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THE ROLE OF FGF23/KLOTHO IN MINERAL METABOLISM AND CHRONIC KIDNEY DISEASE

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Front cover. A reporter strain was used to determine the tissue specificity of Ksp-cadherin Cre. Cre expression (red) was found almost exclusively in the renal distal tubules. LTL (green) is a marker of proximal tubules and DAPI (blue) binds DNA and stains cell nuclei. Photo by Tadatoshi Sato.

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ABSTRACT

Chronic kidney disease (CKD) is a global health burden of growing incidence and prevalence. As renal function declines disturbances in mineral metabolism, such as hyperphosphatemia and secondary hyperparathyroidism, inevitably develop. These metabolic changes are closely associated with poor prognosis and survival. The bone-derived hormone fibroblast growth factor-23 (FGF23) and its co-receptor Klotho represent a novel endocrine axis regulating mineral metabolism in health and disease. FGF23-Klotho signalling inhibits renal phosphate reabsorption and activation of vitamin D, and reduces secretion of parathyroid hormone (PTH). Serum levels of FGF23 rise at early stages of CKD, presumably due to increased phosphate load, and numerous studies identify elevated FGF23 as a predictor of adverse clinical outcome. In contrast, tissue expression of Klotho decreases in parallel with CKD progression and reaches low or undetectable levels in end-stage renal disease. Importantly, mice lacking Klotho develop numerous complications associated with accelerated ageing, and many patients with advanced CKD, a state of Klotho deficiency, display a similar senescence-like phenotype. Altogether, FGF23 excess and lack of Klotho may be key pathogenic factors in CKD. In the present thesis we sought to elucidate the role of renal and parathyroid FGF23-Klotho signalling in physiology and in CKD.

In **Study I** we investigate Klotho levels in surgically resected parathyroid tissue specimen from CKD patients with secondary hyperparathyroidism, and find diminished Klotho expression paralleling the decline in renal function. Further, we demonstrate that FGF23 dose-dependently suppresses Klotho in bovine parathyroid cell culture, indicating a ligand-receptor regulatory process.

In **Study II** we generate parathyroid-specific Klotho knockout mice (*PTH-KL*$^{-/-}$) using Cre-Lox recombination. *PTH-KL*$^{-/-}$ mice display a normal gross phenotype with a preserved calcium-PTH axis. Their PTH response is similar to wild-type mice when treated with FGF23 or challenged with renal failure. Yet, FGF23 treatment activates the MAPK pathway in wild-type mice but not in *PTH-KL*$^{-/-}$ mice. Importantly, blocking of calcineurin with cyclosporine A abolishes the FGF23-mediated PTH suppression in *PTH-KL*$^{-/-}$ mice, whereas wild-type mice remain responsive. Thus, we identify a novel calcineurin-dependent pathway in the parathyroid glands that, in the absence of Klotho, mediates acute suppression of PTH secretion by FGF23.

In **Study III** we develop a novel, non-surgical, mouse model of tubulointerstitial nephropathy. By adding various concentrations of adenine to the diet we define an adjustable protocol for inducing and maintaining uremia in mice.

In **Study IV** we generate distal tubule-specific Klotho knockout mice (*Ksp-KL*$^{-/-}$). In contrast to systemic Klotho knockout mice, *Ksp-KL*$^{-/-}$ mice are fertile with a normal gross phenotype. Adult *Ksp-KL*$^{-/-}$ mice are hyperphosphatemic, indicating attenuated effects of FGF23 on proximal tubular phosphate handling. Further, FGF23 is higher in *Ksp-KL*$^{-/-}$ mice than in wild-type mice with matched serum phosphate, suggesting phosphate-independent regulation of FGF23 in *Ksp-KL*$^{-/-}$ mice.

Collectively, the studies presented in this thesis identify several novel and critical aspects of FGF23-Klotho signalling and function in health and disease, and provide important tools allowing for continuous investigation.

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Parathyroid Klotho and FGF-receptor 1 expression decline with renal function in hyperparathyroid patients with chronic kidney disease and kidney transplant recipients.
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Submitted manuscript

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IV. **Olauson H**, Lindberg K, Amin R, Jia T, Wernerson A, Andersson G, Larsson TE.
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LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25 dihydroxyvitamin D
25(OH)D	25 hydroxyvitamin D
ADHR	Autosomal Dominant Hypophosphatemic Rickets
AKI	Acute Kidney Injury
ARHR1 and 2	Autosomal Recessive Hypophosphatemic Rickets, Type 1 and 2
CaSR	Calcium-sensing receptor
CKD	Chronic Kidney Disease
CKD-MBD	Chronic Kidney Disease-Mineral and Bone Disorder
cKL	Shedded full-length Klotho
CYP24A1	1,25-dihydroxyvitamin D 24-hydroxylase
CYP27B1	25-hydroxyvitamin D 1-alpha-hydroxylase
ESRD	End Stage Renal Disease
FGF	Fibroblast growth factor
FGF23	Fibroblast growth factor-23
$Fgf23^{-/-}$	Fibroblast growth factor-23 knockout mice
FGFR	Fibroblast growth factor receptor
GALNT3	Polypeptide N-acetylgalactosaminyltransferase 3
GFR	Glomerular Filtration Rate
HFTC	Hyperphosphatemic Familial Tumoral Calcinosis
IF	Immunofluorescence
IHC	Immunohistochemistry
$Klotho^{-/-}$	Klotho knockout mice
$Ksp\text{-}KL^{-/-}$	Distal tubule-specific Klotho knockout mice
mKL	Membrane-bound Klotho
PTH	Parathyroid hormone
$PTH\text{-}KL^{-/-}$	Parathyroid-specific Klotho knockout mice
PTH1R	Parathyroid hormone 1 receptor
qPCR	Quantitative real-time polymerase chain reaction
RAAS	Renin-Angiotensin-Aldosterone system
RCT	Randomized Controlled Trial
sHPT	Secondary Hyperparathyroidism
sKL	Truncated Klotho

TRPV5	Transient receptor potential cation channel subfamily V member 5
VDR	Vitamin D receptor
XLH	X-Linked Hypophosphatemia

1 INTRODUCTION

1.1 MINERAL METABOLISM

Calcium and phosphate are vital in a number of biological systems, including bone formation, energy metabolism, different metabolic pathways and intracellular signalling. Accordingly, their endocrine regulation is a tightly controlled process, and involves several hormones and feedback loops. In physiology, mineral homeostasis is achieved through a balance between intestinal absorption, bone influx and efflux, and renal excretion.

Disturbances in mineral metabolism are implicated in various skeletal, metabolic and endocrine disorders[1,2]. In the community, abnormalities in calcium and phosphate are commonly seen in individuals with impaired renal function. Importantly, high serum phosphate and calcium x phosphate product are independent risk factors for cardiovascular morbidity and mortality in patients with chronic kidney disease (CKD), as well as in healthy individuals[3-5].

In recent years, the identification of a novel endocrine axis comprising fibroblast growth factor-23 (FGF23) and αKlotho (Klotho) has lead to a paradigm shift in the understanding of mineral metabolism.

1.1.1 Calcium homeostasis

Calcium (Ca) is the fifth most abundant element in the human body and is critical for a diverse range of biological processes ranging from bone metabolism and muscle contraction to intracellular signalling. It is an essential element and only available through dietary sources. The daily need depends on gender and age, and varies from approximately 600 mg to 1200 mg. In growing people the calcium balance is positive to allow high bone formation, whilst in the elderly the calcium balance is commonly negative with decreased bone formation and reduced bone mass. Calcium homeostasis is regulated through an intricate interplay between factors acting on the intestine, bone, kidneys and parathyroid glands[6]. The main calcium-regulating systems are parathyroid hormone (PTH) acting on the G protein-coupled parathyroid hormone receptor (PTH1R) and 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) acting on the nuclear vitamin D receptor (VDR).

1.1.1.1 Calcium and parathyroid hormone

A decrease in serum calcium rapidly inactivates the parathyroid gland resident calcium-sensing receptor (CaSR), leading to increased secretion of preformed PTH from parathyroid chief cells[7]. PTH is an 84 amino acid polypeptide that acts on distal tubular PTH1R to increase renal calcium reabsorption, and on skeletal PTH1R to increase bone resorption. Low calcium ion concentration also directly inactivates CaSR in the thick ascending limb of the renal tubule to further increase active calcium reabsorption. If the low calcium levels persist for an extended time there is an upregulation in PTH mRNA transcription and eventually an increase in parathyroid cell proliferation. In contrast, high serum calcium activates CaSR and leads to a rapid decrease in secretion of PTH,

subsequently resulting in increased renal calcium loss, decreased bone resorption and decreased intestinal calcium absorption. The relationship between serum calcium and PTH forms a sigmoidal curve where relatively small changes in calcium concentration evoke large responses in PTH secretion.[8]

1.1.1.2 Calcium regulation by vitamin D

Vitamin D can either be ingested with the diet or synthesized in the skin when exposed to UVB radiation. It is subject to 25-hydroxylation in the liver to 25 hydroxyvitamin D (25(OH)D), and later 1-hydroxylated by renal 25-dihydroxyvitamin D 1-alpha-hydroxylase (CYP27B1) to $1,25(OH)_2D$, the biologically active form. In an intricate feedback system CYP27B1 is induced by PTH, hypocalcemia and hypophosphatemia, and repressed by hypercalcemia and hyperphosphatemia. VDR activation in the parathyroid gland decreases PTH synthesis and secretion, and also upregulates CaSR to make the chief cells more susceptible to inhibition by calcium. In the distal tubule $1,25(OH)_2D$ facilitates calcium uptake by increasing the abundance of transient receptor potential cation channel, subfamily V, member 5 (TRPV5) on the apical membrane and by making the tubule cells more susceptible to PTH-mediated calcium reabsorption[9]. VDR activation also facilitates calcium uptake in the small intestine and increases calcium release from bone through activation of osteoclasts, and under conditions of high calcium demand e.g. lactation, also from osteocytes. Of note, also 25(OH)D binds to and activates VDR, although with a 1000-fold lower affinity than $1,25(OH)_2D$[10].

1.1.2 Phosphate homeostasis

Phosphorous (P) is the sixth most common element in humans and comprise approximately 1.4% of the body mass. It is virtually never in its elemental form, and in the body it is predominantly bound to oxygen as phosphate (PO_4^{3-}). Phosphate is required for all forms of life and plays a crucial role in energy metabolism as part of adenosine triphosphate (ATP), in biological molecules such as DNA and RNA and in the cellular membrane as a constituent of phospholipids. The vast majority of phosphate (approximately 80%) is tied to mineralized tissue, predominantly bone and teeth, in the form of hydroxyapatite ($[Ca]_5[PO^4]_3[OH]$). The remainder is distributed in skeletal muscles and in extracellular compartments. Phosphate homeostasis is similarly to calcium maintained by several factors affecting intestinal absorption, renal reabsorption and skeletal metabolism. The physiological range for serum phosphate is wider than for calcium, changes are better tolerated and the adaptive mechanisms much less rapid.

1.1.2.1 Intestinal phosphate absorption

Absorption of phosphate takes place in the small intestine through both passive diffusion and active transport by the sodium-dependent phosphate transporter Npt2b. High dietary phosphate intake increases the passive paracellular uptake while active transport of phosphate is induced by $1,25(OH)_2D$, increasing the abundance of the sodium-dependent phosphate co-transporter Npt2b on the luminal side of enterocytes. The exact contribution of passive versus active transport of phosphate is not known, but recent animal studies emphasize the significance of active transport[11].

1.1.2.2 Renal phosphate handling

The kidneys are key organs in phosphate regulation and adequate renal handling of phosphate is essential in maintaining a neutral balance. Phosphate is filtered freely in the glomerulus and the reabsorption in proximal tubules adapts in response to endocrine regulation. Under physiological conditions, around 70% of the filtered phosphate is reabsorbed. The rate of reabsorption can be increased during low phosphate conditions, and is determined mainly by the apical brush-border abundance of the sodium-dependent phosphate co-transporters Npt2a and Npt2c. Npt2a and Npt2c are expressed predominantly in the early segments of the proximal tubule and accounts for approximately 80% and 20% of the active reabsorption respectively[12].

1.1.2.3 Phosphate and the bone

In addition to the intestine and kidneys, bone plays an important role in phosphate metabolism. Phosphate is a crucial component in the matrix mineralization process by osteoblasts and osteocytes. Conversely, as the main repository for phosphate the skeleton can adapt to changes in the demand of extracellular phosphate through altered bone remodelling.

1.1.2.4 Phosphate regulation

Until recently PTH was considered the principal hormone responsible for maintaining phosphate homeostasis. High serum phosphate increases PTH secretion independently of serum calcium and $1,25(OH)_2D$[13]. In turn, PTH reduces Npt2a and Npt2c at the apical brush-border membrane through internalization and subsequent degradation, thus increasing urinary phosphate loss[14]. On the other hand, PTH signalling leads to increased phosphate efflux from the bone by enhanced resorption. Additionally, PTH increases phosphate uptake in the small intestine indirectly through activation of CYP27B1. Despite its actions on bone and intestine to increase phosphate, the aggregate effect of PTH is a decrease in serum phosphate, and PTH should therefore be regarded as a phosphate-lowering hormone. Although the existence of a phosphate-sensing receptor has been proposed, the mechanism by which phosphate-regulating hormones adapt to changes in serum phosphate remains unclear[15]. The identification of FGF23 and Klotho represents a paradigm shift in the understanding of phosphate regulation, and is described in detail in subsequent chapters.

1.1.3 Fibroblast growth factor-23

1.1.3.1 Fibroblast Growth Factors and Fibroblast Growth Factor Receptors

Fibroblast growth factors (FGFs) are a highly conserved family of genes organized into seven subfamilies. The 22 genes encode molecules with the capability of binding one or several fibroblast growth factor receptors (FGFRs)[16]. This overlap in specificity commonly leads to receptor redundancy, where absence of one FGFR can be compensated by others. Of note, alternative splicing of the four *FGFR* genes results in almost 50 different isoforms of FGFR. Binding of the ligand to a receptor dimer of two FGFRs evokes a signal transduction with a plethora of downstream effects. FGF signalling activates several common pathways, including MAPK and PLCγ[17,18]. In

contrast to other FGFs' intracrine or paracrine actions, the FGF19 subfamily members (FGF19, FGF21 and FGF23) act as endocrine factors[19].

1.1.3.2 FGF23 structure

The *FGF23* gene is located on chromosome 12 and composed of three exons encoding a 251 amino acid protein (Figure 1A). The 32 kDa protein is mainly produced by osteocytes and osteoblasts in bone[20,21]. FGF23 contains several glycosylation sites and is O-glycosylated by Polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3) to prevent preterm proteolytic cleavage. The full-length FGF23 protein is cleaved at position ^{176}RXXR^{179} by an unknown subtilisin-like pro-protein convertase into one 18 kDa N-terminal and one 12 kDa C-terminal fragment[22]. These fragments do not activate FGFRs, but recent data indicate that the C-terminal fragment may act as a competitive inhibitor to full-length FGF23[23]. However, the physiological relevance of the FGF23 fragments is unknown.

1.1.3.3 FGF23 function

In the kidney FGF23 decreases reabsorption of phosphate in the proximal tubule by down-regulating Npt2a and Npt2c[24]. FGF23 also inhibits the vitamin D activating enzyme CYP27B1 and increases the catabolism of vitamin D through activation of 1,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1), altogether resulting in lower levels of circulating $1,25(OH)_2D$[25]. In the parathyroid gland FGF23 decreases the synthesis and secretion of PTH, and in contrast to the kidney induces expression of CYP27B1[26,27].

1.1.3.4 Human disorders

FGF23 was originally identified as the causative factor in patients with autosomal dominant hypophosphatemic rickets (ADHR, OMIM 193100), a rare hereditary disorder characterized by urinary phosphate wasting and reduced bone mineralization[28]. Missense mutations at the cleavage site of FGF23 prevent processing and result in accumulation of the intact protein. Also in X-linked hypophosphatemia (XLH, 307800) and autosomal recessive hypophosphatemic rickets 1 and 2 (ARHR1 and ARHR2, 241520 and 613312) the levels of FGF23 are abnormally elevated[29]. This is caused by mutations in the FGF23-regulating genes *PHEX*, *DMP1* and *ENPP1* respectively, but the molecular mechanisms behind these disorders are incompletely understood. In opposite, inactivating mutations in *GALNT3* lead to enhanced degradation of FGF23 due to defect glycosylation, causing hyperphosphatemic familial tumoral calcinosis (HFTC, 211900), a syndrome characterized by hyperphosphatemia and deposition of calcium-phosphate crystals in the soft tissues secondary to reduced FGF23 activity[30].

