Chapter 1

General introduction
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1.1 Chronic kidney disease

Chronic kidney disease (CKD) is defined as “abnormalities of kidney structure or function, present for more than three months, with implications for health”\(^1\). With a prevalence of 8-16% in general population, CKD has been recognized as a worldwide health problem\(^2\). Regardless the underlying cause, CKD can progress over time to end stage renal disease (ESRD) requiring renal replacement therapy: dialysis or renal transplantation. Furthermore people with CKD are at high risk of developing cardiovascular disease, which is the primary cause of death across stages of CKD\(^3\).

The goal of therapy in CKD is to slow down and possibly halt the progression of renal function loss and structural damage, as in most cases the underlying cause cannot be easily targeted, and/or progressive renal function loss occurs due to a final common pathway independent of the original cause. Current pharmacological strategies for renoprotection are aimed at the reduction of proteinuria and correction of elevated blood pressure\(^4\) along with the control of other risk factors such as hyperlipidemia, smoking, and diabetes mellitus\(^4\). To this purpose, blockade of the renin-angiotensin-aldosterone system (RAAS) has been shown particularly effective\(^5\). In fact, RAAS blockade by angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II receptor 1 blockers (ARB) is currently the cornerstone treatment for CKD. However, in many patients RAAS blockade leads only to reduction but not complete abrogation of proteinuria, with residual proteinuria as a result. The extent of residual proteinuria is considered one of the main risk factors for further renal function decline\(^6\) as well as for cardiovascular complications\(^7\).

Based on the results from experimental studies and the assumption that single agents are insufficiently effective to abolish proteinuria and halt progressive renal function loss, several strategies have been proposed to improve the efficacy of RAAS blockade based treatment regimens. These include titration of a single agent towards an optimal antiproteinuric dose (and above doses used for blood pressure control), dual RAAS blockade (mostly the combination of an ACEi and an ARB) and reduction of dietary sodium intake. These regimens all lead to further reduction of proteinuria, but their long-term benefits are less well established. The effects of dose escalation have not been specifically addressed, and large clinical trials on dual RAAS blockade showed that intensification of RAAS blockade by means of dual blockade does not confer long-term benefits\(^8\)-\(^11\). Importantly, in these trials, dual RAAS blockade was associated with adverse renal and cardiovascular events. On the other hand, post-hoc analysis of the REIN\(^12\), RENAAL and IDNT\(^13\) trials indicated that moderate dietary sodium restriction might have substantial impact on long-term renal outcome. However, the long-term benefits of dietary sodium restriction still need to be confirmed in a prospective trial.

Proteinuria reduction protects against progression of glomerular as well as interstitial damage\(^14\). In CKD patients data on the extent of interstitial damage are not always available, as this requires a renal biopsy. Yet, it is well established that interstitial damage is the best predictor of the subsequent decline in kidney function\(^15\)-\(^17\). Of note, aggressive reduction of blood pressure and proteinuria by RAAS-blockade-based regimens results in improved glomerular protection, but not invariably improves interstitial protection\(^18\), \(^19\). In fact, interstitial damage can even worsen under aggressive antiproteinuric therapy and hence dissociate from the proteinuria reduction\(^20\). If this also holds true in human, the implication would be that in CKD patients receiving RAAS blockade, down-titration of
proteinuria by dual blockade or by strict dietary sodium restriction could be associated with progression of interstitial damage that could go unnoticed, and hence a worse long term outcome. This scenario might perhaps explain the adverse long-term outcomes of dual RAAS-blockade. Thus, treatment strategies for better renoprotection are needed, but further intensification of RAAS-blockade is apparently not warranted. A rational strategy would be to design adjunct therapy that targets pathways of damage that are not susceptible to RAAS-blockade, while preserving the beneficial effects of RAAS blockade. Given the importance of interstitial damage to influence the course of CKD, better tools for non-invasive monitoring of tubulo-interstitial damage, for instance by biomarkers of tubular injury, are warranted. Furthermore, it is relevant to identify novel factors modifying the efficacy of RAAS blockers to protect the tubulo-interstitium. In this respect, the role of hormones regulating mineral metabolism became increasingly recognized over last decade.

1.2 Vitamin D, FGF23 and Klotho in CKD

Normal balance of calcium and phosphorus in the body is maintained by control of their absorption in the intestine, mineralization/resorption in the bone and reabsorption/excretion in the kidney. Regulation of these processes is driven primarily by the active form of vitamin D (1,25(OH)_2D), parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23). The kidney is a target organ for all these hormones but also the main site for the production of 1,25(OH)_2D. Alterations in the factors regulating calcium and phosphorus homeostasis, particularly FGF23, occur relatively early in the course of CKD and progress as kidney function declines. Left untreated, these alterations can result in hyperphosphatemia, vitamin D deficiency, secondary hyperparathyroidism, and ultimately a spectrum of bone abnormalities and vascular and soft tissue calcification. In recognition of the importance of disorders of mineral metabolism that develop in the setting of CKD, the term CKD – mineral and bone disease (CKD-MBD) has been proposed. However, the biological effects of vitamin D, FGF23 and Klotho extend beyond their role in the regulation of mineral metabolism, making them target candidates for therapy in CKD.

