Fibroblast growth factor-23 and Klotho in bone/mineral and parathyroid disorders

TIJANA KRAJISNIK
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Abstract

Fibroblast growth factor-23 (FGF23) is a novel, bone-produced hormone that regulates renal phosphate (P) reabsorption and calcitriol metabolism. Disorders of mineral and bone metabolism, such as autosomal dominant hypophosphatemic rickets (ADHR) and hyperostosis-hyperphosphatemia syndrome (HHS), witness the importance of well-balanced serum levels of FGF23. Patients with chronic kidney disease (CKD) are highly morbid due to P; retention/hyperphosphataemia and calcitriol deficiency, which lead to elevated serum levels of parathyroid hormone (PTH) and secondary hyperparathyroidism (sHPT). As a response to hyperphosphatemia, CKD patients have also remarkably high serum FGF23 levels, which are associated with cardiovascular risk factors and increased mortality in CKD. The overall aim of this dissertation was to discern a possible role of FGF23 in parathyroid biology. Our in vitro experiments on isolated bovine parathyroid cells demonstrate that FGF23 directly and dose-dependently suppresses the PTH production and secretion, while increasing the expression of the 25-hydroxyvitamin D₃-activating enzyme 1α-hydroxylase. We investigated possible expressional changes in the FGF23 receptor co-factor Klotho in hyperparathyroid disorders and found that Klotho expression is decreased or absent and inversely correlated to serum calcium (Ca) in adenomas of primary HPT (pHPT). In the hyperplastic parathyroid glands of sHPT, Klotho expression declines in parallel with the kidney function and correlates with the glomerular filtration rate. Moreover, Klotho expression is suppressed by Ca and FGF23, increased by calcitriol, but unaffected by P, and PTH in vitro. Finally, we identified a novel missense mutation in the gene encoding GALNT3, which is normally involved in the post-translational glycosylation of FGF23, as the cause of aberrant FGF23 processing in a patient with HHS. In summary, we provide evidence for a novel bone/parathyroid axis in which FGF23 functions as a direct, negative regulator of the PTH production. High extracellular Ca is a major determinant of the Klotho expression in pHPT, whereas the Klotho levels in sHPT may be attributed to a combination of the high FGF23 and Ca, and low calcitriol levels associated with CKD. Hence, the decreased Klotho expression in sHPT could explain the concomitantly high FGF23 and PTH levels, as well as the failure of FGF23 to prevent or mitigate the development of sHPT in CKD.

Keywords: calcitriol, chronic kidney disease, CKD, chronic renal failure, fibroblast growth factor 23, fibroblast growth factor-23, FGF23, FGF-23, GalNac-T3, GALNT3, GFR, hyperostosis-hyperphosphatemia syndrome, HHS, Klotho, parathyroid hormone, PTH, hyperparathyroidism, pHPT, sHPT, uremic, vitamin D₃

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urn:nbn:se:uu:diva-107456 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-107456)
To all the patients and their families,

to the cows of Swedish Meats,

and the rats and mice whose days were never described in any paper.

I think of you.
The Moirae or the Three Fates *Clotho, Lachesis* and *Atropos* were initially worshipped as goddesses of birth in the Greek mythology. The three spinners of the thread of life were believed to be daughters of Themis and Zeus. The sisters of destiny are here embodied on a Flemish tapestry from the beginning of the 16th century (© V&A Images/Victoria and Albert museum, London [Museum No: 65-1866]).
List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals.


*These authors contributed equally.