1.1.3.5 Mouse models

In support of FGF23's phosphaturic and vitamin D suppressive properties, overexpression of FGF23 in transgenic mice or intravenous administration of recombinant FGF23 causes a phenotype similar to that seen in human disorders of FGF23 excess with hypophosphatemia, reduced $1,25(OH)_2D$ levels and impaired skeletal mineralization[31-33]. Conversely, FGF23 knockout mice (*Fgf23*$^{-/-}$) display severe hyperphosphatemia secondary to reduced renal phosphate excretion, elevated $1,25(OH)_2D$ and widespread soft tissue calcifications[34].

1.1.3.6 Regulation of FGF23

The regulation of FGF23 has proven to be more complex than first anticipated, and is still incompletely understood. All key components of mineral metabolism, namely phosphate, calcium, vitamin D and PTH, stimulate the expression of FGF23. In addition, recent studies have found that iron, estrogen, leptin and glucocorticoids also alter FGF23 synthesis and secretion[35]. Activation of a vitamin D-responsive element in the FGF23 promoter is the most potent known stimulus for FGF23 expression, thus forming a classical endocrine feedback loop between FGF23 and vitamin D. Administration of $1,25(OH)_2D$ dose-dependently increases transcript and serum levels of FGF23, which in turn suppresses $1,25(OH)_2D$[25]. Conversely, when vitamin D activity is abrogated through dietary means or by genetic targeting, serum FGF23 is virtually undetectable[16,36]. Since FGF23 is a phosphaturic hormone, phosphate was long considered to be one of its main inducers. Indeed, dietary phosphate does regulate FGF23, although the effects in healthy individuals are modest and have slow onset[37]. Of note, the intestine seems to play a part in phosphate-mediated FGF23 induction since intravenous administration of phosphate does not lead to elevated FGF23, despite a similar increase in serum phosphate as for dietary loading[38]. When summarizing available data there is little evidence for a direct regulation of FGF23 by phosphate, suggesting regulation via indirect mechanisms; speculatively through intestinal factors and altered bone metabolism. Emerging data indicates a central role for iron in FGF23 processing and secretion. C-terminal FGF23 is markedly elevated in patients with iron deficiency, and infusion of iron-containing compounds decreases the c-terminal FGF23 levels while intact FGF23 increases or remains unchanged[39]. This is supported by a recent study where wild-type mice exposed to a low-iron diet had elevated mRNA levels of FGF23 but maintained normal intact FGF23 levels through enhanced intracellular degradation. In contrast, mice carrying an ADHR mutation fed an iron-deficient diet suffered from hypophosphatemia and osteomalacia secondary to increased levels of intact FGF23[40]. The cellular mechanism(s) governing transcriptional and posttranslational regulation of FGF23 are still largely unknown and merits further investigation.

1.1.4 Klotho

1.1.4.1 Discovery of the Klotho gene

The *Klotho* gene was identified by Kuro-o et al in 1997 when studying transgenic mice overexpressing a sodium proton exchanger[41]. By accident a locus of a neighbouring gene was interrupted, and the mice (*Klotho*$^{-/-}$) displayed a striking phenotype resembling human ageing with reduced activity, osteoporosis, vascular and soft tissue calcifications, pulmonary emphysema, skin atrophy and shorter lifespan. Conversely, overexpression of Klotho in mice led to an extended lifespan, and *Klotho* was therefore considered to be an anti-ageing gene[42]. The name derives from the goddess Clotho, daughter of Zeus and the youngest of the Three Fates or Moirai. In Greek mythology, Clotho spins the thread of life and decides over birth and death.

1.1.4.2 Structure and isoforms of Klotho

The *KLOTHO* gene is located on chromosome 13 and has five exons encoding a 1014 amino acid protein. The Klotho protein has a short intracellular domain and two

extracellular tandem repeats (KL1 and KL2) with homology to beta-glucosidases. Membrane-bound Klotho is predominantly expressed in the kidneys distal tubule, the parathyroid glands and the choroid plexus of the brain[43]. Expression has also been reported in other cell types such as sinoatrial cells of the heart, monocytes and mesenchymal stem cells, although at low absolute levels and of uncertain physiological relevance[44,45]. The Klotho protein exists in three distinct isoforms; the 130 kDa full-length membrane-bound form (mKL), soluble Klotho produced by ectodomain shedding of mKL from the cell surface (cKL), and a truncated form produced through alternative splicing at exon 3 that only contains the KL1 domain (sKL)(Figure 1B)[43].

1.1.4.3 Klotho as a co-reptor for FGF23

Unlike most other FGFs, FGF23 lacks a heparan-sulfate-binding motif and therefore has low affinity to FGFRs. Interestingly, *Fgf23*$^{-/-}$ and *Klotho*$^{-/-}$ mice share almost identical phenotypes with reduced body size, organ atrophy, extensive soft tissue calcifications, reduced bone mineralization and shortened life-span. In 2006, a Japanese group identified mKL as an essential co-factor for FGF23 signalling, enabling high-affinity binding to FGFR1c, 3c and 4 and subsequent activation of the MAPK pathway (Figure 1C)[46]. The indispensable role of mKL as a co-receptor for FGF23 is supported by the fact that *Klotho*$^{-/-}$ mice suffer from hyperphosphatemia, elevated $1,25(OH)_2$ D and reduced urinary phosphate excretion despite extreme levels of circulating FGF23. A similar phenotype is seen in a patient with an inactivating mutation in the *KLOTHO* gene where a markedly elevated FGF23 is unable to correct the biochemical disturbances due to end-organ resistance[47]. Since the FGFRs are ubiquitously expressed the tissue-specificity for FGF23 action is determined by the limited distribution of Klotho.

1.1.4.4 FGF23-independent functions of Klotho

In addition to its role as a co-receptor for FGF23 mKL has been proposed to facilitate PTH secretion at low calcium conditions through recruitment of the Na^+/K^+-ATPase to the surface of parathyroid cells[48]. Similarly, Klotho was found to increase abundance of the Na^+/K^+-ATPase in the collecting duct to prevent renal salt wasting and hypovolemia[49]. Although interesting, these data are controversial and the mechanisms of action have been challenged[50].

1.1.4.5 Regulation of Klotho

Hypophosphatemia and $1,25(OH)_2D$ both induces the renal expression of mKL[51,52]. In contrast, continuous exposure to high FGF23 in *Fgf23* transgenic mice reduces mKL, although it is unknown if this is a direct or indirect effect[53]. The upstream regulators of Klotho shedding and alternative splicing are still largely unknown.

1.1.4.6 Soluble Klotho

cKL is cleaved off the cell surface by membrane-anchored proteases, including ADAM10 and 17, and acts as a hormone and an enzyme[54]. In an FGF23-independent fashion cKL reduces the proximal tubule phosphate reabsorption through enzymatic actions leading to enhanced endocytosis and degradation of Npt2a[55]. In addition, cKL can hydrolyze sugar residues on the renal calcium channel TRPV5 and the potassium channel ROMK1[56]. The removal of sugar residues triggers interaction with Galectin-1 and prevents endocytosis, thereby increasing the abundance of these cation channels at

the cell surface[57]. cKL also inhibits insulin and insulin-like growth factor I activity, corroborating with the observation that *Klotho*$^{-/-}$ mice are hypoglycemic with increased sensitivity to insulin[42]. The receptor responsible for binding cKL has yet not been identified, but could speculatively be a FGFR or even mKL. cKL doesn't function as a co-receptor for FGF23, but has proven to be a potent stimulator of FGF23 expression. In a patient with a translocation in the *KLOTHO* gene causing increased serum cKL, FGF23 was markedly elevated resulting in hypophosphatemic rickets[58]. Likewise, mice overexpressing cKL have increased FGF23 levels despite persistent hypophosphatemia[59]. The notion of cKL as an inducer of FGF23 is somewhat paradoxical, and the physiological relevance remains uncertain.

sKL is present in serum, urine and cerebrospinal fluid of healthy individuals, but the functions of sKL are not well characterized. Recent data suggest that sKL as well as cKL are antagonists of endogenous Wnt/β-catenin signalling, and ameliorate the development of renal fibrosis[60]. However, further investigation to determine the role of sKL is warranted.

1.1.4.7 Quantification of soluble Klotho

Methods for quantification of soluble Klotho (cKL and sKL) have recently been developed. A sandwich ELISA was established in 2010 and the first report revealed a graded decline with increasing age in healthy individuals. Soluble Klotho also correlated to serum phosphate and inversely to creatinine and FGF23[61]. However, soluble Klotho levels were unaltered in patients with XLH and elevated FGF23[62]. In a comparison of two different ELISA for soluble Klotho the outcome differed substantially, underlining the uncertainty of current methods and the need for precaution when interpreting the results[63].

A schematic overview of the regulation of mineral metabolism is found in Figure 2.

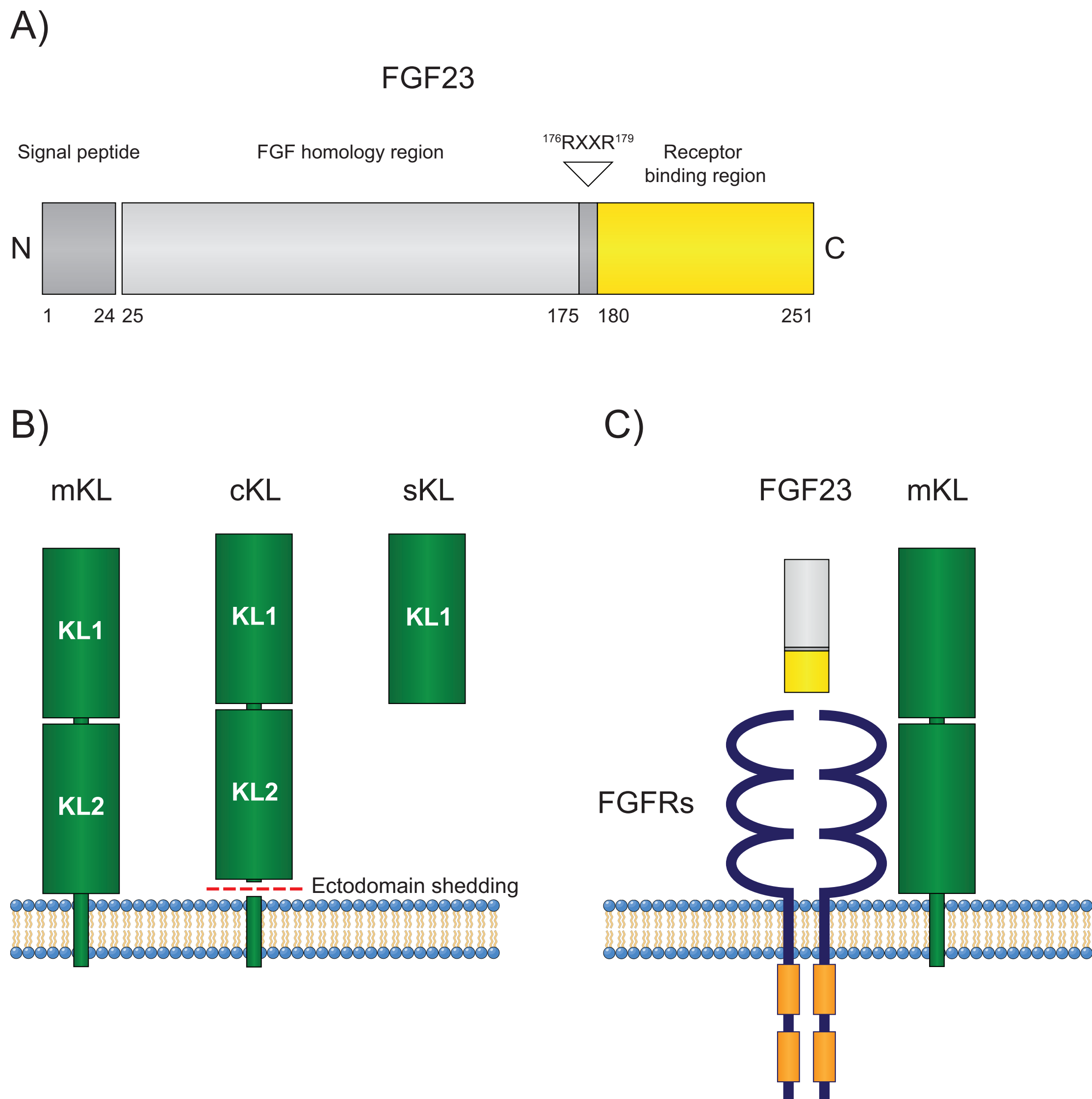


Figure 1. Fibroblast growth factor-23 (FGF23), the three isoforms of Klotho and the FGF23–FGF receptor (FGFR)–Klotho complex. A) Structure of the 251 amino acid FGF23 protein, with the N-terminal FGF homology region and the unique C-terminal receptor-binding region, allowing interaction with the co-receptor Klotho. The active full-length form of FGF23 can be cleaved into two inactive fragments at the position ^{176}RXXR^{179}. B) Membrane-bound Klotho (mKL) can be cleaved of at the cell surface by secretases to form soluble Klotho (cKL). Another form of soluble Klotho (sKL) is generated through alternative splicing at exon 3. C) Intact FGF23 binds a receptor complex of Klotho and a FGFR dimer to activate downstream signalling, most importantly through the MAPK pathway. Adapted from Hu MC et al[64].

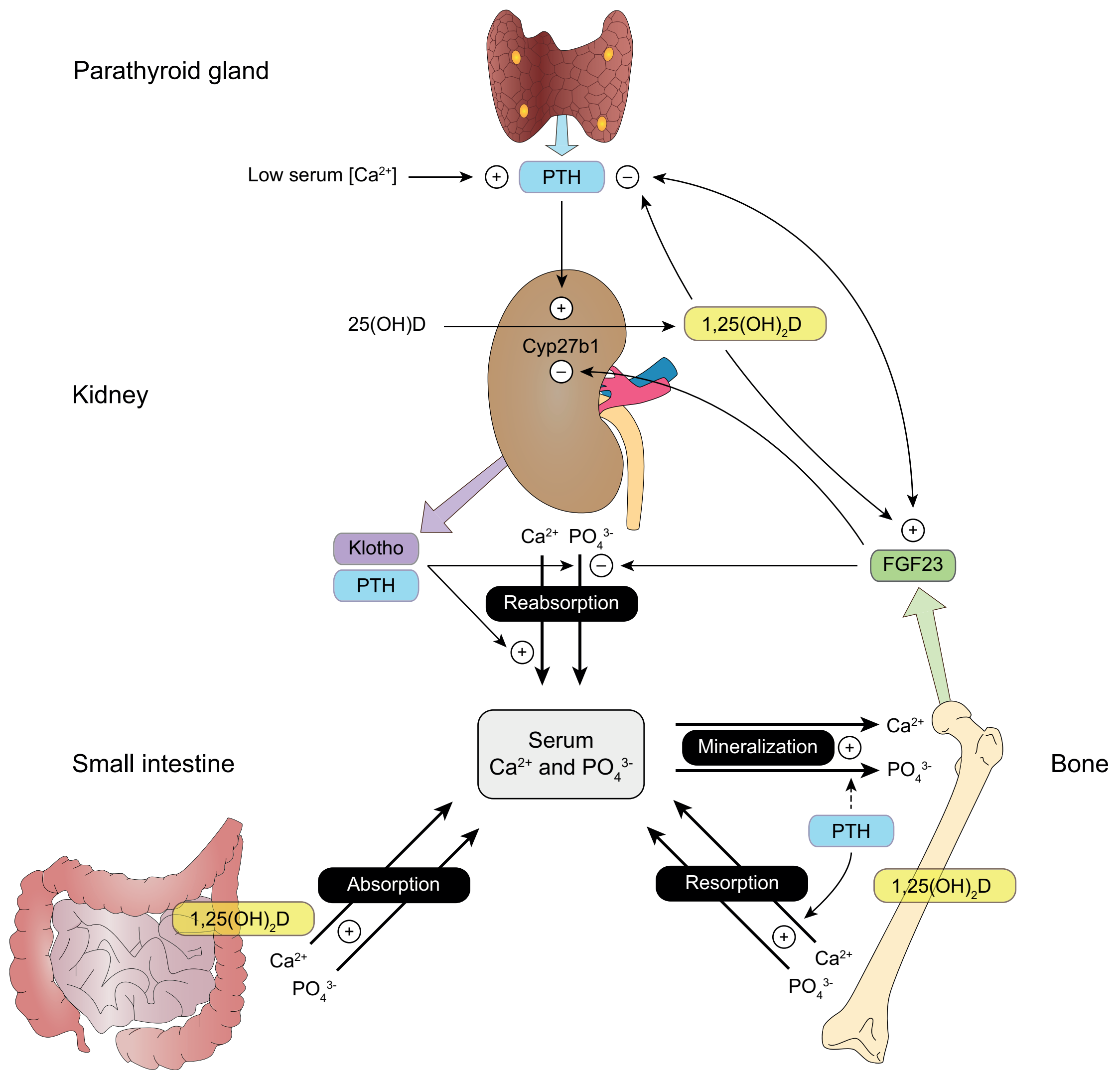


Figure 2. Endocrine regulation of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) metabolism. Calcium and phosphate are regulated through a complex interplay involving the parathyroid glands, kidneys, intestine and bone, and the key hormonal regulators; parathyroid hormone (PTH), fibroblast growth factor-23 (FGF23), soluble Klotho and 1,25 dihydroxyvitamin D (1,25$(OH)_2$D). PTH is released from the parathyroid glands when serum calcium is low, and increases bone resorption and renal calcium reabsorption directly, and intestinal absorption indirectly through activation of 1,25$(OH)_2$D. Klotho is released from the kidney and act as a phosphaturic and calciotropic hormone in the renal tubules. High serum phosphate induces FGF23 secretion, which in turn reduces renal phosphate reabsorption. PTH, FGF23, Klotho and 1,25$(OH)_2$D also regulate each other through negative feedback loops.