1.2.1 Vitamin D

The majority of the inactive form of vitamin D known as cholecalciferol is produced in the skin from 7-dehydrocholesterol under the influence of UV light irradiation (Figure 1). To achieve its active form, cholecalciferol has to go through two hydroxylation steps in the liver and in the kidney. The active, 1,25(OH)_2D or calcitriol is produced in the kidney by the 25-hydroxyvitamin D 1α-hydroxylase (CYP27b1). This enzyme is also present in non-renal tissues such as the skin, immune system and intestine. Biological actions of 1,25(OH)_2D are mediated by the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily. The widespread expression of the VDR in tissues and cells that are not involved in mineral metabolism forms a basis for broad biological actions of vitamin D and VDR activators. These so-called non-classical functions of vitamin D include regulation of cellular proliferation, inflammation, the immune system and the endocrine system including the RAAS, insulin resistance and lipid metabolism. Many
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Figure 1.1: Metabolism and biological actions of vitamin D. Vitamin D is produced in the skin by exposure to UVB radiation or is ingested in the diet. In the liver, both vitamin D2 and vitamin D3 are converted to 25(OH)D by the vitamin D-25-hydroxylase. 25(OH)D is then converted to active form (1,25(OH)$_2$D) by 1-α hydroxylase. Active vitamin D formed in the kidney regulates calcium and phosphorus metabolism. In addition, it stimulates the expression of the 25(OH)D-24-hydroxylase. 25(OH)D can be activated at extra renal sites as well. Active vitamin D produced in this way regulates immune function and different cellular processes. The induction of this enzyme results in the deactivation of 1,25(OH)$_2$D into a water-soluble inactive metabolite calcitroic acid.

of these pathways depend on local autocrine/paracrine activation of the VDR. Vitamin D deficiency, on the other hand, can have an array of detrimental effects not only on bone but also on other organs such as the kidney and cardiovascular system$^{27}$. On the other hand, treatment with vitamin D and/or VDR activators may provide benefits to each of these organ systems. Experimental studies provided insights into mechanisms of renal protection of vitamin D and its analogues. In a number of models of renal damage, vitamin D analogues showed anti-proteinuric, anti-fibrotic and anti-inflammatory effects in the kidney. These were mediated via negative regulation of renin expression$^{28, 29}$, the NF-kB pathway$^{30-32}$, the Wnt/β-catenin pathway$^{33}$ and up-regulation of slit diaphragm proteins such as nephrin$^{34}$. In addition, one of the vitamin D analogues, maxacalcitol, has been shown to specifically down regulate the TGF-β pathway independently of the RAAS$^{35}$. When given on top of RAAS blockade, vitamin D analogues exerted additional antiproteinuric effect and better preservation of the tubulo-interstitium$^{36-39}$.

In CKD patients, observational studies have demonstrated a link between vitamin D deficiency, impaired glomerular filtration rate (GFR), and increased mortality in patients with CKD and an association between treatment with vitamin D (analogues) and im-
proved survival in both predialysis and ERSD populations\textsuperscript{40-43}. These encouraging data, as well as the promising findings in the preclinical setting, resulted in a number of clinical trials with the use of active vitamin D analogues targeting mostly residual proteinuria. The results so far have been inconclusive, with studies reporting significant\textsuperscript{44,45}, borderline significant\textsuperscript{46-48} or non-significant findings\textsuperscript{49,50}. However, a recent meta-analysis of these RCTs reported an overall of 15% reduction of proteinuria when given on top of RAAS blockade\textsuperscript{51}. Trials addressing hard end points are still lacking. What also needs to be determined is to what extent the potential beneficial effects of vitamin D supplementation go beyond the correction of vitamin D deficiency, known to be a risk factor for CKD progression\textsuperscript{52}. Interestingly, in the VITAL study, reduction of albuminuria with paricalcitol was independent of baseline vitamin D levels (unpublished data).