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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>$1\alpha,25(OH)_2D_3$</td>
<td>$1\alpha,25$-dihydroxyvitamin D$_3$ (calcitriol)</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ABD</td>
<td>adynamic bone disease</td>
</tr>
<tr>
<td>ADHR</td>
<td>autosomal dominant hypophosphatemic rickets</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>CaSR</td>
<td>calcium-sensing receptor</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CKD-MBD</td>
<td>chronic kidney disease mineral bone disorder</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DCT</td>
<td>distal convoluted tubule</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMP1</td>
<td>dentin matrix protein-1</td>
</tr>
<tr>
<td>DTEC</td>
<td>distal tubular epithelial cell</td>
</tr>
<tr>
<td>Egr-1</td>
<td>early growth response-1</td>
</tr>
<tr>
<td>ESRD</td>
<td>end-stage renal disease</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FD/MAS</td>
<td>fibrous dysplasia of bone/McCune-Albright syndrome</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GALNT3</td>
<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>GNAS1</td>
<td>stimulatory $\alpha$-subunit of guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>HapMap</td>
<td>haplotype map (of the human genome)</td>
</tr>
<tr>
<td>HFTC</td>
<td>hyperphosphatemic familial tumoral calcinosis</td>
</tr>
<tr>
<td>HHS</td>
<td>hyperostosis-hyperphosphatemia syndrome</td>
</tr>
<tr>
<td>iFGF23</td>
<td>intact fibroblast growth factor-23</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>kbp</td>
<td>kilo-basepair</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo-Dalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEN1</td>
<td>multiple endocrine neoplasia-1</td>
</tr>
<tr>
<td>mKlotho</td>
<td>membrane-bound Klotho</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NaP$_i$</td>
<td>sodium-dependent phosphate co-transporter</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>proximal convoluted tubule</td>
</tr>
<tr>
<td>PHEX</td>
<td>P&lt;sub&gt;1&lt;/sub&gt;-regulating gene with homologies to endopeptidases on the X-chromosome</td>
</tr>
<tr>
<td>pHPT</td>
<td>primary hyperparathyroidism</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PTEC</td>
<td>proximal tubular epithelial cell</td>
</tr>
<tr>
<td>PTGs</td>
<td>parathyroid glands</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RANK(L)</td>
<td>receptor activator for nuclear factor κ B (ligand)</td>
</tr>
<tr>
<td>ROMK1</td>
<td>renal outer medullary potassium channel-1</td>
</tr>
<tr>
<td>sCa</td>
<td>serum calcium</td>
</tr>
<tr>
<td>s[Ca&lt;sup&gt;2+&lt;/sup&gt;]</td>
<td>serum calcium ion concentration</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>sHPT</td>
<td>secondary hyperparathyroidism</td>
</tr>
<tr>
<td>SIFT</td>
<td>sorting intolerant from tolerant</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>TG</td>
<td>transgenic</td>
</tr>
<tr>
<td>TIO</td>
<td>tumor-induced osteomalacia</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TRPV</td>
<td>transient receptor potential cation channel V</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D&lt;sub&gt;3&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>VDRAs</td>
<td>vitamin D&lt;sub&gt;3&lt;/sub&gt; receptor activators</td>
</tr>
<tr>
<td>VDRE</td>
<td>vitamin D&lt;sub&gt;3&lt;/sub&gt;-responsive element</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless-type</td>
</tr>
<tr>
<td>XLH</td>
<td>X-linked hypophosphatemia</td>
</tr>
</tbody>
</table>
Introduction

The organ triad involved in mineral metabolism

Kidney

The kidney is necessary for keeping the organism free from hydrophilic drug metabolites, endogenous toxins and waste fluid, as well as for the regulation of electrolyte balance and blood pressure. The purification of blood and production of filtrated urine occurs in the nephron, the smallest functional part of the kidney (Figure 1). It consists of two components: a renal corpuscle and tubules. The former constitutes a glomerulus which is located within the capsule of Bowman and resembles a twisted ball of arterioles through which blood is pressed. The fine filtrate-collecting prolongation of the capsule is called proximal convoluted tubule (PCT) and leads further down through the loop of Henle to the distal convoluted tubule (DCT) and the collecting duct (1).

The filtrating property of the glomerulus lies in its semi-permeability, allowing soluble agents and excess water to exit the circulation stream and enter the capsule as primary urine, without causing proteinuria. The filtered blood is returned by efferent arterioles leading back to the circulation while secondary urine is excreted from the DCTs into the collecting duct system (1). Besides the modification of urinary electrolyte concentration, the regulation of renal hormone production, blood volume, pressure and pH is governed by hormones (1).

