification\textsuperscript{100-102}. Vitamin K\textsubscript{1} and vitamin K\textsubscript{2} serve as crucial cofactors to activate MGP by converting specific protein-bound glutamate residues into g-carboxyglutamate (Gla). Vitamin K insufficiency therefore results in increased plasma levels of uncarboxylated (uc) inactive MGP proteins, including desphosphorylated-ucMGP (dp-ucMGP)\textsuperscript{103,104}. High circulating dp-ucMGP levels have been associated with vascular calcification and increased cardiovascular risk in CKD\textsuperscript{105,106}. The majority of stable kidney transplant recipients are vitamin K insufficient, reflected by increased dp-ucMGP levels\textsuperscript{107}. Moreover, a recent preclinical study found a cause-effect relationship between decreased kidney function and vitamin K insufficiency through reduced endogenous vitamin K recycling\textsuperscript{108}. Through renal function loss, deregulated RAAS-vitamin D-FGF23-klotho cross-talk could therefore reduce endogenous calcification inhibitors such as MGP. Furthermore, the RAAS could directly influence MGP metabolism\textsuperscript{109}. However, the clinical implications of functional vitamin K insufficiency after kidney transplantation have not been addressed so far.

\textbf{Figure 3. Inhibitors and promoters of vascular calcification}

An imbalance between promoters and inhibitors of mineralization results in vascular smooth muscle cells developing an osteoblast-like phenotype. These cells lay down mineralized matrix resulting in vascular calcification.
A drawback of current modalities quantifying vascular calcification, e.g. coronary CT scans, is that these provide information on prevalent calcification, rather than calcification development or progression. This limits their potential to identify renal transplant recipients with no ongoing vascular calcification after transplantation. Since vascular calcification is the net result of several calcification-promoting and -inhibiting factors (Figure 3), a single component such as serum phosphate cannot serve as a marker of overall calcification propensity. Importantly, a test was recently developed that monitors the calcification propensity in serum, i.e. the transformation time ($T_{50}$) of primary to secondary calciprotein particles (CPPs). In serum, fetuin-A can form a complex with calcium and phosphate in the circulation, which protects soft tissues from the precipitation of supersaturated calcium and phosphate by the formation of primary CPPs (reviewed by Jahnen-Dechent et al\textsuperscript{111}). Primary CPPs spontaneously undergo topological rearrangement and convert to secondary CPPs. A long delay of transformation time indicates a high residual capacity of the patient’s serum to prevent the formation of secondary CPPs and is therefore indicative of an intact endogenous defense against calcification. Serum calcification propensity defines the future calcification risk largely related to common mineral and bone factors. The balance of potentiating and inhibitory factors present in each serum sample governs the transformation time. Furthermore, serum calcification propensity has a marked between-individual variance but apparent stability over time in the same individual, which makes it a favorable predictive risk marker. In CKD patients increased calcification propensity was independently associated with progressive aortic stiffness and an increased risk of all-cause mortality. Thus, serum $T_{50}$ could be a valuable tool to evaluate the impact of (deregulations in) the cross-talk between RAAS and vitamin D-FGF23-klotho axis on calcification propensity.
AIMS OF THE THESIS

The general aim of this thesis is to translate the implications of the cross-talk between the RAAS and vitamin D-FGF23-klotho axis into its clinical implications. In the first part we explore the impact of modulation of the RAAS and the vitamin D-FGF23-klotho axis on albuminuria development and on renoprotective treatment. In the second part we study the clinical impact of novel clinical readouts and factors downstream of the cross-talk, in renal transplantation.

OUTLINE OF THE THESIS

Part I - Clinical impact of targeting cross-talk: albuminuria and renoprotective treatment.

Vitamin D deficiency and excessive sodium intake are both associated with increased albuminuria and progressive renal function loss in CKD. In chapter 1 we investigate whether plasma 25(OH)D or 1,25(OH)_2D levels are associated with the risk of developing CKD, estimated glomerular filtration rate (eGFR) below 60 mL/min and/or urinary albumin excretion above 30 mg per day, and whether these associations depend on sodium intake. Both VDRA treatment and dietary sodium restriction potentiate the efficacy of renin-angiotensin-aldosterone system (RAAS)-blockade to reduce albuminuria. These previous – independent – observations led us to investigate in chapter 2 whether a VDRA (paricalcitol) in combination with dietary sodium restriction provides further albuminuria reduction in non-diabetic CKD patients on top of single RAAS-blockade.

In experimental CKD, renal klotho expression is decreased and soluble klotho in urine and blood is barely detectable. Preclinical observations imply that proteinuria decreases renal klotho expression and that the renoprotective effects of ARB therapy may be partially explained through – indirectly or directly - preserving klotho expression. Dietary sodium restriction addition and/or a second RAAS blocker (dual blockade) potentiate the antiproteinuric efficacy of single RAAS blockade. In chapter 3 we explore in a post-hoc analysis whether serum klotho is affected by addition of dietary sodium restriction or ARB, or their combination, in non-diabetic CKD patients receiving background treatment with ACEi.

Part II - Clinical impact of cross-talk: novel clinical readouts and downstream factors

In renal transplant recipients, the elevated post-transplant cardiovascular risk is mainly due to premature and markedly accelerated vascular calcification. Biomineralization is the result of several promoting and suppressing factors of calcification, together determining calcification propensity. In chapter 4 we report on the association between serum calcification propensity and graft and patient survival in stable renal transplant recipients. Furthermore, we set out to identify potentially modifiable determinants of serum calcification propensity in this population. One of the most powerful endogenous inhibitors of vascular calcification is MGP, which is activated through carboxylation by vitamin K. In renal transplant recipients, vitamin K insufficiency
is common, but the implications for renal and patient survival after kidney transplantation are unclear. To provide more insight in the clinical implications of functional vitamin K insufficiency, we explore in chapter 5 whether plasma dephosphorylated-uncarboxylated MGP (dp-ucMGP), indicating vascular vitamin K deficiency, is an independent risk factor of clinical outcomes in renal transplant recipients.

Vitamin D deficiency is considered one of the main clinically relevant consequences of deregulations in the vitamin D-FGF23-klotho axis, and is associated with adverse cardiorenal outcomes. Vitamin D status could be a clinically relevant readout of the deregulated cross-talk. Therefore, we investigate whether circulating 25(OH)D or 1,25(OH)₂D are associated with long-term patient or graft outcomes in chapter 6. The adverse renal effects of vitamin D deficiency might be mediated by loss of Wnt/β-catenin suppression. Preclinical data suggest that vitamin D receptor activators improve podocyte function and reduce albuminuria, at least in part, by blocking the increased Wnt/β-catenin signaling pathway. The Wnt/β-catenin pathway controls the expression of several pro-inflammatory and pro-fibrotic genes. Dickkopf-1 (Dkk-1), a soluble endogenous inhibitor of Wnt signaling pathway, may be considered as an anti-fibrotic factor detectable in the circulation. Thus, in chapter 7 we study the association between serum Dkk-1 and renal fibrosis as well as the risk of developing graft failure. Besides acting as a marker of renal fibrosis and graft failure, Dkk-1 may serve as a novel target for renoprotective therapy in kidney transplantation.
REFERENCES