Since the kidney is the main source of 1α-hydroxylase, the enzyme needed to convert 25(OH)D to its active form, the widely accepted view is that vitamin D deficiency in CKD is caused primarily by impaired synthesis of 1,25(OH)\textsubscript{2}D in the kidney. Until recently, decreased activity of 1α-hydroxylase has been attributed to diminished functional renal mass leading to progressive loss of enzyme in proximal tubular cells. However, this concept cannot explain why the decline in 1,25(OH)\textsubscript{2}D is seen early in the course of kidney disease\textsuperscript{53-55} when there is unlikely to be sufficient loss of nephron mass to substantially impair synthesis of 1,25(OH)\textsubscript{2}D. The discovery of fibroblast growth factor 23 and Klotho and their functions provided valuable insights into mechanisms of disordered vitamin D metabolism in CKD, and the potential consequences for cardio-renal outcomes.

\subsection*{1.2.2 FGF23/Klotho}

FGF23 is a bone-derived hormone, secreted by osteoblasts and osteocytes. FGF23 belongs to fibroblast growth factor superfamily of peptides that exert pleiotropic effects on a broad range of biological functions\textsuperscript{56}. The FGF superfamily consists of twenty-two members identified in humans, most of which act as paracrine/autocrine factors. Oppositely, FGF23 along with FGF19 and FGF21 forms the FGF19 subfamily. These FGFs lack conserved heparin binding-domains necessary for the interaction with extracellular matrix and activation of FGF receptors\textsuperscript{57}. Because of this feature, endocrine FGFs can be secreted from the sites of their synthesis and act in endocrine manner, but also for the activation of specific receptors require co-receptors belonging to the Klotho family of proteins\textsuperscript{58, 59}. These are single pass transmembrane proteins, with extracellular domain mediating their functions. Alpha-klotho (Klotho further in the text) is the specific co-receptor for FGF23\textsuperscript{60}. Since most tissues express FGF receptors, tissue-specific expression of Klotho determines FGF23 target organs. Indeed, the highest expression of Klotho is found in the kidney and parathyroid gland, which are the main target organs for FGF23 actions\textsuperscript{61,62}. The primary and the best-studied physiological functions of FGF23 include regulation of phosphate excretion and vitamin D synthesis (Figure 2)\textsuperscript{63,64}. In the kidney FGF23 induces urinary phosphate excretion via down-regulation of sodium-phosphate cotransporters and decrease of 1,25(OH)\textsubscript{2}D levels via inhibition of renal 1α-hydroxylase and induction of catabolic enzyme CYP24a1. In the parathyroid glands, FGF23 may act as a negative regulator of PTH production\textsuperscript{62,65}. Klotho exists in two forms: as a transmembrane and a secreted form. The transmembrane form of Klotho, due to its extremely short intracellular domain, most probably serves only to stabilize the interaction between
Figure 1.2: Biological functions of FGF23. FGF23 is produced in bone in response to increased levels of phosphate, 1,25(OH)₂D, and PTH. In the kidney, FGF23 decreases the expression of sodium–phosphate co-transporters Npt2a and Npt2c and decreases production of 1,25(OH)₂D, resulting in hypophosphatemia. Potentially, FGF23 might down-regulate PTH in a feedback loop. Figure adapted from: E.G. Farrow & K.E. White, 2010, Recent advances in renal phosphate handling, 2010, Nature Reviews Nephrology 6, 207-217.

FGF23 and the specific FGF receptor. The secreted form is produced either by alternative splicing of the Klotho mRNA or by ectodomain shedding and can function as a humoral factor independently of FGF23. Presently, it is not clear if secreted Klotho acts also as a co-receptor for FGF23, however, it has been shown that it can regulate ion transport in the kidney and suppresses activity of IGF-1, Wnt and TGF-beta.

1.2.3 FGF23 and Klotho in CKD

Through the prism of disturbed mineral metabolism, CKD may be seen as a state of high FGF23, high PTH and low vitamin D and Klotho levels. Serum levels of FGF23 rise early in the course of CKD and these changes occur before alterations in phosphate, PTH and (active) vitamin D can be measured. The reason of the early rise of FGF23 is still not completely clear. Two mechanisms are proposed: decreased renal clearance, although a recent study suggested that impaired renal clearance of PTH exceeds FGF23 clearance in CKD, or end organ resistance due to deficiency of Klotho. Indeed, lower
Klotho expression has been found in CKD patients. In any case, the initial rise in FGF23 might be an adaptive response to promote phosphate excretion in the face of failing renal function. Elevated FGF23 levels have been identified as the earliest detected serum abnormality in CKD-MBD, and are strongly and independently associated with worse clinical outcomes in kidney transplant recipients, CKD and ESRD patients. It is thought that these associations indicate a causal role of FGF23 in the pathophysiology of CKD, making it a potential target for future interventions. However, it is unclear if (and to which extent) FGF23 is a causative factor or merely a marker for different pathological processes. While studies addressing renal pathophysiological effects of FGF23 are only beginning to emerge, protective effects of Klotho have already been reported. Similar to CKD patients, various animal models of CKD are characterized by considerable down regulation of Klotho mRNA and protein in the kidney and by low plasma or urine soluble Klotho levels. Conversely, data from experimental studies suggest that correction of Klotho deficiency either by genetic means or by treatment with recombinant protein has multiple protective effects. Soluble Klotho has been shown to protect against (acute) kidney injury and renal fibrosis but also against cardiovascular complications of CKD such as vascular calcification and endothelial dysfunction. Although vasculoprotective effects of Klotho can hardly be disputed, it is still a matter of debate whether only soluble Klotho mediates these effects or a membrane form of Klotho is also expressed in the vessel wall, which could be involved as well. If this would be the case, then the presence of the functional co-receptor for FGF23 in the vessel wall would provide a link between the epidemiological evidence that connects high levels of FGF23 in CKD, and increased all-cause and cardiovascular mortality.