1.2 CHRONIC KIDNEY DISEASE

1.2.1 Background

The term chronic kidney disease (CKD) is defined by the presence of renal damage (often quantified by albuminuria) and/or decreased glomerular filtration rate (GFR below 60 mL/min/1.73 m^2) for more than three months, irrespective of underlying aetiology. It is classified into five stages based on GFR[65]. The most common underlying pathology of CKD is diabetic glomerulosclerosis, followed by vascular disease/hypertensive nephrosclerosis and glomerular disease. Today CKD is a major health concern affecting 5-10% of the population globally, and as many as 15% in Europe and in the United States (Table 1)[66,67]. The global incidence and prevalence of CKD is growing, mainly attributed to the ageing population and the concomitant increase in CKD risk factors, such as hypertension and diabetes. The age-adjusted mortality risk is increased already in early stages of CKD and increases further as the deterioration in renal function progresses[68]. In patients with CKD stage 5, also called end stage renal disease (ESRD), the five-year survival rate is approximately 50%[5]. The incidence and prevalence of cardiovascular disease (CVD) is dramatically increased in patients with renal dysfunction, and CVD is one of the leading causes of mortality in CKD[69]. Current therapies in CKD are targeted at the multiplicity of factors known to be associated with disease progression and mortality, including albuminuria, hypertension, hyperphosphatemia, secondary hyperparathyroidism (sHPT) and activation of the Renin-Angiotensin-Aldosterone system (RAAS). Renal replacement, i.e. dialysis or kidney transplantation, are life saving therapies in end stage CKD. Yet, the reduced long-term survival and risk for cardiovascular complications in the uremic setting are discouraging and new effective treatment strategies for patients in all stages of CKD are much needed.

<table>
<tr><th rowspan="2">Stage</th><th rowspan="2">Description</th><th rowspan="2">GFR (mL/min/1.73 m²)</th><th colspan="2">Albuminuria (mg/g)</th></tr>
<tr><th><30</th><th>>30</th></tr>
<tr><td>1</td><td>Normal or increased GFR</td><td>≥90</td><td rowspan="2">87.9%</td><td>3.7%</td></tr>
<tr><td>2</td><td>Mild decrease in GFR</td><td>60-89</td><td>3.4%</td></tr>
<tr><td>3</td><td>Moderate decrease in GFR</td><td>30-59</td><td colspan="2">4.7%</td></tr>
<tr><td>4</td><td>Severe decrease in GFR</td><td>15-29</td><td colspan="2">0.2%</td></tr>
<tr><td>5</td><td>Kidney failure</td><td><15 (or dialysis)</td><td colspan="2">0.0%</td></tr>
</table>

Table 1. Stages of chronic kidney disease (CKD) and prevalence in the US population. CKD is defined as either kidney damage (usually quantified by albuminuria) or glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for more than three months. Adapted from the revised KDIGO Guidelines[65].

1.2.2 Chronic kidney disease – mineral and bone disorder

1.2.2.1 Disturbances in mineral metabolism

As renal function declines there is a progressive derangement in mineral homeostasis. FGF23 increases in early stages of CKD and its rise precedes the reduction in 1,25(OH)$_2$D and elevation in PTH[70,71]. Initially, adaptive mechanisms compensate for the reduced number of nephrons and maintain serum calcium and phosphate within normal ranges. However, starting at CKD stage 3-4 the response to FGF23 and PTH is insufficient to compensate for the loss of GFR and phosphate retention and hypocalcemia begin to develop[72].

Along with the new insights into the dysregulation of mineral metabolism in CKD-MBD, the sequence of events has been redefined as; increased FGF23 – 1,25(OH)$_2$D-deficiency – sHPT – hyperphosphatemia. The temporal changes in mineral metabolism during CKD progression are summarized in Figure 3.

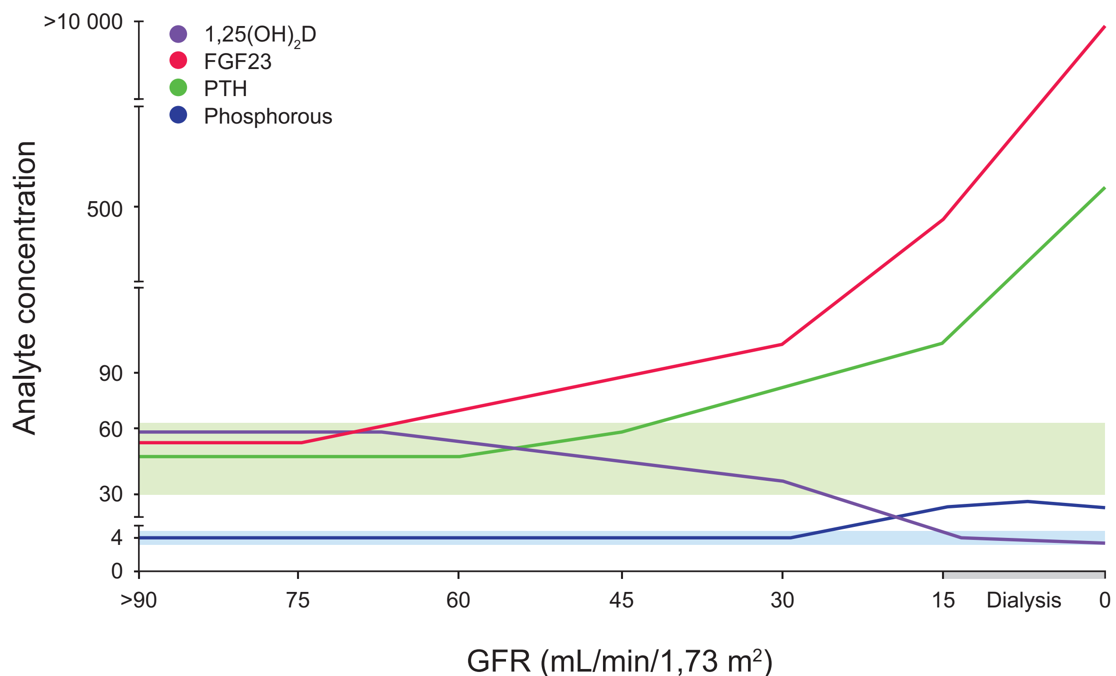


Figure 3. Temporal changes in mineral metabolism during development of chronic kidney disease-mineral and bone disorder (CKD-MBD). The rise in fibroblast growth factor-23 (FGF23) is an early event during CKD progression, and prevents hyperphosphatemia at the expense of decreased levels of 1,25 dihydroxyvitamin D (1,25(OH)$_2$D) and a subsequent development of secondary hyperparathyroidism. The markedly reduced tissue concentration of the FGF23 co-receptor Klotho in late stages of CKD induces end-organ resistance, and FGF23 levels rise exponentially. Adapted from Wolf M[72].

1.2.2.2 Renal osteodystrophy

The bone, as one of the main organs for handling calcium and phosphate, is dependent on a well-regulated mineral metabolism to maintain its normal function, and as a consequence bone abnormalities are frequently found in the CKD population. The alterations in bone morphology associated with CKD are termed renal osteodystrophy, and encompasses changes in bone turnover, mineralization and bone volume. Renal

osteodystrophy may result in fractures, bone pain and impaired linear growth in children[73].

1.2.2.3 Vascular calcification

In parallel with bone abnormalities there is an increase in extraosseous calcifications in CKD, most importantly in the vasculature. Vascular calcification develops as a result of an imbalance between inducers and inhibitors of the calcification process, and several known inhibitors, including Fetuin-A and matrix Gla-protein, are reduced in CKD patients[74,75]. Accordingly, vascular calcification is a major concern in CKD patients with increased prevalence already at CKD stage 3 (40% compared to 13% in healthy individuals), and is a universal phenomenon in ESRD (approximately 80%). Vascular calcification is closely related to and considered to be a valid surrogate for the longitudinal risk of CVD and mortality[76]. Although the molecular mechanism behind vascular calcification is not fully elucidated it appears to be a combination of passive deposition and an active cellular process. The passive deposition is caused by precipitation of high circulating concentrations of calcium and phosphate into the vascular wall, while the active process is commonly viewed as a dedifferentiation of vascular cells into bone-like cells, triggered by various stimuli[77].

1.2.2.4 Chronic kidney disease-mineral and bone disorder

Numerous epidemiological studies have shown a strong correlation between CKD-related disturbances in mineral metabolism to bone abnormalities, CVD and overall mortality[3,78]. This constellation of features is collectively referred to as chronic kidney disease-mineral and bone disorder (CKD-MBD) and defined by one or a combination of a) abnormalities in calcium, phosphate, PTH and vitamin D metabolism, b) abnormalities in bone metabolism, and c) vascular or other soft tissue calcifications (Figure 4)[79].

1.2.2.5 Treatment of CKD-MBD

Current treatment strategies in CKD-MBD are primarily aimed at correcting the aforementioned biochemical abnormalities to limit their assumed negative impact on clinical outcome. To this end, hyperphosphatemic patients in advanced stages of CKD are recommended to receive phosphate-binding agents to reduce the oral phosphate load and achieve a neutral phosphate balance. Further, dialysis patients with sHPT despite adequate supplementation of calcium and/or vitamin D commonly receive treatment with vitamin D analogues or calcimimetics to reduce PTH. However, there are to date no large randomized clinical trials (RCTs) that have demonstrated benefit of such treatment in terms of hard clinical endpoint (i.e. CVD or mortality)[79].

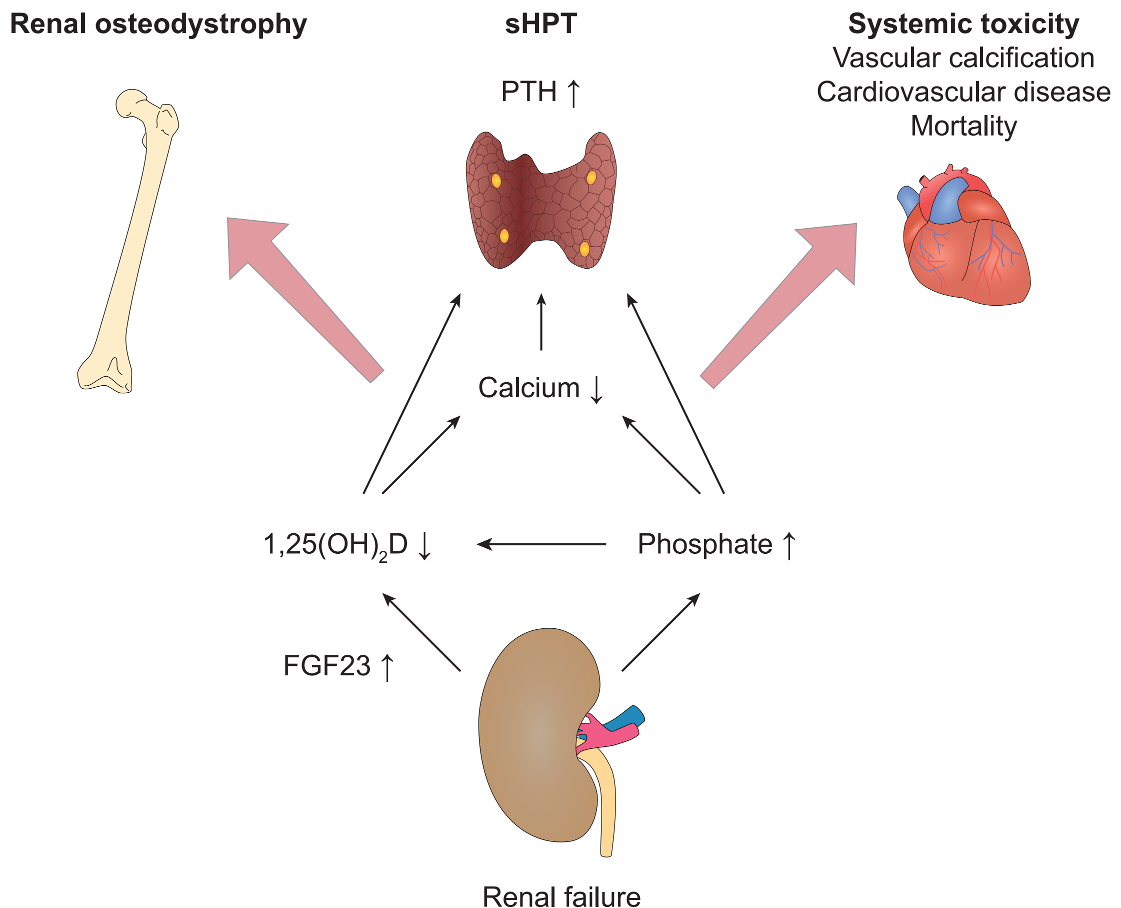


Figure 4. Development of chronic kidney disease-mineral and bone disorder (CKD-MBD). When renal function declines there is a progressive derangement in mineral homeostasis. FGF23 increases in early stages of CKD and is followed by $1,25(OH)_2D$ deficiency, hyperphosphatemia and a rise in PTH (secondary hyperparathyroidism (sHPT)). The alterations in mineral metabolism is closely linked to renal osteodystrophy, ectopic calcification and mortality.

1.2.3 FGF23 in CKD

1.2.3.1 Regulation of FGF23 in CKD

Phosphate retention due to decreased renal clearance was long considered as the main trigger for the increase in FGF23 accompanying CKD progression. Emerging evidence, however, emphasize the importance of other factors in initiating and sustaining the high FGF23 expression during CKD. Although additional factors likely remains to be identified, iron deficiency, vitamin D supplementation, high PTH and hypocalcemia have all been implicated in the regulation of FGF23 in CKD[35].

1.2.3.2 FGF23 as a predictor of adverse outcome

In 2008 the first prospective study of FGF23 provided evidence for a graded relation between circulating FGF23 and mortality in hemodialysis patients[80]. The association was independent of other established risk factors, and FGF23 had stronger predictive value than serum phosphate. This was followed by a number of epidemiological studies supporting that FGF23 is associated with adverse clinical outcomes, most importantly cardiovascular morbidity, mortality and CKD progression rate, in diverse populations

ranging from healthy individuals to various strata of CKD patients and renal transplant recipients[81-90].