There are several ways of determining renal function. Glomerular filtration rate (GFR) represents the flow rate of produced urine per unit area (normal ≥100 mL/min/1.73cm²; decreases with age). Measurements of endogenous creatinine or cystatin C in blood are most commonly used by clinicians for GFR approximations.
Figure 1. (A) A sketch of a nephron. (B) Renal corpuscle – afferent and efferent blood vessels (right), filtration site or glomerulus (middle), and PCT (left). The proximal and distal tubular epithelial cells (PTECs; DTECs) are responsible for further modification of the primary urine by reabsorption of fluid and electrolytes, and its ultimate excretion. (C) PTEC – The sodium-dependent phosphate-co-transporters type IIa and IIc (NaPi IIa; NaPi IIc), that are located apically on the PTEC, drive the luminal Na$^+$ and P$_i$ into the cell using the free driving force in the form of a Na$^+$ gradient produced by Na$^+$/K$^+$ ATPase pumps on the basolateral side of the cell, which actively drive the Na$^+$ reabsorption in exchange for K$^+$ ions. With the help of Na$^+$, P$_i$ is easily reabsorbed back to the circulation (2). CaSR is located on the luminal side of the PTEC (3) where increase in filtrated Ca$^{2+}$ stimulates P$_i$ reabsorption. Ca$^{2+}$ itself mainly diffuses paracellularly in the PCTs. (D) DTEC – From the filtrate, Ca$^{2+}$ reenters the epithelial cell lining DCTs through the apical TRPV5, is transported across the cell bound to calbindin-D$_{28k}$ and is finally released back into circulation via the basolateral pumps PMCA1b and NCX1 (4). CaSR is located on the basolateral side of the DTEC for further fine-tuning of the [Ca$^{2+}$] (3). See text and abbreviations for further understanding.
Parathyroid gland

Normally there are two superior and two inferior parathyroid glands (PTGs) usually located dorsally on the thyroid gland. The exact location can vary from pharynx/esophagus and larynx, to thyroid gland and thymus, complicating surgical exploration of the neck (5). The approximate size of a normal PTG is the size of a rice grain and inadvertent removal of such during thyroidectomy occurs in 9–22% of the cases without clinical consequences (6).

The gland is bordered by a capsule of connective tissue that intersperses the parenchyma as fibrous septae, dividing it into lobules. Histologically, the tissue is richly supplied by capillaries and consists of chief cells, oxyphil cells, clear cells and adipocytes. The three parenchymal cell populations may be morphological variants of the same cell (5). The predominant chief cells have a relatively high nucleus/cytoplasm area ratio, and are the sole source of the 84-aa-peptide parathyroid hormone (PTH) that is stored in secretory granulae. The oxyphil cells have an eosinophilic, larger, granular cytoplasm, while clear cells are chief cells with excessive cytoplasmic stores of glycogen. The proportion of fat cells in a normal PTG is about 30%, with individual variations of lower rate being normal. Active chief cells contain little intracellular lipids, whereas in developed adenomas even absence of lobules and intercellular fat is expected (5).

The main function of PTH is to maintain the serum calcium level constant. PTH is mainly secreted as a full-length (1–84) peptide, even though fragments of varying lengths; agonistic (1–34)PTH (7), antagonistic (7–84)PTH (7, 8), and shorter (9) can be detected in serum. Serum measurements of biointact PTH refer to the full-length peptide of 9.4 kDa.

![Figure 2](image.png)

Figure 2. Normal parathyroidea: chief cells (c), oxyphil cells (o), adipocytes (a), erythrocyte (e).
Bone

Except for the maintenance of the erected posture and the provided organ protection, bones are involved in the great wheel of mineral metabolism machinery directly by their composition and storage of inorganic hydroxyapatite, and indirectly by being an endocrine source of hormones. The endocrine functions of bone are managed by bone-forming cells of the mesenchymal lineage, osteoblasts and osteocytes (10, 11), whereas the more direct involvement of bone in mineral metabolism is embodied by bone-resorbing giant cells of phagocytic origin named osteoclasts (12). Together, these cells govern the coupling mechanism of paracrine and autocrine communication paralleling the process of bone remodeling, i.e. formation and resorption of bone (13). The retiring mature osteocytes and the surrounding collagenous matrix constitute the organic, elastic part of the bone, providing a physical foundation for the inorganic mineralization layer crucial for its stiffness and strength. The level and magnitude of both parts are normally regulated by hormones as well as by many micro-environmental factors such as cytokines (13), keeping the turnover of the bone at a constant and balanced rate.