FGF23 has been associated with myocardial infarction, vascular calcification, and endothelial dysfunction, although FGF23 has been even more strongly associated with volume overload and heart failure and may be involved in the development of left ventricular hypertrophy. However, the detrimental effects of FGF23 may not be limited to the cardiovascular system. Several studies reported association between FGF23, proteinuria and renal disease progression but also with increased expression of inflammatory markers in CKD patients. Higher FGF23 levels have been linked with a resistance to RAAS blockade based therapies as well. Post-hoc analysis of the Ramipril Efficacy In Nephropathy trial has shown that high serum phosphate is associated with reduced efficacy of ACE-inhibitors to retard the development of ESRD. However, since FGF23 levels were not determined in this study, it is tempting to speculate that attenuation of the efficacy of ACEi might be also related to higher FGF23. In line, studies from our group indicate that higher FGF23 levels were associated with a worse response to intensified RAAS-blockade-based treatment of proteinuria. As already mentioned, it is still not completely clear if FGF23 represents a marker of renal damage or it is an actual contributing factor to progression of renal damage and/or therapy resistance. Presuming the causative role of FGF23 then three possible underlying mechanisms may be envisaged, based on the current knowledge. High levels of FGF23 could directly promote the progression of renal damage by regulating the expression of genes and pathways involved in tubular damage and fibrosis such as lipocalin-2, TGF-β and TNF-α. Second, via down-regulation of the Ace2 gene, FGF23 might lead to activation of the RAAS (i.e. persistent high angiotensin II levels), but also to downregulation of vitamin D. Lastly, FGF23 could promote volume retention thus contributing to therapy resistance. If and to which extent
these possible effects of FGF23 depend on Klotho still remains to be determined.

1.3 Thesis outline

The overall aim of this thesis is to gain more insight in the role of mineral hormones in the development and treatment of chronic renal disease. In Chapter 2 an overview of renoprotective effects of vitamin D and its analogues is presented. Vitamin D has anti-proteinuric, anti-inflammatory and anti-fibrotic effects. Activation of the vitamin D receptor also provides additional reduction of proteinuria and renal damage when given on top of RAAS blockade. Optimization of RAAS blockade efficacy by dietary sodium restriction has been demonstrated by many studies, both in the experimental and the clinical setting. In Chapter 3 we take a step further and investigate if in the experimental model of adriamycin nephropathy addition of vitamin D analogue paricalcitol could provide additional protection when given on top of RAAS blockade during a low sodium diet. Moreover, we explore the potential of vitamin D analogue to overcome resistance to RAAS blockade during a high sodium diet. Although proteinuria can be lowered to almost control levels as evidenced by animal studies this does not always translate into full protection of renal tubulo-interstitium. Besides proteinuria, the severity of tubulo-interstitial damage is also correlated with subsequent renal function decline. Identification of non-invasive markers of tubulo-interstitial damage may allow better titration and monitoring of renoprotective therapy. In Chapter 4 we identify urinary vitamin D binding protein (VDBP) as a marker of interstitial inflammation and fibrosis. Development of interstitial renal fibrosis is a final common pathway of renal disease regardless the initial cause. Currently available renoprotective strategies are not sufficiently effective in lowering interstitial fibrosis, and this may at least in part be through (deleterious) cross talk between FGF23-Klotho and the RAAS. In Chapter 5 we explore the interaction between FGF23 and RAAS blockade in a mouse model of renal fibrosis. Finally, in Chapter 6 we investigate the vascular expression of membrane bound Klotho. Presently it is not completely clear if this form of Klotho is expressed in human vessels and if it is whether exerts same protective effects as soluble form of Klotho. These observations are important to understand the mechanisms behind vascular calcification in CKD but also with respect to the development of future treatments, including replacement/substitution of the potentially missing hormone. In Chapter 7 the results of the studies described in this thesis are summarized and perspectives are discussed.
General Introduction

References