1.2.4 Klotho in CKD

1.2.4.1 Reduced tissue expression of Klotho in CKD

Many features of aging, such as osteoporosis, oxidative stress, insulin resistance, infertility and cognitive dysfunction also characterize the phenotype of renal failure, and CKD has accordingly been proposed as a clinical model of premature ageing[91]. Notably, dialysis patients share many biochemical and histological features with *Klotho*$^{-/-}$ mice, with hyperphosphatemia, elevated FGF23 levels, bone abnormalities, vascular calcification and reduced survival. Thus, Klotho deficiency could be a functional link between *Klotho*$^{-/-}$ mice and CKD patients explaining their senescent phenotype. Due to its tissue residing properties invasive methods are required to quantify mKL, which have effectively limited the number of studies available in humans. One study using renal biopsies reported on a gradual decrease in mKL as renal function decline, with the most severe reduction in patients with diabetic nephropathy[92]. In another study comprising patients with ESRD that had undergone nephrectomy, both renal mRNA and protein levels of mKL were nearly undetectable[93]. A large number of animal studies unequivocally confirm the marked reduction of renal mKL in renal failure[64]. Collectively, these data support the concept of CKD as a state of Klotho deficiency.

1.2.4.2 Soluble Klotho in CKD

The relation between soluble Klotho (cKL and sKL) and mKL in CKD is of definite interest, but the measurement of soluble Klotho yet suffers from methodological shortcomings as previously discussed. In some minor cohort studies using a commercially available ELISA, it was reported that soluble Klotho decline in parallel with the progression of CKD, although the associations were rather weak[94-96]. Conversely, in a larger study by Seiler et al, soluble Klotho was not associated with renal function and in contrast to FGF23 it did not predict adverse outcome in patients with CKD stage 2-4[97]. Using a different approach, Hu et al assessed cKL in humans and rodents by immunoprecipitation and immunoblotting, and found a marked downregulation of serum and urinary cKL as early as in CKD stage 1 and 2, presumably reflecting the renal tissue concentration[98]. In sum, there are conflicting data on the levels of soluble Klotho in CKD and additional studies are warranted.

1.2.4.3 Regulation of Klotho in CKD

The mechanism(s) behind reduced Klotho expression in CKD are not fully understood, but are likely to be multifactorial. Several factors associated with CKD, such as RAAS activation, oxidative stress, increased levels of tumor necrosis factor and interferon-γ, have been shown to decrease Klotho expression in cell culture[64]. The regulation is rapid since also acute kidney injury (AKI) potently decreases renal and circulating concentrations of Klotho

2 AIMS

The overall aim of this thesis was to further enhance the understanding of renal and parathyroid FGF23-Klotho function in health and disease. Specifically, our objectives were:

- To define the role of parathyroid FGF23-Klotho signalling in physiology and in the development of renal secondary hyperparathyroidism (Study I and II).
- To investigate the regulation of parathyroid Klotho by factors involved in mineral metabolism (Study I).
- To develop a novel, non-surgical model of renal failure in mouse (Study III).
- To explore the function of distal tubular Klotho in renal phosphate handling, and its putative role in the regulation of FGF23 (Study IV).

3 METHODOLOGICAL CONSIDERATIONS

3.1 ETHICAL APPROVAL

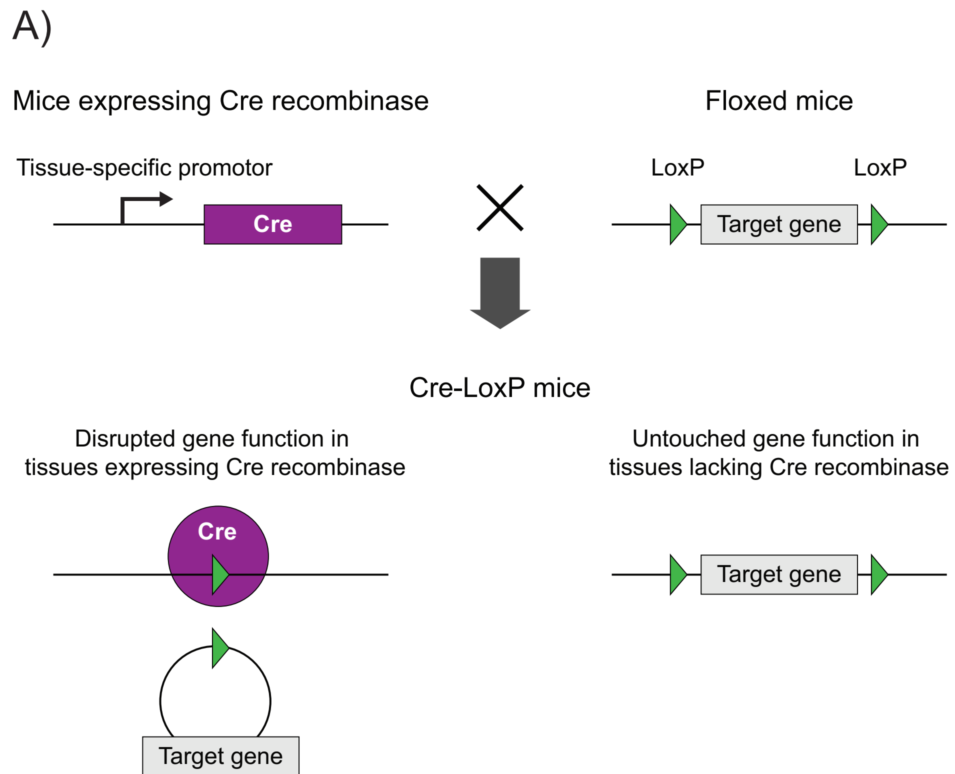
All studies in this thesis adhere to the declaration of Helsinki and/or the 3 Rs principle (replacement, reduction, refinement), for human and animal studies respectively. Study I was approved by the institutional ethical committee at Uppsala University, and written informed consent was obtained from all study participants prior to inclusion in the study. Study II-IV were conducted in compliance with the guidelines of animal experiments of Karolinska Institutet and approved by a regional ethical committee. Ethical approval numbers are Uppsala University 00-128 (Study I) and Stockholm South ethical committee S38-09, S68-10, S184-10, S118-12 and S19-13 (Study II-IV).

3.2 STUDY PARTICIPANTS

All patients (n=31) included in Study I had undergone surgical parathyroidectomy between 1998 and 2008. The study was performed on stored material thus eliminating the need for additional interventions. Inclusion criteria were GFR <90 ml/min/1.73 m^2 and access to at least one frozen parathyroid tissue sample. 21 patients were kidney transplant recipients, and had undergone transplantation at least one year prior to the parathyroidectomy. Parathyroid tissue samples obtained from parathyroid glands unintentionally removed during thyroid surgery served as controls. Serum biochemistries were measured at the Department of Clinical Chemistry, Uppsala University Hospital.

3.3 CRE-LOX RECOMBINATION

Cre-Lox recombination is an efficient and widely used technology to generate tissue-specific and conditional knockout mice and reporter strains. The mechanism was first identified in bacteriophages that use Cre-Lox recombination to circularize and facilitate replication of its genomic DNA. It is based on two components: 1) Cre recombinase, a site-specific DNA recombinase, and 2) LoxP sites, specific 34 base pair sequences allowing for recombination. Neither Cre recombinase nor LoxP sites are native to the mouse genome and must be introduced by transgenic techniques. When LoxP sites are inserted into the genome, recombination occurs between the LoxP sites in cells expressing Cre recombinase, resulting in deletion of a specific DNA sequence or gene (Figure 5A). There are also inducible Cre strains available, allowing for studies of age dependent gene functions. A drawback of the Cre-Lox system is the variable potency of the promoters driving Cre recombination, commonly resulting in less efficient gene deletion compared to conventional knockout techniques. Another potential problem is the specificity of the Cre promoter, and it is therefore recommended to use reporter strains to confirm that Cre expression is restricted to the target tissue[99].



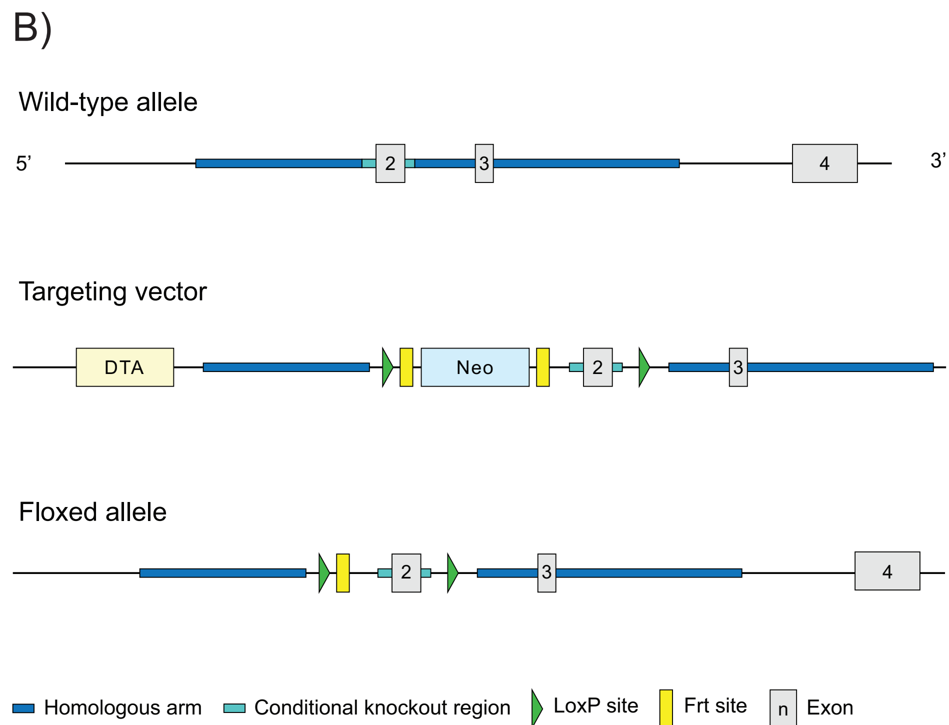


Figure 5. Cre-Lox recombination as a system for tissue-specific gene deletion. A) Mice expressing Cre recombinase under a tissue-specific promotor is crossed with floxed mice to generate targeted knockout mice. B) Schematic representation of the wild-type Klotho allele (top), targeting vector (middle), and floxed allele with deleted Neo cassette (bottom). LoxP sites are flanking exon 2 of the *Klotho* gene enabling targeted deletion by Cre-Lox recombination. Adapted from Olauson H et al[100].

Since *Klotho*$^{-/-}$ mice display extensive morphological abnormalities and dysregulated mineral metabolism, we decided to generate floxed Klotho mice to enable dissection of tissue-specific effects of Klotho in a more physiological setting. Hence, in Study II and IV we use mice with LoxP sequences introduced in the flanking regions of exon 2 of the *Klotho* gene, resulting in disrupted gene function in tissues expressing Cre recombinase (Figure 5B). Briefly, the sequence of mouse chromosome 5 was retrieved from the Ensembl database (http://www.ensembl.org). The RP23-434H9 BAC clone was used for generation of homology arms and the conditional knockout region of the targeting vector. The fragments were cloned in the LoxFtNwCD or pCR4.0 vector and electroporated into C57BL/6 embryonic stem cells. Male chimeras were generated and subsequently bred with wild-type females to generate Klotho-LoxP heterozygotes (Klotho$^{flox/+}$). Floxed Klotho mice were crossed with mice expressing Cre recombinase under different promotors, to achieve tissue-specific deletion of Klotho. In Study II we used mice expressing Cre recombinase under the human PTH promotor (129;FVB-Tg(PTH-cre)4167Slib/J; Jackson laboratory, ME, US)[101] to generate parathyroid-specific Klotho knockout mice. In Study IV we used mice expressing Cre recombinase under the Ksp-cadherin promoter (B6.Cg-Tg(Cdh16-cre)91Igr/J; Jackson laboratory, ME, US)[102], to generate mice with a distal tubule-specific deletion of Klotho. In both studies homozygous mice without Cre (Klotho$^{flox/flox}$) served as wild-type controls. In Study IV, mice with a systemic Klotho deletion were generated using mice expressing Cre under the human beta-actin promotor (FVB/N-Tg(ACTB-cre)2Mrt/J, Jackson laboratory). All Cre strains had previously been crossed to reporter mice to verify tissue specificity of Cre expression. Mice with a C57BL/6 background were used for maintenance breeding.

3.4 TRANSCRIPT ANALYSIS

Quantitative real time PCR (qPCR) is a common and sensitive method to amplify and quantify specific gene products. The method can use either DNA binding dyes such as SYBR Green, or sequence specific fluorescent reporter probes. In this thesis, total RNA was extracted and reverse-transcribed into cDNA, and qPCR subsequently performed using gene-specific primers in SYBR Green based assays, except otherwise stated. The relative gene expression was calculated with the $2^{-\Delta\Delta}$ Cq or Ct method normalizing the gene of interest to the reference genes GAPDH (Study I) or β-actin (Study II-IV) in the same sample.

In Study II mouse parathyroid tissue was microdissected using laser capture microscopy and mRNA expression profiles were obtained with the nanostring nCounter system (Nanostring technologies, Inc.). Nanostring uses confocal microscopy to count fluorescently bar-coded probes, detecting and quantitating RNA molecules without amplification or introduction of position-dependent effects. Data were processed using different normalization strategies, including quantile normalization and six reference genes.

3.5 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Immunohistochemistry (IHC) and immunofluorescence (IF) utilize antibodies for detection of specific target antigens. The techniques are widely used in both scientific research and clinical practice. Adequate preparation of the tissue sample is crucial to maintain tissue morphology and antigenicity of the target epitopes. After fixation and sectioning additional steps, including antigen retrieval, may be necessary to allow the antibody to bind its epitope. The antibodies used for IHC and IF may be monoclonal or polyclonal. Polyclonal antibodies are a mix of antibodies recognizing several different epitopes, whereas monoclonal antibodies bind a single epitope and are therefore considered to be more specific. Further, antibodies are classified into primary and secondary antibodies. Primary antibodies are raised against the epitopes of interest, and secondary antibodies target immunoglobulins on the primary antibodies, and are usually conjugated with a linker molecule or directly bound to a reporter molecule. The reporter molecules differs between IHC and IF. While IHC uses chromogenic reporters yielding a colour that can be seen with a regular light microscope, IF uses fluorophores requiring a fluorescence microscope for detection. The different wavelength of the fluorophores permits simultaneous staining with several different antibodies in IF.

IHC and IF are excellent techniques for protein detection and can show precisely where a specific protein is located within the tissue sample, or even the subcellular location within a single cell. Main drawbacks of IHC and IF is the risk for unspecific staining and that they only provide a semi-quantitative assessment of protein expression.

The specificity of all antibodies used for IHC or IF in this thesis was verified by immunoblot or other techniques. IHC and IF techniques were primarily used to investigate the expression pattern and/or localization of specific proteins, and secondarily for semi-quantification when transcript analysis or immunoblotting were not available.

3.6 STATISTICAL ANALYSIS

GraphPad Prism version 5.0 or higher (GraphPad Software Inc, CA, US) was used for statistical analysis. Biochemistries in Study I are presented as means ± SD. Non-normally distributed parameters were logarithmized before analysis. In Study II-IV data is presented as arithmetic means ± SEM or median (range), unless otherwise stated. Gaussian distribution was tested using D'Agostino and Pearson omnibus normality test. Variables fulfilling the criteria for normal distribution were compared with two-tailed unpaired t-test. Treatment results were evaluated using paired t-test. Non-normally distributed variables were compared using Mann-Whitney test. Correlations between variables were tested with Pearson or Spearman correlation test. P-values <0.05 were considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 PARATHYROID KLOTHO AND FGFR1 DECLINE IN PARALLEL WITH RENAL FUNCTION IN CKD PATIENTS WITH SECONDARY HYPERPARATHYROIDISM (STUDY I)

The concomitant increase in FGF23 and PTH during CKD progression is a paradoxical phenomenon, given that FGF23 was shown to inhibit PTH synthesis and secretion. Study I was designed as an exploratory study to test the hypotheses of parathyroid FGF23 resistance in CKD due to downregulation of the responsible receptors. To this end, we investigated the transcript and protein expression of Klotho and FGFR1 in 88 parathyroid glands from 31 patients with CKD and sHPT[103]. Overall, parathyroid Klotho and FGFR1 levels were markedly decreased in patients with sHPT, although there was a large intraglandular variation. Mean Klotho levels correlated positively to renal function ($r=0.42$; $p<0.05$), and declined significantly over CKD stages (Figure 6). Also parathyroid FGFR1 correlated to renal function ($r=0.50$; $p<0.01$) and accordingly decreased across CKD stages. Serum phosphate but not calcium or PTH associated to Klotho levels, whereas phosphate and PTH correlated negatively to FGFR1 expression. Unfortunately, lack of serum prevented us from determining FGF23 and $1,25(OH)_2D$ levels.