One of the biomarkers of osteoblast activity is bone alkaline phosphatase (bALP), which is measured for estimation of bone turnover and is often increased in bone diseases such as bone-involving malignancies, fractured bone and rickets/osteomalacia. Osteocalcin is another biomarker of bone formation whose levels provide an insight in the effectiveness of anti-osteoporotic drugs. Several biomarkers of osteoclastic activity, including cross-linked N- and C-terminal telopeptidases of type I collagen (NTx and CTx) and TRAP, are being used for estimation of bone resorption.

Storage and intra-/extracellular transport of minerals

To a certain extent, calcium can directly affect its own extracellular levels as well as those of phosphate, and vice versa. However, an inverse relationship between the two generally prevails in normal physiology (14-17).

Calcium

Ninety-nine percent of all calcium (Ca) in the body is stored in the skeleton and has a Ca:phosphorus ratio of 2:1. The remaining 1% is found in bodily fluids and soft tissues (18). The reference range of total serum\(^1\) Ca (sCa) is 2.15–2.5 mmol/L (8.6–10 mg/dL) with the free s\([Ca^{2+}\]) accounting for about half of the above indicated. The other half of total measured sCa is mainly

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\(^1\) Routinely, Ca\(^{2+}\) is measured in plasma before coagulation starts. The use of serum instead will be consistent throughout the text and refer merely to the blood compartment.
albumin-bound and eventual alterations in albumin are therefore corrected for. Only the free Ca$^{2+}$ is bioavailable, and is involved in extraskeletal processes such as: enzyme activity, blood clotting, cell division, intracellular signaling, muscle contraction and intercellular (nervous) communication.

The selective Ca$^{2+}$ ion channels `transient receptor potential cation channels V5/V6´ (TRPV5/6) are membrane-spanning proteins mainly expressed in transcellular Ca$^{2+}$ transport-mediating tissues; small intestine, renal DCTs, exocrine pancreas, prostate, and salivary/sweat/mammary glands (19). They are constitutively active at physiological s[Ca$^{2+}$]. In the kidney, the predominant TRPV5 channel is co-localized with other proteins involved in Ca$^{2+}$ transport like Na$^+$/Ca$^{2+}$ exchanger type 1 (NCX1), plasma membrane Ca$^{2+}$ ATPase type 1b (PMCA1b), as well as Ca$^{2+}$-binding protein calbindin-D$_{28k}$ (Figure 1) (20). The basolateral Na$^+$/K$^+$ ATPase pumps in PTGs, brain and kidney were recently associated with indirect Ca$^{2+}$ regulation (Figure 1) (21). The TRPV5 channel activity and thereby Ca$^{2+}$ transport can be modulated through feedback inhibition by intracellular Ca$^{2+}$ (22).

**Phosphate**

Phosphorus (P) is the 6th most common element in the body, after calcium. One percent of the total bodily P is located in the extracellular fluids, 14% in the cellular compartments and the remaining majority of 85% comprises the hydroxyapatite crystals of the skeleton together with calcium (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$). Most of the inorganic P in the fluids is free from binding proteins and exists in varyingly reduced forms of phosphate (H$_2$PO$_4^-$/HPO$_4^{2-}$/PO$_4^{3-}$) (18). Therefore, circulating free P will herein be denoted as inorganic phosphate, or P$_i$. Being an integral part of nucleic acids, energy-carrying ATP molecules, intracellular second messengers and plasma membranes, P$_i$ is essential for the basic functions of cells. Its involvement in cellular signaling includes modulation of intermediates in glycolysis and activation states of receptors and kinases (18, 23).

Normal serum P$_i$ concentration is 0.7–1.5 mmol/L (2.17–4.65 mg/dL). Appropriate balancing of serum P$_i$ requires involvement of intestinal absorption, active renal reabsorption/excretion in the PCTs and active release from skeletal hydroxyapatite stores.

The microvillus-lining enterocytes of duodenum/jejunum/ileum express the sodium-dependent P$_i$ co-transporter (NaP$_i$) IIb which is required for the active, hormone-responsive part of intestinal P$_i$ absorption (24). The remaining passive, diffusion-based, mechanism of intestinal P$_i$ uptake is located to the jejunum/ileum and is predominant only during high P$_i$ intake (18).

Basolateral Na$^+$/K$^+$ ATPase pumps of the renal PTECs help drive the NaP$_i$ IIa/c-dependent reabsorption of P$_i$ from the lumen (Figure 1) (2).

Finally, the release of P$_i$ from bone depends on osteoclastic resorption.
Major mineral-regulating hormones

Parathyroid hormone

**Ca\(^{2+}\)-regulating properties**

Keeping the s[Ca\(^{2+}\)] within the mentioned narrow range is largely controlled by the Ca\(^{2+}\)-sensing receptor (CaSR). This 120 kDa G-protein-coupled transmembrane receptor is expressed throughout the renal nephron, PTGs and gastrointestinal tract, among many other organs.

CaSR is an important mediator of s[Ca\(^{2+}\)] regulation of PTH secretion (25). Normal/high concentration of extracellular Ca\(^{2+}\) keeps the inhibitory, inositol triphosphate (IP\(_{3}\)) -mediated intracellular signaling of the ligand-bound CaSR activated, and thereby the PTH mRNA stability and protein secretion balanced/suppressed (25-28). The instant, dose-dependent release of PTH in response to s[Ca\(^{2+}\)] can be illustrated by the steep slope of a sigmoidal curve (29) – small deviations in s[Ca\(^{2+}\)] with changes in the CaSR activation state that follow, lead to rapidly adjusted PTH secretion. The short half-life of PTH peptide allows its rapid decline in serum after secretion from the granular stores of PTGs (30). PTH receptors in target tissues like bone and kidney mediate the converging PTH effects towards elevating the s[Ca\(^{2+}\)] back to normal.

The most prominent effects of PTH on the Ca homeostasis are exerted in bone. Although PTH plays a certain anabolic role (31, 32) it is mainly associated with bone resorption through osteoclast activation and inhibition of the osteoblastic collagen synthesis which result in decreased bone matrix formation. The loss of matrix leads to less foundation on which to build the mineralization layer, which stimulates the maintenance of Ca\(^{2+}\) in the serum (1).

In the kidney, PTH increases the levels of the key players in Ca\(^{2+}\) transcellular transport, TRPV5, NCX1 and calbindin-D\(_{28k}\) (Figure 1) (33). This results in active Ca\(^{2+}\) retention and elevated sCa\(_{2+}\) levels. The indirect PTH-induced and active vitamin D\(_{3}\)-mediated Ca\(^{2+}\) retention accounts for a minor calcitropic effect of the hormone (see below) (1).

PTH does not appear to have any major direct role on mineral absorption in the gastrointestinal tract, other than via active vitamin D\(_{3}\). PTH does although, and active vitamin D\(_{3}\) does not, stimulate the levels of duodenal calbindin-D\(_{9k}\) needed for transcellular Ca\(^{2+}\) transport (34, 35).

**P\(_{1}\)-regulating properties**

Parathyroid hormone receptors (PTHr) are expressed in kidney, intestine, bone and other organs (36). Similar to CaSR, PTHr1 is a 66 kDa G-protein-coupled transmembrane receptor that signals via several pathways (25, 37).

In the kidney, ligand-bound PTHr1 (that is interestingly expressed both apically and basolaterally on PTECs (38)) directly induces downregulation
and retrieval of the apical NaP$_i$ IIa (Figure 1) (39), resulting in a reduced P$_i$ reabsorption and thereby increased urinary excretion and lowering of serum P$_i$ levels. The internalized co-transporters are subsequently degraded both via lysosomal proteolytic and non-lysosomal ubiquitin/proteasome-mediated pathways (40). This property of PTH classifies it into a phosphaturic group of proteins. However, the phosphaturic role of PTH is dispensable since parathyroidectomized rats maintain appropriate urinary P$_i$ excretion in response to dietary P$_i$ load (41).