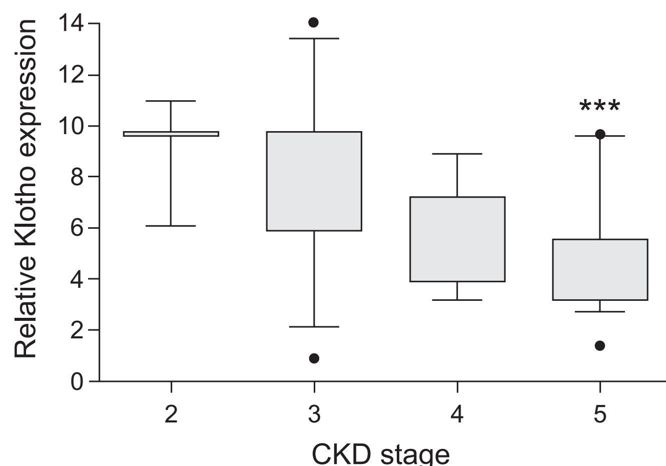


Figure 6. Parathyroid Klotho expression declined over chronic kidney disease (CKD) stages. CKD 3 was set as reference. ***p<0.001 versus reference. Error bars represent the 5th and 95th percentiles. Outliers are represented by dots.

To further explore the regulation of parathyroid Klotho we performed *in vitro* experiments using isolated bovine parathyroid cells. As previously shown, calcium potently suppress Klotho expression at 24 hours[104]. Likewise, treatment with recombinant FGF23 at physiological concentrations decreased the expression of Klotho. Conversely, treatment with $1,25(OH)_2D$ or a vitamin D analogue (EB1089) dose-dependently increased Klotho expression. There was no effect on Klotho levels by treatment with phosphate or PTH. Co-treatment with EB1089 could not mitigate the suppressive effects of FGF23 or calcium on Klotho expression (Figure 7). All treatment effects were blunted in human hyperplastic parathyroid cell culture.

The downregulation of parathyroid FGFR1-Klotho receptor complex seen in patients with impaired renal function and sHPT corroborates the hypothesis of FGF23 parathyroid resistance in CKD. Other groups have also presented similar results[105,106]. In further support of this notion, rats with adenine-induced renal failure had decreased parathyroid expression of Klotho and FGFR1, and treatment with FGF23 failed to inhibit PTH secretion[107].

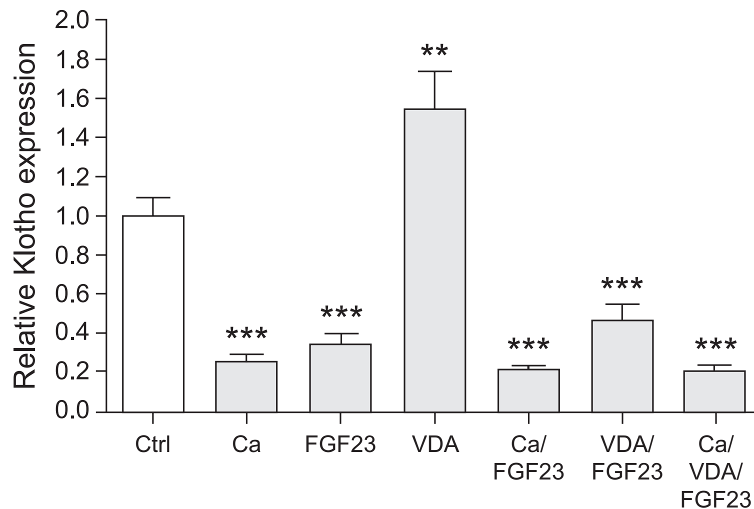


Figure 7. Klotho regulation by calcium (Ca), vitamin D analogue (VDA), and fibroblast growth factor-23 (FGF23) in cultured bovine parathyroid cells. Calcium and FGF23 treatment decreased Klotho expression at 24 h, whereas treatment with the vitamin D analogue EB1089 increased Klotho mRNA levels. Co-treatment with EB1089 did not attenuate the suppressive effects of FGF23 or calcium on Klotho expression. The concentrations used were 4 mmol/l calcium, 2000 pg/ml FGF23, and 10^{-7} mol/l EB1089. Data is presented as median ± SEM. N=2-4. Ctrl; control medium. **$p<0.01$, ***$p<0.001$ versus control.

Several potential mechanisms underlying the suppression of parathyroid Klotho and FGFR1 in CKD can be envisioned. First, it might be attributed to the loss of parathyroid cell phenotype as hyperplasia progresses. Second, it could speculatively be caused by CKD-related alterations in serum biochemistries, most importantly $1,25(OH)_2D$ deficiency and FGF23 excess, as supported by our *in vitro* data. Although FGF23 suppresses PTH secretion in the short-term, sustained exposure to high FGF23 could potentially in the long-term decrease the abundance of the FGFR1-Klotho receptor complex. Third, additional circulating and tissue factors associated with the progression of renal failure may be implicated. In this regard, uremic toxins have been shown to decrease renal Klotho expression *in vitro* and *in vivo* through hypermethylation of the promoter region[108,109]. Similarly, we found increased promoter methylation paralleling the decreased expression of parathyroid Klotho in hyperplastic parathyroid glands (unpublished data).

Collectively, parathyroid expression of Klotho and FGFR1 declines with renal function, and may provide an explanation to the putative parathyroid resistance to FGF23 in advanced stages of CKD and sHPT.

4.2 GENETIC EVIDENCE FOR A NOVEL, KLOTHO-INDEPENDENT, FGF23-SIGNALLING PATHWAY IN THE PARATHYROID GLANDS (STUDY II)

As previously shown, parathyroid resident Klotho mediates FGF23 suppression of PTH secretion. Thus, its reduced abundance in CKD might be a pathogenic factor in the development of sHPT. To test this hypothesis and to test the physiological role of parathyroid Klotho we generated parathyroid-specific Klotho knockout mice (*PTH-KL*$^{-/-}$) using Cre-Lox recombination. Successful deletion of parathyroid Klotho was confirmed with immunohistochemical staining (Figure 8) and analysis of mRNA transcripts. *PTH-KL*$^{-/-}$ mice had a normal gross phenotype and unaltered serum PTH and calcium levels. Notably, serum levels of 1,25$(OH)_2$D were significantly increased in *PTH-KL*$^{-/-}$ mice compared to wild-type mice. Parathyroid size and histology was unchanged in *PTH-KL*$^{-/-}$ mice and there were no apparent changes in protein expression of PTH, CaSR or VDR. Analysis of parathyroid mRNA transcripts for >90 genes essential for parathyroid function revealed significant expressional changes for several genes, including *Cfd* (Entrez Gene: 11537), *Fabp4* (11770) and *Smad4* (17128).

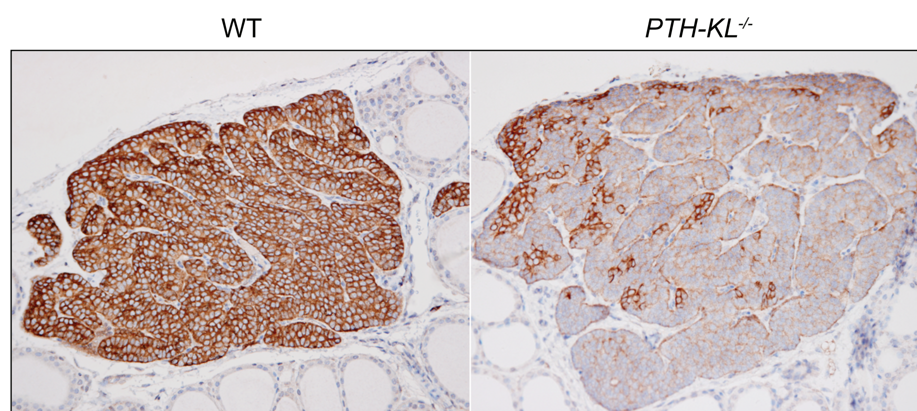


Figure 8. Immunohistochemical staining revealed successful deletion of parathyroid Klotho in parathyroid-specific Klotho knockout mice (*PTH-KL*$^{-/-}$). Efficiency of deletion varied, and was up to >90%. Wild-type (WT)

The parathyroid response to acute alterations in serum calcium did not differ between *PTH-KL*$^{-/-}$ mice and wild-type mice, and *PTH-KL*$^{-/-}$ mice challenged with renal failure developed sHPT of similar magnitude as wild-type mice. Notably, serum FGF23 levels increased by approximately 50-fold in mice challenged with renal failure, yet it did not suppress PTH secretion neither in *PTH-KL*$^{-/-}$ nor in wild-type mice. Conversely, intravenous FGF23 administration decreased PTH equally in *PTH-KL*$^{-/-}$ and wild-type mice. Importantly, the suppressive effect of FGF23 on PTH secretion was not mediated by the MAPK pathway in *PTH-KL*$^{-/-}$ mice since phosphorylation of ERK1/2 was abrogated in Klotho-deficient cells. To identify the pathway responsible for FGF23 suppression of PTH in *PTH-KL*$^{-/-}$ mice we examined if the calcineurin/NFAT pathway, another major downstream signalling pathway of FGFR activation, was activated. Indeed, NFAT2C had a partial nuclear localization in *PTH-KL*$^{-/-}$ mice contrasting the cytoplasmatic localization in wild-type mice, and MCIP1, a facilitator of calcineurin activity, was markedly upregulated in *PTH-KL*$^{-/-}$ mice. To provide *in vivo* evidence that

the calcineurin/NFAT pathway mediated FGF23 actions, *PTH-KL*$^{-/-}$ and wild-type mice were treated with the calcineurin inhibitor cyclosporine A prior to injection of FGF23. As shown in Figure 9, pre-treatment with cyclosporine A nearly abolished the suppressive effect of FGF23 on PTH secretion in *PTH-KL*$^{-/-}$ mice, whereas wild-type mice remained responsive to FGF23 actions. These observations were confirmed in thyroparathyroid explants *ex vivo*.

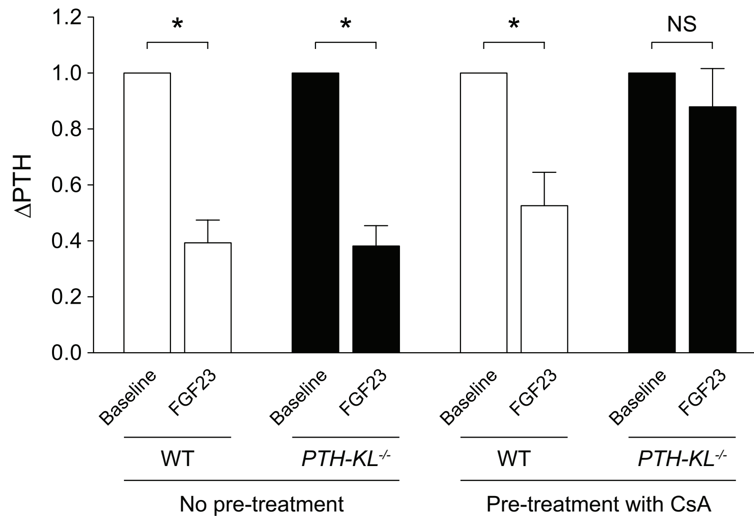


Figure 9. Parathyroid hormone (PTH) response to a single intravenous injection of fibroblast growth factor-23 (FGF23) with and without pretreatment by cyclosporin A (CsA). Wild-type mice (WT) pretreated with the calcineurin inhibitor CsA had a preserved responsiveness to FGF23 as evidenced by a significant reduction in serum PTH 15 minutes after a single FGF23 injection i.v. In contrast, the FGF23-mediated inhibition of serum PTH was blunted in *PTH-KL*$^{-/-}$ mice pretreated with CsA. *p<0.05.

We conclude that deletion of parathyroid Klotho does not alter the sensitivity to acute fluctuations in serum calcium, or aggravates the sHPT in renal failure, as previously suggested. This is presumably explained by compensatory activation of the calcineurin/NFAT pathway in the absence of Klotho. The calcineurin/NFAT pathway has previously been implicated in FGF23 effects on cardiomyocytes that lack endogenous Klotho expression, resulting in increased cell growth and left ventricular hypertrophy[110]. Our findings support FGF23 activation of the calcineurin/NFAT pathway also in parathyroid glands, a tissue normally expressing Klotho. The discovery of Klotho-independent FGF23 signalling in the parathyroid glands and heart presents the possibility of FGF23 effects in tissues that so far may have been overlooked. This could be particularly relevant in CKD patients who suffer from markedly elevated FGF23 levels, which in turn have been independently linked to adverse clinical outcomes. Reduced abundance of parathyroid Klotho in CKD is currently a generally endorsed concept for parathyroid FGF23 resistance and thus for aggravation of sHPT. However, our data clearly demonstrate that the parathyroid glands remain responsive to FGF23 despite absence of Klotho, and that parathyroid Klotho deficiency does not translate into aggravated sHPT in renal failure. Thus, the observed parathyroid FGF23 resistance in CKD is more likely to be attributed to reduced FGFR expression.

In sum, our data suggest that the calcineurin/NFAT pathway is activated by FGF23 in the parathyroid glands of *PTH-KL*$^{-/-}$ mice to suppress PTH secretion. The presence of Klotho-independent effects of FGF23 in a Klotho expressing tissue could represent a paradigm shift in the understanding of FGF23 signalling.

4.3 A NOVEL, NON-INVASIVE, MODEL OF EXPERIMENTAL RENAL FAILURE IN MICE (STUDY III)

The use of *in vivo* models of renal failure is essential to study a number of aspects related to acute renal injury and CKD. However, most existing models in mice require invasive surgical procedures or are based on single injections with nephrotoxic substances, thus preventing the possibility to temporally adjust the level of uremia[111,112]. To bypass these limitations we developed a novel, non-invasive, model of renal failure through dietary delivery of adenine[113]. This technique has previously been established in rats[114], but has not been possible to adapt in mice due to their reluctance to consume adenine. We were able to circumvent this problem by mixing the adenine in a casein-based diet, masking its repugnant smell and taste. Through a series of pilot studies we determined the "optimal" urea range to be between 80 and 100 mg/dL in terms of presence of disturbances in mineral metabolism without any mortality. Next we performed an eight weeks long proof-of-concept study, comprising an induction phase and a maintenance phase. During the 10-day induction phase the adenine concentration was set to 0.3%, causing a rapid increase in serum urea, phosphate, PTH and FGF23. When the optimal urea range was reached, the adenine concentration was lowered to maintain a stable level of renal dysfunction and biochemical disturbances. During the maintenance phase the adenine concentration was modified in the interval 0.15-0.2%. Lowering the dose to 0.15% resulted in a rapid decline in urea, phosphate and PTH, suggesting at least partial reversibility of the model. Interestingly, FGF23 levels increased continuously throughout the entire study, indicating reduced renal clearance or that renal injury *per se* stimulates FGF23 expression. Of note, mice on adenine did not have increased proteinuria. This is likely explained by the limited glomerular damage in this model, and the known resistance of C57BL/6 mice towards developing proteinuria[115]. Serum markers of bone metabolism were assessed and indicated an increased bone formation and a reduced bone resorption.

Renal histology showed mainly tubulointerstitial damage with peritubular leukocyte infiltration and interstitial/peritubular edema. However, in some but not all adenine-exposed mice also the glomeruli were affected. Ladewig staining revealed a mild interstitial fibrosis, and in von Kossa stain extensive calcification of tubular structures was observed. Histological evaluation of the parathyroid glands revealed no overt hypertrophy although proliferation rate was markedly increased. Bone analysis showed extended bone trabeculae and increased bone marrow adiposity in the femurs, corroborating with the serum markers of bone metabolism. No vascular calcification was found in the thoracic aortas of adenine-treated mice.