In the small intestine, the NaP$_i$ IIb co-transporter is insensitive to PTH (42) which instead only mediates indirect absorption of dietary P$_i$, again by affecting the levels of active vitamin D$_3$ (see below).

Although the principal function of PTH in bone is to release Ca$^{2+}$ from the mineralized matrix, the induction of osteoclastic resorption is accompanied by a release of P$_i$. Since skeletal PTHr1 is mainly expressed by osteoblasts, this is mediated in a locally paracrine fashion through osteoblast (RANKL)/osteoclast (RANK)-communication (43, 44).

**Vitamin D$_3$**

**Metabolism, signaling and parathyroid regulation**

1α,25-dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$; calcitriol) is the active metabolite of this hormone, although additional less hydroxylated metabolites have also been shown to exert biological effects (45). The steroid precursor cholecalciferol (vitamin D$_3$) is provided by dietary means and is also synthesized from a cholesterol backbone with the help of UV light in the skin (46). Figure 3 demonstrates a simplified flowchart of the synthesis and elimination of the vitamin D$_3$ metabolites.

The vitamin D$_3$ receptor (VDR) is a nuclear transcription factor with expression in many tissues (47-49). In the PTGs, the ligand-bound VDR binds directly to vitamin D$_3$-responsive elements (VDREs) in the PTH promoter (47) and suppresses the parathyroid cell proliferation and hormone production in a feedback-regulatory manner (50-52). This implicates calcitriol in an indirect regulation of both Ca and P$_i$ via changes in PTH.
Figure 3. In the liver, vitamin D₃ is further hydroxylated by the 25-hydroxylase (CYP27A1) or the microsomal CYP2R1 to become 25-hydroxyvitamin D₃ (or 25(OH)D₃) (53, 54), which later gains another hydroxyl-group in the kidney. This renal reaction is catalyzed by the 1α-hydroxylase (CYP27B1) which is expressed in both PCTs and DCTs (55) and produces the active 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃, calcitriol) (56). The latter feedback-autoregulates its own serum level by inhibiting the 1α-hydroxylase (57). Degradation of calcitriol is initiated by the renal 24-hydroxylase (CYP24) that calcitriol itself strongly induces (58, 59). PTH-induced net activation of 25(OH)D₃ involves both induction of the 1α- and suppression of the 24-hydroxylase and takes a few hours (29, 57, 60). Both enzymes are also expressed extrarenally, for instance in PTGs (61), bone and skin (62-65). Extrarenally produced calcitriol is believed to function in an autocrine/paracrine fashion (62, 66).

Ca²⁺-regulating properties

Induction of enterocytic Ca²⁺ transport is the most important direct calcitropic action of calcitriol, since it mediates 95% of the intestinal Ca²⁺ uptake (1). For instance, experimental hypercalciuria caused by inactivation of the TRPV5 in murine kidneys is compensated by increased intestinal Ca²⁺ absorption by the action of elevated calcitriol levels and TRPV6 expression (22, 67). Although, the lethality of PMCA1b gene knock-out animals (68), together with the recent data reporting a functional intestinal Ca²⁺ uptake in TRPV6 null mice (69), and the implications of importance of tight junction proteins like claudins (70, 71), make it clear that important information on intestinal Ca²⁺ absorption is still missing.

Calcitriol is strongly stimulated by hypocalcemia and raises the levels of sCa also by upregulating the expression of the renal TRPV5 channel (72). Calcitriol administration restores the decreased renal levels of TRPV5, cal-
bindin-D28k, NCX1 and PMCA1b in hypocalcemic mice deficient of 1α-hydroxylase (73).

The bone-resorptive and Ca\(^{2+}\)-releasing actions of calcitriol are relatively minor. They may slightly reinforce the bone resorption mainly induced by PTH, but are outbalanced by its similarly minor anabolic actions (74–76).