Further, we analysed the expression of a number of renal genes associated with inflammation or fibrosis. Inflammatory markers *Mmp3* (Gene ID: 17392), *Mmp9* (17395), *Il7rα* (16197), *Ccl20* (20297) and *Ccl5* (20304) were all significantly upregulated in adenine-exposed mice. Corresponding markers of fibrosis *Tgfb1*

(21803), *Col1a1* (12842) and *Ccl2* (20296) were also elevated compared to mice on a control diet.

In sum, we have developed a novel adenine-based protocol to induce tubulointerstitial nephropathy in mice mimicking several features of human CKD-MBD, i.e. disturbances in mineral metabolism and bone abnormalities. One of its main advantages is the possibility to adjust the urea levels by altering the adenine concentration, thus allowing for adaptive studies in terms of severity of renal failure over time. Another major benefit over other common models is that there is no need for surgical intervention, making it available also to groups with limited experience of surgical techniques. It should be pointed out that there are several advantages of using rats instead of mice, including higher tolerance towards adenine and larger blood sample volumes. However, since genetically modified strains mainly exist in mice, experimental models of renal failure in mice are a requisite to study the impact of specific genes in the uremic setting. In that context, our model will serve as an important complement to existing models for studies of gene function in the setting of renal failure.

4.4 PARTIAL DELETION OF DISTAL TUBULAR KLOTHO INCREASES FGF23 AND DISRUPTS MINERAL METABOLISM (STUDY IV)

Renal Klotho is obligate to maintain a normal mineral homeostasis, both through direct regulation of calcium- and phosphate-transporters, and by mediating the effects of FGF23 on phosphate and vitamin D metabolism. However, the mechanism by which FGF23 binds Klotho to increase phosphate wasting is debated. Klotho is predominantly expressed in the distal tubule, while phosphate reabsorption occurs in the proximal tubule. On one hand it has been reported that there is sufficient Klotho expression in the proximal tubule to allow FGF23 signalling[116], while on the other hand the initial activation of downstream signalling events upon FGF23 administration resides exclusively in the distal tubule[117]. To enhance the understanding of renal FGF23-Klotho signalling and function, we generated distal tubule-specific Klotho knockout mice (*Ksp-KL*$^{-/-}$) by crossing floxed Klotho mice with mice expressing Cre-recombinase under the Ksp-cadherin promoter[100]. We validated the specificity of the Ksp-cadherin promotor by crossing Cre-mice to a reporter strain (unpublished data, see cover image). Partial deletion of distal tubular Klotho was confirmed with immunohistochemistry (Figure 10), and analysis of renal transcripts revealed that residual Klotho expression in *Ksp-KL*$^{-/-}$ mice varied between 26% and 100%.

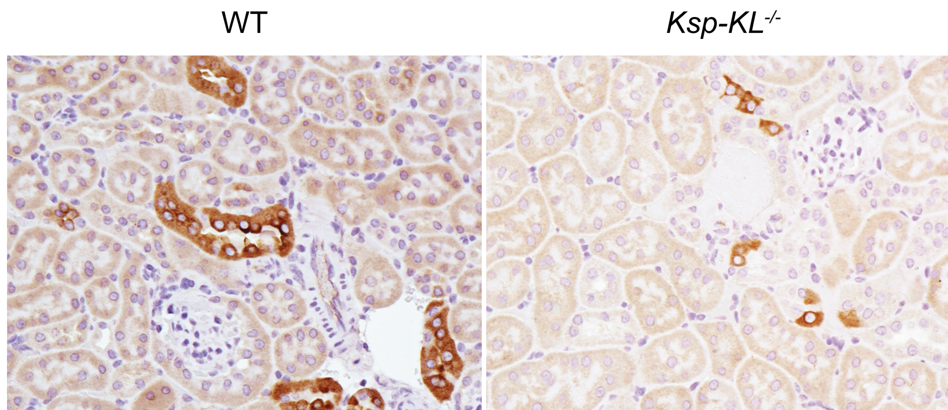


Figure 10. Immunohistochemical staining revealed partial deletion of distal tubular Klotho in *Ksp-KL*$^{-/-}$ mice. Efficiency of deletion varied between 0-74% compared to wild-type mice. WT; wild-type

In contrast to systemic Klotho knockout mice, *Ksp-KL*$^{-/-}$ mice were viable, fertile and had normal longevity. However, *Ksp-KL*$^{-/-}$ mice were hyperphosphatemic with elevated FGF23 levels, corresponding to the degree of residual Klotho expression. PTH was decreased whereas calcium and 1,25(OH)$_2$D remained normal. Urinary calcium excretion was increased and urinary phosphate excretion unchanged. The increased calciuria could speculatively be due to decreased activity of TRPV5, as a downstream effect of reduced Klotho. In a subgroup of *Ksp-KL*$^{-/-}$ mice with normal serum phosphate, FGF23 levels were higher than in wild-type mice. This indicates that factors other than serum phosphate stimulate FGF23 expression in *Ksp-KL*$^{-/-}$ mice. Since there was a high variability in Klotho expression, we categorized the *Ksp-KL*$^{-/-}$ mice into three groups based on residual expression; >70% (mean Klotho expression 88%, n=7), 30-79% (59%, n=6) and <30% (28%, n=2). There was a gradual increase in serum phosphate and calcium with lower Klotho levels, whereas a threshold was seen for FGF23 with extremely elevated levels when renal Klotho was <30% (Figure 11).

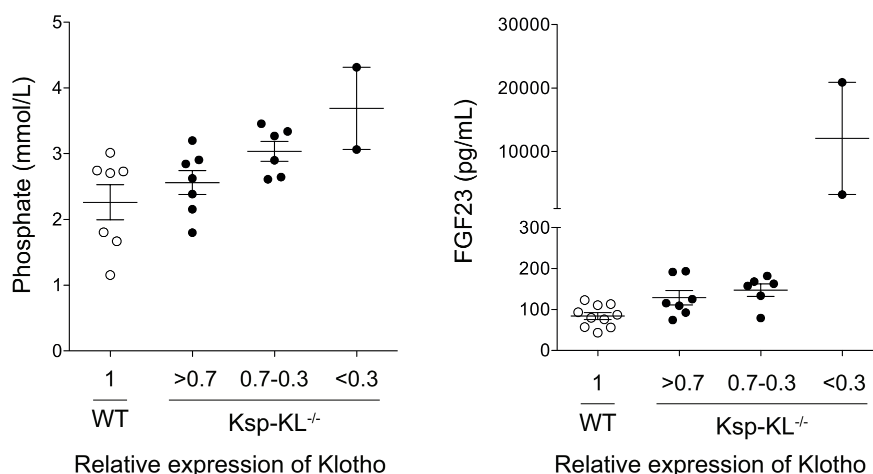


Figure 11. Serum phosphate and fibroblast growth factor-23 (FGF23) levels in wild-type and *Ksp-KL*$^{-/-}$ mice categorized according to residual Klotho expression. There was a gradual decline in serum phosphate with lower Klotho expression, whereas a threshold was seen for FGF23, with a marked elevation when Klotho was <30%. WT; wild-type

No major differences were found in renal or parathyroid histology between wild-type and *Ksp-KL*$^{-/-}$ mice. To determine the impact of Klotho deletion on key factors regulating mineral metabolism, we examined renal protein and transcript expression. Immunostaining revealed increased abundance of the sodium phosphate co-transporter Npt2a on the apical membrane, in concordance with the observed hyperphosphatemia in *Ksp-KL*$^{-/-}$ mice. Immunoblotting showed increased renal levels of VDR and decreased levels of TRPV5 in *Ksp-KL*$^{-/-}$ mice. Transcript levels of renal Cyp27B1 were increased in *Ksp-KL*$^{-/-}$ mice, and Klotho transcripts correlated to Cyp27B1, VDR, Npt2a, and FGFR1.

Collectively, we present genetic and functional evidence that partial deletion of Klotho in the distal tubules has a major impact on renal phosphate handling in the proximal tubules. The factor(s) responsible for this proposed distal-to-proximal tubule signalling are currently unknown, but could speculatively be soluble Klotho shedded from the cell surface upon binding of FGF23. Although distal tubular Klotho appears essential to renal phosphate handling, our results indicate a limited effect on vitamin D metabolism. Importantly, our data does not exclude a role for Klotho in the proximal tubule, and this should be further examined in future studies.

The variable efficiency of deletion allowed us to study dose-dependent effects of Klotho on mineral metabolism. Indeed, we found a graded relationship between residual Klotho expression and serum phosphate, calcium and PTH. Conversely, serum FGF23 increased exponentially when Klotho expression was below 30%. This could represent a compensatory response due to renal FGF23 resistance. In support, the *Ksp-KL*$^{-/-}$ mice with the most efficient Klotho deletion displayed pronounced hyperphosphatemia in the face of markedly elevated FGF23 levels.

The elevated FGF23 raises another important question; how does reduced renal Klotho translate into increased expression of FGF23 in bone? Secondary mediators such as hyperphosphatemia are likely to account for part of the increase. However, also in in *Ksp-KL*$^{-/-}$ mice with normal phosphate levels, FGF23 levels was elevated, implying that additional factors are involved. Again, soluble Klotho acting on the bone is a plausible factor. Further studies are needed to explore this potential relationship.

In stark contrast to *Klotho*$^{-/-}$ mice, *Ksp-KL*$^{-/-}$ mice are viable and fertile with an essentially normal gross phenotype. This contradicts that Klotho deficiency *per se* causes systemic toxicity. However, the knockout is incomplete and the residual Klotho might be sufficient to maintain a normal phenotype. Similarly, the lack of renal fibrosis and vascular calcification argues against direct paracrine effects of Klotho on maintaining cellular integrity, at least during low cellular stress. These characteristics might be more pronounced in *Ksp-KL*$^{-/-}$ mice with ageing or by induction of renal failure, and should be tested in subsequent studies.

A summary of the phenotypes seen in various models of Klotho deficiency is found in Table 2.

Parameter	*Klotho$^{-/-}$ mice*	*PTH-KL$^{-/-}$ mice*	*Ksp-KL$^{-/-}$ mice*	CKD
Blood chemistry				
Phosphate	↑↑↑	Normal	↑	↑-↑↑
GFR	↑	Normal	Normal	↓-↓↓↓
FGF23	↑↑↑	Normal	↑-↑↑	↑-↑↑↑
PTH	↓↓	Normal	↓	↑-↑↑
Renal Klotho	0	Normal	↓-↓↓	↓-↓↓↓
Parathyroid Klotho	0	↓↓↓	Normal	↓↓
Soluble Klotho	0	Normal?	↓-↓↓?	↓-↓↓↓?
Gross phenotype				
Body weight	↓↓	Normal/↓	Normal	↓
Growth	↓↓	Normal	Normal	↓ (in children)
Physical activity	↓↓	Normal	Normal	↓
Fertility	0	Normal	Normal	↓
Life span	↓↓	Normal	Normal	↓↓
Cardiovascular disease				
Heart	Hypertrophy and fibrosis	Normal	Normal	Hypertrophy and fibrosis
Vasculature	Calcification	Normal	Normal	Calcification
Blood pressure	↑	?	?	↑↑
Anemia	Mild	?	?	Severe
Bone	Osteomalacia/ Osteoporosis	Normal	Normal	Renal bone disease

Table 2. Characteristics of various states of Klotho deficiency. Systemic Klotho knockout mice (*Klotho$^{-/-}$*), parathyroid-specific Klotho knockout mice (*PTH-KL$^{-/-}$*), distal tubule-specific Klotho knockout mice (*Ksp-KL$^{-/-}$*) and chronic kidney disease (CKD). Pathological parameters are described as absent (0), mild (↓/↑), severe (↓↓/↑↑) or extreme (↓↓↓/↑↑↑). In part adapted from Hu MC et al[64].

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The identification of FGF23 (2000) and Klotho (1997) represents a paradigm shift in the conceptualization of mineral metabolism in health and disease. Despite major advances over the last decade there are still fundamental gaps in the understanding of the regulation and function of this endocrine axis.

5.1 NOVEL FINDINGS AND IMPLICATIONS

The studies presented in this thesis contribute substantially to the knowledge of FGF23-Klotho function and regulation, both in physiology and in CKD. We provide evidence that parathyroid Klotho and FGFR1 declines in parallel with decreased GFR in patients with CKD and sHPT; that reduced Klotho level can be directly mediated by hypercalcemia, vitamin D deficiency and elevated FGF23; and that suppression of the FGFR1-Klotho receptor complex may provide the functional basis for parathyroid FGF23 resistance in CKD. To further define the role of parathyroid Klotho we generated parathyroid-specific Klotho knockout mice. Contradictory to the current paradigm, parathyroid Klotho ablation does not impact the calcium sensitivity or aggravate renal uremic sHPT. Further, despite absence of Klotho and loss of functional MAPK signalling the parathyroid glands retain FGF23 responsiveness. On these premises, we identified a novel, calcineurin-dependent, pathway that mediates the suppressive effects of FGF23 on PTH secretion. The existence of a Klotho-independent FGF23 signalling pathway in a Klotho-expressing organ represents a paradigm shift in the conceptual framework of FGF23 endocrine function. Speculatively, high FGF23 may negatively impact clinical outcomes in CKD through enhanced calcineurin signalling. Other implications include the possibility of iatrogenic FGF23 resistance and aggravation of sHPT after kidney transplantation when serum FGF23 remains elevated in conjunction with the use of calcineurin inhibitors as immunosuppressive therapy.

To allow studies of specific gene function and pharmacological intervention in a uremic milieu, we developed a novel non-surgical mouse model of renal failure by adding adenine to the diet. Mice exposed to adenine developed tubulointerstitial lesions and secondary changes in bone and mineral metabolism, resembling human CKD-MBD. Adenine-induced renal failure thus constitutes a novel and useful tool for studies of genetic and pharmacological manipulations in CKD-MBD.

Furthermore, by generating mice with a distal tubule-specific deletion of Klotho we provide *in vivo* evidence for a vital and dose-dependent role of distal tubular Klotho on renal phosphate handling in the proximal tubule. This supports the presence of a distal-to-proximal tubule signalling mechanism. Elevated levels of FGF23 despite normal serum phosphate in mice with a partial deletion of renal Klotho also suggest that renal derived factors such as soluble Klotho may directly regulate FGF23 expression in bone. Finally, the lack of fibrosis, calcification or other age-related histological abnormalities in Klotho-deleted cells argues against a direct role for Klotho in regulation of cellular senescence.

5.2 LIMITATIONS

Several limitations in the work of this thesis should be mentioned. Study I is largely exploratory with regard to Klotho and FGFR1 analysis in human tissue specimen. External factors such as age, co-morbidities, medication, renal replacement therapy etc. are likely to influence Klotho and FGFR1 levels. Importantly, a majority of the patients were renal transplant recipients and on immunosuppressive therapy, which may impact the expression of parathyroid Klotho and FGFR1. However, the patient sample was too small to perform a robust sensitivity analysis. Also, lack of serum prevented us from analysing FGF23 and $1,25(OH)_2D$, which would have been enlightening given that they directly regulate Klotho expression in bovine parathyroid cells. Consequently, some results from our regression models should be interpreted with caution due to residual confounding factors.

In Study II and IV, the impact of tissue-specific Klotho deletion was examined in mice. Although endocrine regulation of mineral metabolism is a highly preserved system across mammal species, results derived from animal studies cannot be translated into humans without careful consideration. Yet, mice have been valuable in previous studies in terms of predicting human FGF23-Klotho physiology. Also some potential methodological shortcomings deserve mentioning. The efficiency of Klotho deletion in Study II and IV varied, and was incomplete. To partially overcome this problem we performed subgroup analysis comparing animals with different degrees of deletion. Nevertheless, we cannot rule out that a complete deletion of Klotho in the specific tissue would have yielded different results.

In Study III, the underlying aetiology of adenine-induced nephropathy is tubular toxicity, which contrasts human CKD where the most common pathology is glomerular scarring. Thus, this model should not be regarded as a model of CKD-MBD *per se* but rather as model of tubulointerstitial disease. Adenine may also have extra-renal effects that modify the phenotype independently of uremia. Further, we did not examine reversibility of renal failure or long-term impact of adenine administration.