Calcitriol also increases the CaSR expression in both PTGs and kidney by interacting with the VDREs in the CaSR promoters (77, 78) contributing thereby to a better sensing and control of s[Ca\(^{2+}\)].

\[ \text{Pi-regulating properties} \]

Low-P\(_i\) diet induces calcitriol production (79, 80), which in turn raises the serum levels of P\(_i\) through direct stimulation of NaP\(_i\) IIa, NaP\(_i\) IIb and the osteoclast activator RANKL in kidney, intestine (24, 81) and bone (82-84), respectively. However, similarly to the dispensability of the phosphaturic actions of PTH, the calcitriol status does not affect renal P\(_i\) handling in response to dietary P\(_i\) load (81, 85). The action on enterocytes is therefore considered the major direct action of calcitriol in P\(_i\) homeostasis as well (1).

In addition, calcitriol treatment increases the expression of the suggested parathyroid ‘P\(_i\) sensor’, Pit-1 (NaP\(_i\) III) (86-88) which might be an additional mechanism for PTH suppression (88). However, the downregulation of PTH leads to an indirect reduction of renal P\(_i\) excretion, adequate during low-P\(_i\) intake.

\[ \text{Fibroblast growth factor-23} \]

**A member of the endocrine FGF19-subfamily**

Fibroblast growth factor-23, or FGF23, is the 22\(^{nd}\), latest and largest member of the FGF-protein family. FGF23 constitutes 251 aa and has a molecular weight of 32 kDa. Together with FGF19 (human ortholog of the murine Fgf15) and FGF21, FGF23 comprises the FGF19-subfamily of endocrine FGFs, distinguished from the intracellular (FGF11-14) and the canonical (remaining) non-circulating family members (89). The affinity to glycosaminoglycans (GAGs) such as heparin is low compared to that of the canonical members allowing them to freely circulate in the vasculature. The target specificity of FGF19-subfamily members is instead determined by the tissue distribution of co-receptors, whereas the canonical FGFs do not require such to signal (90). The primary function of the FGF19-subfamily trio is regulation of adult metabolism, whereas regulation of development and morphogenesis is the main function of the other FGFs (90). While FGF19 and FGF21 are mainly involved in bile acid regulation, and glucose uptake and lipolysis, respectively, FGF23 is important in mineral metabolism (91).
Maturation and activity
In order to be transported from the Golgi apparatus through the cytoplasm and finally secreted, FGF23 requires O-linked glycosylation of the threonine residue at position 178, which can be seen as several bands on a Western blot (92). The enzyme responsible for the post-translational modification of FGF23 is GALNT3 (UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3) (93). The sequence recognition motif \textsuperscript{176}RHTR\textsuperscript{179} is also an important proteolytical cleavage site for furin-like protease-mediated degradation of the bioactive, intact FGF23 (iFGF23) (94), and accurate glycosylation at the Thr\textsuperscript{178} protects FGF23 from inactivation (95, 96). The motif is conserved among species and the mouse/rat/bovine ortholog is ~70/71/78% identical to the human sequence.

Discovery and physiological function
FGF23 is a relatively novel phosphaturic hormone (a phosphatonin) involved in multiple disorders of mineral metabolism (Table 1). Being secreted mainly by osteoblasts and osteocytes (97, 98), it includes the skeleton among active endocrine organs. FGF23 was first identified among other proteins secreted from tumors of tumor-induced osteomalacia (TIO; also called oncogenic osteomalacia (OOM)) giving rise to hypophosphatemia and low serum calcitriol levels, as well as rickets/osteomalacia in both men and mice (99-101). The biochemical features returned to normal after resection of the TIO tumors (102). Transplantation of the tumor-derived cells (and later also FGF23-producing CHO cells) into healthy mice reproduced the effects, confirming the cause to be a circulating factor (103, 104). The iFGF23 levels can reach as high as 10 000 pg/mL in TIO (105), compared to the normal 10–50 pg/mL (94). The phosphaturic property of FGF23 lies in suppressing the NaPi IIa expression in the kidney (106). FGF23 also suppresses the renal 1α-hydroxylase expression while stimulating that of the 24-hydroxylase, leading to a net suppression of serum calcitriol levels (106).