5.3 GENERAL DISCUSSION

5.3.1 PTH regulation

The calcium-PTH sigmoidal curve defines that relatively small changes in systemic calcium ion concentrations evoke large responses in PTH secretion. This is regulated through activation or inactivation of CaSR expressed at the surface of parathyroid chief cells. In addition to calcium, several other components of mineral metabolism regulate PTH synthesis and secretion. Local VDR activation inhibits PTH release whereas high phosphate promotes PTH secretion and parathyroid gland hyperplasia. sHPT is a universal feature of CKD patients and has traditionally been attributed to the triad of $1,25(OH)_2D$ deficiency, hypocalcemia and hyperphosphatemia[118]. The discovery of FGF23 and Klotho has prompted a re-evaluation of parathyroid physiology and sHPT pathophysiology. In physiology, FGF23-Klotho signalling inhibits PTH secretion whereas Klotho was reported to mediate PTH secretion during hypocalcemia. However our studies contradict that Klotho is essential for PTH secretion during hypocalcemia, at least in the short-term. Further, we provide evidence that Klotho is not critical for

FGF23 suppression of PTH since calcineurin-NFAT signalling can mediate the same effects in the absence of Klotho. Similar "rescue pathways" are also seen for other vital biological systems, and its existence emphasizes the importance of fine-tuning systems for maintaining calcium homeostasis. This leaves us with the questions what the true function of parathyroid Klotho is, and to what extent parathyroid FGF23 signalling is relevant in physiology. We propose that FGF23, in analogue with $1,25(OH)_2D$, has a fine-tuning role subordinate to CaSR in the regulation of PTH secretion. The proposed endocrine regulation of PTH in physiology is depicted in Figure 12. In sHPT the situation might be somewhat different. The parallel rise in FGF23 and PTH was initially perceived as an enigma assuming a negative feedback from FGF23 on PTH secretion. There are however several plausible explanations for this observation. In advanced sHPT, the parathyroid glands are unresponsive to FGF23 signalling, and our data support that this is due to suppression of FGFR rather than loss of Klotho. At early and intermediate stages of sHPT FGF23 likely has an inhibitory effect on PTH, but that may be overshadowed by the FGF23-mediated decrease in systemic $1,25(OH)_2D$ levels. This is supported by studies showing improved biochemical profile, including higher $1,25(OH)_2D$ and lower PTH levels, in uremic rats treated with FGF23 neutralizing antibodies[119]. The dominant role of $1,25(OH)_2D$ deficiency over FGF23 excess on PTH regulation is further evidenced by the high PTH and parathyroid gland hyperplasia in transgenic mice overexpressing FGF23[31].

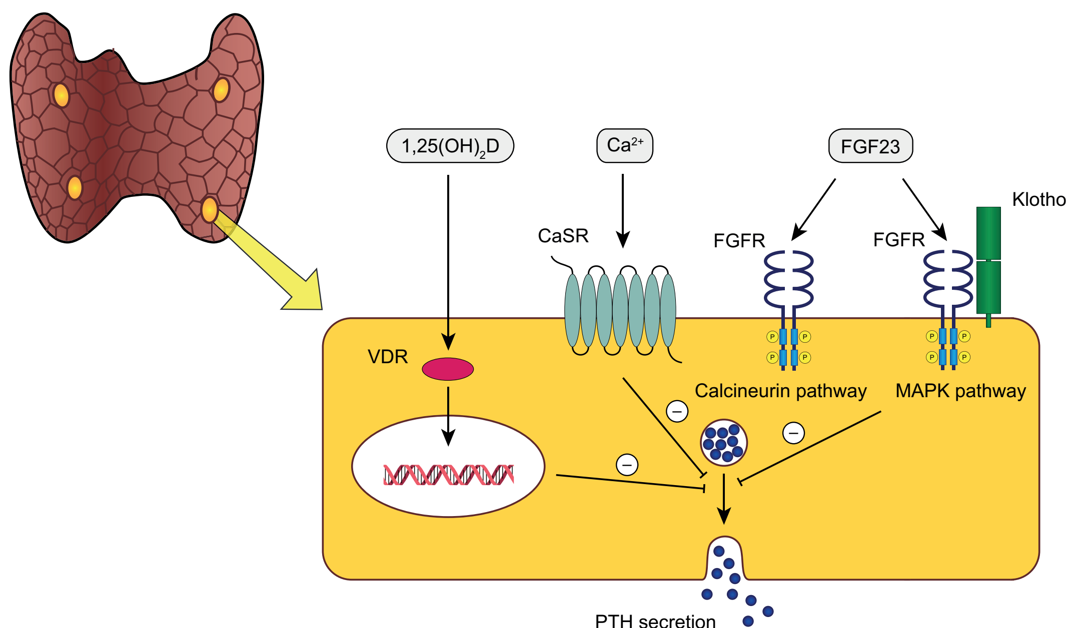


Figure 12. Proposed regulation of parathyroid hormone (PTH) by vitamin D, calcium and fibroblast growth factor-23 (FGF23) in physiology. Active vitamin D ($1,25(OH)_2D$) acts on the nuclear vitamin D receptor (VDR) to inhibit PTH synthesis. Activation of the calcium sensing receptor (CaSR) by high serum calcium also suppresses PTH secretion. Conversely, low serum calcium inactivates CaSR and facilitates PTH release. FGF23 has been shown to suppress PTH synthesis and secretion, through both Klotho-dependent and -independent pathways.

5.3.2 Regulation of FGF23 in CKD

Although an extensive amount of epidemiological and *in vivo* data convincingly argues that increased FGF23 is an early and sensitive biomarker of renal damage, some fundamental questions need to be answered before measurement of FGF23 can be applied in the clinical practice. First, there is some controversy on the processing of FGF23 in CKD. While some report on accumulation of c-terminal fragments in ESRD[120], others found almost exclusively intact bioactive FGF23 in dialysis patients[121]. Another central issue is how and why the increase in FGF23 expression is initiated. Phosphate retention due to decreased renal clearance was initially believed to be the main trigger for FGF23 in CKD. However, clinical data suggest a biphasic profile in serum phosphate in CKD that begins with a hypophosphatemic phase, arguing against hyperphosphatemia as the principal stimuli of FGF23 in early CKD[71]. In support, in a recent study dietary phosphate restrictions alone could not prevent the rise in FGF23 in a mouse model of progressive CKD[122]. Another study showed a marked increase in FGF23 within hours after induction of acute kidney injury (AKI), independent of dietary phosphate[123]. Further, treatment with phosphate-binding agents in patients with CKD stage 3-4 reduces the levels of serum phosphate, but do not normalize FGF23[124,125]. In a recent review, Kuro-o presents the intriguing hypothesis that the elevated FGF23 in early CKD reflects an increased phosphate load per nephron, rather than high serum phosphate[126]. Recent studies also promote expressional changes in DMP1, PHEX and Sclerostin as intrinsic regulators of FGF23 expression in CKD[127]. Other newly identified regulators of FGF23 in bone include FAM20C and Entpd5, which when deleted in mice reduces mineralization of bone and stimulates FGF23 synthesis[128,129]. Additional upstream factors such as local FGFR activation, inorganic polyphosphates, sex steroids, retinols and osteoprotegerin have also been implied. It is plausible that also the failing kidney itself promotes FGF23 expression, either by secreting a stimulating factor or by reduced production of an inhibitor. Indeed, as supported by the findings in this thesis, renal but not parathyroid Klotho deficiency *per se* appears to induce FGF23 expression. Finally, accumulation due to decreased urinary clearance of FGF23 in CKD has been proposed as a possibility, but is unlikely to be a major determinant according to a recent study[123]. Taken together, it appears unlikely that one single factor will be identified as responsible for the increased FGF23 expression in CKD, but rather that numerous factors are involved in a complex interplay.

5.3.3 Regulation of Klotho in CKD

Downregulation of Klotho in CKD appears to be equally multifaceted as the increase in FGF23. In addition to reduced renal mass, numerous factors such as ionized calcium, high glucose, inflammation, oxidative stress, activation of RAAS, uremic toxins, TGF-β1 and FGF23 have been shown to reduce Klotho[130]. High phosphate has also been implicated, and healthy mice on a high phosphate diet show significantly reduced renal Klotho levels (unpublished data from Study IV). Importantly, the downregulation of Klotho in CKD may at least in part be due to epigenetic changes as uremic toxins were shown to induce hypermethylation of the *KLOTHO* gene promoter and reduce renal Klotho expression both *in vitro* and *in vivo*[108,109].

As previously mentioned, activation of vitamin D responsive elements in the *Klotho* gene promotor stimulates increased expression[51]. In mice with CKD, treatment with a vitamin D receptor agonist increases Klotho and decrease vascular calcification[131]. Also hypophosphatemia increases Klotho, as hypomorph *Klotho*$^{-/-}$ mice fed a low phosphate diet regain some Klotho expression[52]. In a rat model of ageing, treatment with a PPARγ agonist increase renal Klotho expression, reduce proteinuria, improves GFR, and alleviates cell senescence[132]. Similarly, correction of blood glucose in a murine model of diabetic nephropathy resulted in improved renal function and higher levels of Klotho[133]. Finally, treatment with an angiotensin II inhibitor blocked the RAAS-mediated suppression of Klotho, independently of its effects on blood pressure[134]. Whether these are direct effects on Klotho expression or mediated by improved renal function is currently unknown.

5.3.4 FGF23-Klotho dysregulation

The reduction in Klotho appears to temporally coincide with the rise in FGF23, and this chicken-and-egg conundrum in CKD is not yet solved. Regardless of which comes first, the mutual regulation by high FGF23 and low Klotho forms a vicious spiral that results in extreme FGF23 concentrations and severe Klotho deficiency in advanced stages of CKD. The disturbances in the FGF23-Klotho axis may in turn aggravate abnormalities in mineral metabolism, including $1,25(OH)_2D$ deficiency, hyperphosphatemia and development of sHPT. All these factors, individually and in combination, contribute to CKD progression and development of associated complications such as bone disease, vascular calcification and increased mortality[135].

5.3.5 FGF23 as a pathogenic factor

In addition to promoting secondary changes in mineral metabolism, a fundamental question is if the high FGF23 in CKD is toxic and contribute directly to adverse clinical outcome. A couple of recent studies have evaluated the potential impact of FGF23 excess on end-organ damage. Faul et al showed that intramyocardial and intravenous administration of FGF23 results in cardiomyocyte proliferation and left ventricular hypertrophy. Importantly, cardiomyocytes does not express Klotho and the effects on cell growth were mediated by the PLC pathway in a Klotho-independent manner[110]. However intriguing, these findings need to be validated in additional studies. In a study by Lim et al *in vitro* treatment with FGF23 decreased vascular calcification in human vascular smooth muscle cells in a Klotho-dependent fashion[136]. Notably, these data are controversial and other reports do not see Klotho expression in the vasculature, or any direct effects on the calcification process by FGF23[137,138]. In a recent study by Kawai et al FGF23 was shown to suppress chondrocyte proliferation and linear growth of metatarsals in the presence of soluble Klotho[139]. This could be of potential clinical importance as pediatric CKD patients with high FGF23 levels suffer from disturbed longitudinal growth. Finally, it was reported that FGF23 inhibits CYP27B1 in monocytes, thus decreasing the local conversion of 25(OH)D to $1,25(OH)_2D$, which may be a key determinant of immune response in CKD patients[45]. In conclusion, high FGF23 predicts mortality and poor clinical outcome in CKD, and emerging evidence

indicates a direct pathogenic role in end-organ dysfunction. Given that FGF23 evokes organ toxicity, lowering FGF23 levels in CKD may be a viable therapeutic option. Indeed, treatment with neutralizing FGF23 antibodies improved the bone phenotype and attenuated the sHPT in a rat model of CKD-MBD, but simultaneously resulted in hyperphosphatemia, increased vascular calcification and associated mortality[140]. Of note, treatment with neutralizing antibodies leads to undetectable FGF23 levels, and there may still be beneficial effects by a moderate lowering of FGF23 in advanced CKD. In this regard, Moe recently presented preliminary data that treatment with c-terminal FGF23, in its proposed role as a competitive inhibitor to intact FGF23, improved the biochemical phenotype and increased survival in 5/6 nephrectomised mice (oral presentation, ISN Nexus 2012, Copenhagen).

5.3.6 Klotho and adverse outcome

The impact of Klotho deficiency on CKD progression, and the potential role of its replacement, has been extensively investigated in rodents. In 2007 Haruna et al crossbred mice overexpressing Klotho with a glomerulonephritis mouse model[141]. Mice overexpressing Klotho had markedly prolonged survival and dramatic improvements in renal function with fewer morphological lesions, compared to mice with a wild-type background. Similarly, viral delivery of the Klotho gene lead to improved creatinine clearance, decreased proteinuria and amelioration of the tubulointerstitial damage in a model of angiotensin II-induced renal failure[134]. Expression of Klotho is severely reduced in spontaneous hypertensive rats, and viral delivery decrease blood pressure and improves kidney histology[142]. Hu et al induced renal injury in mice with low, normal or high Klotho expression[143]. Mice with low Klotho expression (*Klotho*$^{+/-}$) were more susceptible to renal damage and developed a more severe renal dysfunction compared to wild-type mice. Conversely, mice overexpressing Klotho were more resistant to acute renal damage and maintained renal function to a higher extent. A similar protective effect against renal dysfunction was seen in mice given recombinant Klotho protein shortly after induction of AKI. In a recent study by Zhou et al, Klotho was found to be an endogenous antagonist of Wnt/β-catenin-signalling[60]. Loss of Klotho in CKD was closely associated to increased fibrosis and β-catenin activity. Conversely, *in vivo* overexpression of soluble Klotho (both cKL and sKL) inhibited the activation of renal β-catenin and ameliorated renal fibrosis in two different mouse models of renal failure. Finally, Hu et al showed that overexpression of Klotho decreases CKD-associated vascular calcification, both by lowering serum phosphate and by directly acting on the vasculature[98]. In conclusion, these results indicate that Klotho deficiency aggravates and Klotho overexpression attenuates renal injury and associated complications, both in AKI and in CKD.

An updated summary of the endocrine effects of FGF23 and soluble Klotho is shown in Figure 13, and a proposed model of FGF23–Klotho dysregulation in CKD, and its adverse effects, is presented in Figure 14.

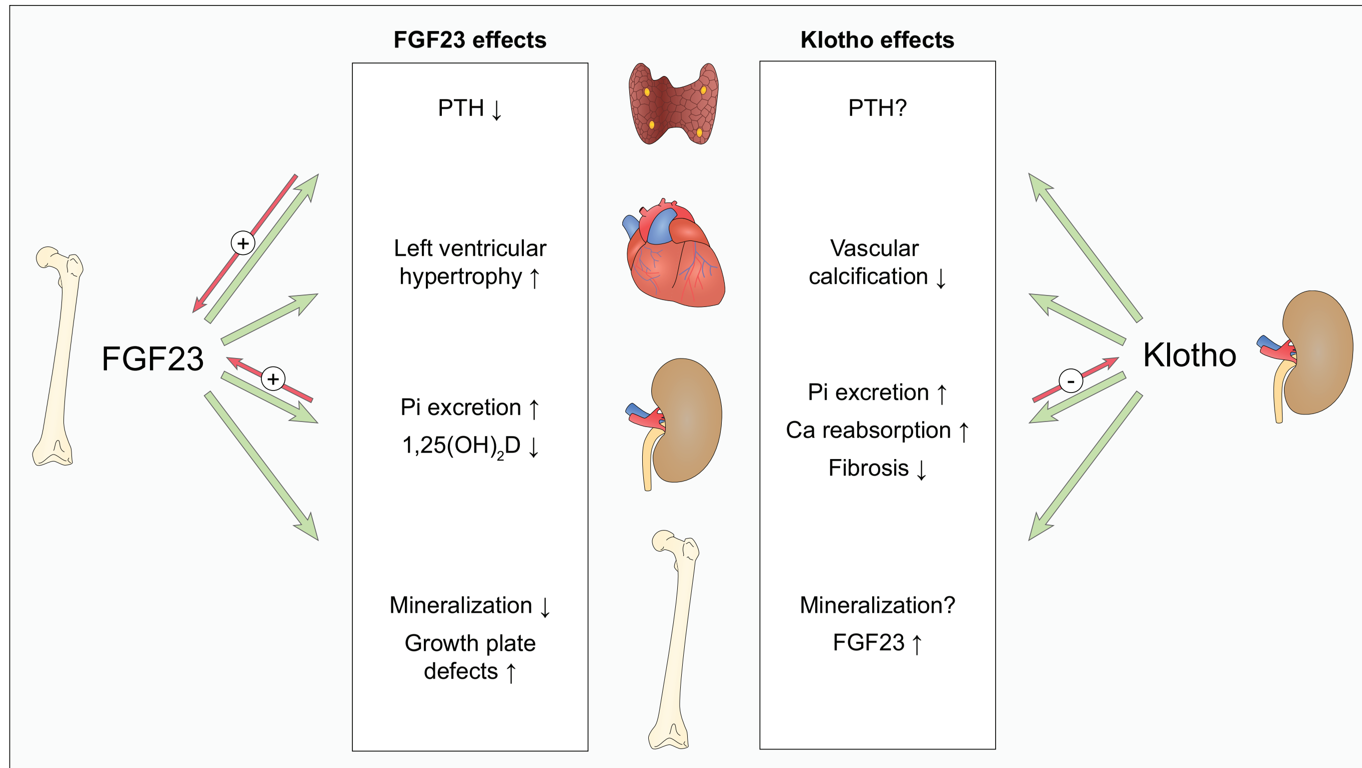


Figure 13. Endocrine functions of fibroblast growth factor-23 (FGF23) and soluble Klotho. Bone and kidney are the principal sources of FGF23 and soluble Klotho, respectively. FGF23 regulates mineral metabolism in a Klotho-dependent fashion, whereas soluble Klotho modulates renal calcium and phosphate transport through FGF23-independent effects. The heart and vasculature were recently identified as novel targets for FGF23 and Klotho action. Experimental data suggest that FGF23 and Klotho modulate bone mineralization in opposite directions, and that soluble Klotho can increase FGF23 production in the osteocytes. Adapted from Olauson H and Larsson TE[135].

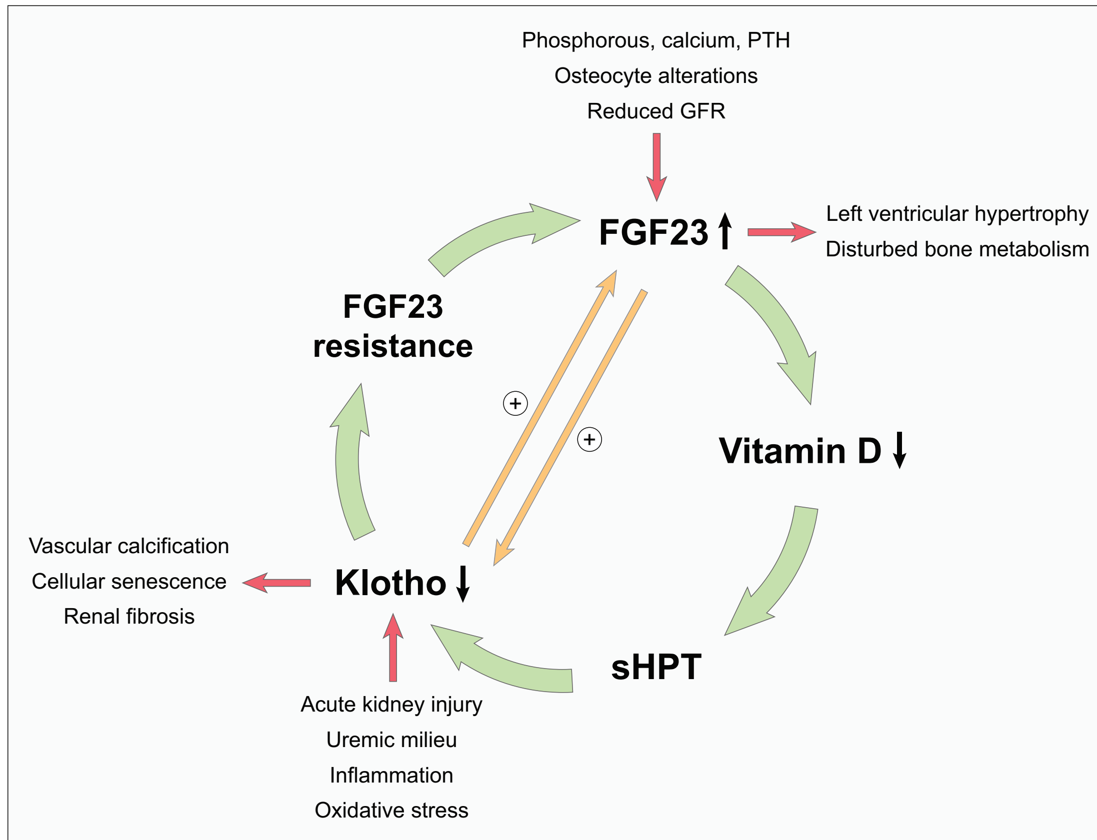


Figure 14. The vicious circle of fibroblast growth factor-23 (FGF23)-Klotho dysregulation in CKD-MBD. Circulating FGF23 increases whereas tissue level of Klotho decreases in parallel with declining kidney function. The link between reduced renal function and increased FGF23 is unknown but may involve local mechanisms in bone as well as kidney-derived factors including reduced soluble Klotho. Multiple factors in the uremic milieu contribute to Klotho suppression. The loop of FGF23–Klotho dysregulation may also be aggravated by a putative FGF23-mediated suppression of Klotho. High FGF23 is associated with cardiovascular disease, mortality and CKD progression rate in a number of epidemiological studies. A causal relationship is supported by experimental studies linking both FGF23 excess and Klotho deficiency to end-organ damage. Adapted from Olauson H and Larsson TE[135].

5.3.7 Phosphate toxicity

Disturbances in FGF23 and Klotho cannot be excluded as potential targets for intervention in CKD. However, the adverse effects of a disrupted FGF23-Klotho axis are at least partially mediated by secondary changes in mineral metabolism. Both *Fgf23*$^{-/-}$ and *Klotho*$^{-/-}$ mice suffer from hypercalcemia, hyperphosphatemia and elevated 1,25(OH)$_2$D levels. In order to dissect the impact of these abnormal parameters, several "rescue experiments" have been conducted. Both dietary and genetic disruption of 1,25(OH)$_2$D activity ameliorates the phenotypes in *Fgf23*$^{-/-}$ and *Klotho*$^{-/-}$ mice[144-147]. However, these interventions simultaneously lower serum calcium and phosphate, making it difficult to assess the contribution of the individual factors. In mice where both Klotho and Npt2a is deleted (*Klotho*$^{-/-}$/*Npt2a*$^{-/-}$), the hyperphosphatemia is reversed through increased renal phosphate wasting, whereas serum calcium and 1,25(OH)$_2$D are even further elevated compared to in *Klotho*$^{-/-}$ mice[148]. Despite persistent high levels of calcium, 1,25(OH)$_2$D and FGF23, the lowering of serum phosphate results in regained fertility, increased body weight, suppressed ectopic calcification and prolonged survival. Importantly, when hyperphosphatemia is reintroduced in these mice through dietary means the premature ageing phenotype reappears. Excessive dietary phosphate has also been shown to directly impair renal function by inflicting tubulointerstitial damage, in animals as well as in humans[149,150]. The mechanism by which phosphate induce renal damage is not clear, but it has been speculated that high phosphate in the tubular fluid forms insoluble crystals together with calcium, and that these crystals promote tubular injury and progression of CKD. Calcium-phosphate crystals are also deleterious to vascular smooth muscle cell function and induce vascular calcification[151]. In conclusion, there is compelling evidence that phosphate is a potent toxin that contributes to adverse outcome. This is further supported by numerous epidemiological studies showing that high phosphate, even within the normal range, is associated with increased cardiovascular morbidity and mortality[3,4,152,153].

5.3.8 Targeting hyperphosphatemia

Lowering serum phosphate by restricting dietary intake or using phosphate-binders is a well-established therapy in late CKD[154]. Although epidemiological data indicates improved survival by the use of phosphate-binders in CKD[155,156], it has been difficult to prove beneficial effects in RCTs[157]. There may be several reasons for this. Current guidelines recommend the use of phosphate-binders to treat hyperphosphatemia in advanced stages of CKD[79]. As previously discussed, prevalent hyperphosphatemia is a rather late occurring event in the development of CKD-MBD, and a single intervention at that point may be insufficient to attenuate the poor outcome. Further, compliance is rather low, and as many as >50% of the patients don't take the medication as prescribed[158]. Finally, the phosphate-binding capacity of current agents in relation to gastrointestinal side effects is suboptimal, and high doses are often required to achieve and maintain normophosphatemia. To overcome these obstacles, earlier initiation of treatment, development of more efficient phosphate-lowering modalities such as Npt2b inhibitors and simultaneous intervention against several targets may be feasible strategies to improve outcome.

Also, measurement of FGF23 has been proposed for patient enrichment in clinical trials, i.e. that individuals with high FGF23 are most likely to benefit from early intervention and should be targeted at earlier stages[126]. However, the benefits of monitoring FGF23, and to use it for selection of treatment and clinical decision-making, remain to be proven.

5.3.9 Klotho and cancer

It should be mentioned that *Klotho*, in addition to its role in mineral metabolism, has been implicated as a tumour suppressor gene in various cancers, including breast[159], pancreatic[160], lung[161] and gastric[162] cancers. Briefly, Klotho is downregulated through epigenetic silencing in many tumours, and its restoration inhibits proliferation and induces apoptosis of tumour cells. However, the potential role for Klotho in cancer was not the scope of the present thesis, and is discussed in detail elsewhere[163].

5.4 FUTURE PERSPECTIVES

5.4.1 Exploring parathyroid signalling

Given the results from Study I and II, further investigation of the role of the calcineurin-NFAT pathway in PTH regulation is warranted to elucidate its physiological significance. This could be achieved through studies of human parathyroid tissue, and/or by generating parathyroid-specific calcineurin or NFAT knockout mice. If proven relevant it could affect the choice of immunosuppressive therapy, i.e. non-calcineurin inhibitors, in transplanted patients with persistent sHPT to avoid blocking the calcineurin/NFAT pathway.

The function of parathyroid Klotho should also be further examined. Standard rodent chows contain calcium and vitamin D far exceeding the recommended daily intake, which may mask a potential impact of parathyroid Klotho on calcium metabolism. Thus, long-term sustained challenges, such as exposure to calcium and vitamin D depleted diets, would be informative.

5.4.2 A distal-to-proximal tubular mechanism

The initial FGF23 binding and signalling occurs in distal tubules whereas phosphate reabsorption is confined to proximal tubules. The sequence of events from distal tubule FGF23 signalling to proximal tubule phosphate transport is unclear. Although our data in Study IV clearly indicates regulation of brush-border membrane abundance of Npt2a by distal tubular Klotho, the factors involved in such proposed distal-to-proximal tubule mechanism are unknown. Also, some studies report on expression of Klotho in the renal proximal tubule[55,116], although the biological significance is unclear. Further studies are warranted to elucidate this putative paracrine mechanism.

5.4.3 Shedding and alternative splicing of Klotho

The circulatory level of Klotho defines its endocrine actions, and is the sum of the two soluble isoform (sKL and cKL). However, the alternative splicing of the *Klotho* gene and the shedding of mKL are poorly characterized events. The ratio of these isoforms in humans is currently unknown. Accordingly, it is of major importance for the understanding of endocrine Klotho to define the regulatory processes behind alternative splicing and mKL shedding respectively. This could be achieved by employing a combination of different methods. First, *in vitro* studies of cell lines expressing Klotho, endogenously or through transfection, would shed light over the cellular mechanism(s) governing shedding and alternative splicing. Second, *ex vivo* studies of kidney slices, from healthy subjects and subjects with CKD, could be used to translate these results into a whole-organ setting. Third, transgenic mice with point mutations in the *Klotho* gene to prevent alternative splicing and cell surface shedding could be generated to differentiate the impact of the two circulatory isoforms of Klotho *in vivo*. Finally, novel techniques to accurately determine the circulatory levels of sKL and cKL in health and in CKD are warranted.

5.4.4 Future pharmacological studies

There is an unacceptable high morbidity and mortality in CKD, and compelling evidence point to disturbances in mineral metabolism as key contributing factors. Previous RCTs have targeted hyperphosphatemia, $1,25(OH)_2D$ deficiency and sHPT mainly in dialysis patients, but despite correction of the biochemical parameters the studies have failed to demonstrate beneficial effects on hard clinical outcomes[157,164,165]. Klotho deficiency may be an alternate target of interest. To this end, two main options emerge; direct substitution of Klotho protein or treatment with drugs increasing endogenous Klotho expression. Among the compounds known to induce Klotho several are already in use to treat CKD patients. It is plausible that part of the potential beneficial effects by RAAS inhibition, vitamin D receptor activators and reduced dietary phosphate uptake is due to increased Klotho levels. Additional treatment options including PPARγ agonists and suppressors of hypermethylation need to be further examined. Several pharmaceutical companies are currently evaluating the therapeutic potential of soluble Klotho in CKD, although still only at the experimental stage. Nevertheless, if proven safe and efficient, soluble Klotho could be an important and much needed addition to the current therapeutic arsenal in CKD.

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7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kronisk njursjukdom (chronic kidney disease; CKD) är ett samlingsnamn för bestående njurskada med eller utan minskad filtrationsförmåga, oavsett bakomliggande orsak. Såväl nyinsjuknandet som förekomsten av CKD har ökat i takt med att befolkningen åldrats och att andra riskfaktorer förknippade med CKD, t.ex. diabetes och högt blodtryck, blivit allt vanligare. Upp till 15 % av befolkningen i västvärlden beräknas idag vara drabbade av CKD. Trots att CKD för de allra flesta är ett symtomfritt tillstånd medför även en måttligt nedsatt njurfunktion en ökad risk för bl.a. hjärt-kärlsjukdom. En bidragande orsak till detta är de störningar i kalcium- och fosfatbalansen som uppstår då njurarnas filtrationsförmåga avtar. Parallellt med att njurfunktionen försämras utvecklas även sekundära hormonella rubbningar, såsom brist på vitamin D och förhöjda nivåer av bisköldkörtelhormon (sekundär hyperparathyroidism).

Hormonet FGF23 och dess ko-receptor Klotho är två nyligen identifierade faktorer med centrala roller i mineralmetabolismen. FGF23 ökar utsöndringen av fosfat i urinen, hämmar aktiveringen av vitamin D och minskar sekretionen av bisköldkörtelhormon. Vid CKD ökar nivåerna av FGF23 kraftigt, och vävnadsnivåerna av Klotho sjunker. Kliniska studier har visat ett starkt samband mellan förändrade nivåer av FGF23 och Klotho vid CKD och risken för komplikationer som hjärt-kärlsjukdom, snabbare förlust av njurfunktion och försämrad överlevnad.

Syftet med denna avhandling har varit att klargöra FGF23s och Klothos funktioner i njurarna och i bisköldkörtlarna, såväl i normal fysiologi som vid CKD. Vi har tillämpat en translationell forskningsmetodik och kombinerat kliniska studier med molekylärbiologiska metoder och framtagande av genetiskt modifierade djurmodeller.

Sammanfattningsvis visar vi att vävnadsuttrycket av Klotho är minskat i bisköldkörtlarna hos patienter med CKD och sekundär hyperparathyroidism, och att höga nivåer av FGF23 direkt bidrar till att sänka Klotho-uttrycket. Vi påvisar en ny mekanism för hur FGF23 signalerar i bisköldkörtlarna oberoende av Klotho, vilket har stor principiell betydelse för andra biologiska effekter av FGF23. Vi utvecklar även en ny modell av njursvikt hos mus som utgör ett värdefullt verktyg för experimentella studier av rubbningar i kalcium- och fosfatomsättningen vid CKD. Avslutningsvis påvisar vi centrala funktioner för Klotho i njurarna beträffande det renala återupptaget av fosfat och regleringen av FGF23.

Denna avhandling bidrar till en ökad förståelse för kroppens hantering av kalcium och fosfat och hur denna förändras vid CKD genom ändrade nivåer och funktioner av FGF23 och Klotho. Vidare har vi tagit fram ett antal metodologiskt viktiga redskap för framtida studier inom detta forskningsfält.

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