Animal models
Hypophosphatemic FGF23 transgenic (TG) mice early develop severe osteomalacia (107). Yet, the skeletal phenotypic anomalies of several Pi-wasting disorders do not seem to be a consequence of hypophosphatemia alone, since hypophosphatemic NaPi IIa null mice do not develop rickets/osteomalacia (108). Interestingly, hyperphosphatemic FGF23 null mice also exhibit abnormal bone phenotype such as reduced bone mineralization (109). The enigma of direct autocrine/paracrine effects of FGF23 on bone is slowly becoming solved. Expression of the FGF23 receptors in osteoblasts and osteoclasts supports this possibility (110, 111). A genetic study on FGF23\textsuperscript{+/−}/NaPi IIa\textsuperscript{−/−} double-mutant mice recently demonstrated that despite reversal of the hyperphosphatemia of FGF23\textsuperscript{+/−} mice into hypophosphatemia
by NaPi IIa ablation, the skeletal phenotype remained unchanged (109). This suggests that either an unknown factor, the temporal aspect of Pi disturbance, the irreversibility of a bone phenotype, or the aberrant levels of FGF23 itself, rather than the Pi levels per se, may be the actual cause of the bone phenotypes observed in Pi disorders. In addition, FGF23 treatment of isolated osteoblasts has recently been reported to result in impaired mineralization (109, 111). However, the possibility remains that the aberrant bone morphology and mineralization may partly be an intrinsic bone defect due to abnormal FGF23 expression, which could affect other bone-produced factors of relevance for bone health (111-114).

FGF23-involving mineral disorders

Another disorder of elevated iFGF23 levels is autosomal dominant hypophosphatemic rickets (ADHR), caused by stabilizing mutations (R176Q, R179Q, R179W) in the cleavage site motif which render the hormone resistant to degradation (95, 96). The biochemical symptoms in ADHR are hyperphosphaturia and hypophosphatemia, inappropriately normal/low serum calcitriol, and normal PTH and Ca²⁺ levels (Table 1).

On the contrary, reduced levels of iFGF23 due to loss-of-function mutations in the FGF23 gene cause soft-tissue and vascular calcifications in a rare autosomal recessive disorder called hyperphosphatemic familial tumoral calcinosis (HFTC) (115-117).

Multiple other mineral disorders are rather due to mutations in proteins neighboring the FGF23 functions; HFTC (118, 119), HHS (hyperostosis-hypophosphatemia syndrome) (120, 121), XLH (X-linked hypophosphatemia) (112, 122, 123), ARHR (autosomal recessive hypophosphatemic rickets) (113, 124), HHRH (hereditary hypophosphatemic rickets with hypercalciuria) (125), and FD/MAS (fibrous dysplasia of bone/McCune-Albright syndrome) (97, 126) (Table 1).

Table 1. FGF23-involving disorders of mineral metabolism

<table>
<thead>
<tr>
<th>No.</th>
<th>Disorder (OMIM #)</th>
<th>Mutated gene</th>
<th>Mut. iFGF23 levels</th>
<th>Serum biochemistry /bone involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ADHR (#193100)</td>
<td>FGF23 (+)</td>
<td>↑ P₃; ~↓ vitD₃; ~ PTH; ~ Ca²⁺/yes</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>HFTC (#211900)</td>
<td>FGF23 (-)</td>
<td>↓ P₃; ~↑ vitD₃; ↑ PTH; ~↑ Ca²⁺/no</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GALNT3 (-)</td>
<td>↓ P₃; ~↑ vitD₃; ~ PTH; ~ Ca²⁺/no</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Klotho (-)</td>
<td>↑ P₃; ↑ vitD₃; ↑ PTH; ~↑ Ca²⁺/yes</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>HHS (#610233)</td>
<td>GALNT3 (-)</td>
<td>↓ P₃; ~vitD₃; ~ PTH; ~↑ Ca²⁺/yes</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>XLH (#307800)</td>
<td>PHEX (-)</td>
<td>↑ P₃; ~↓ vitD₃; ~ PTH; ~↑ Ca²⁺/yes</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>ARHR (#241520)</td>
<td>DMP1 (-)</td>
<td>~↓ P₃; ~vitD₃; ~ PTH; ~ Ca²⁺/yes</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>HHRH (#241530)</td>
<td>NaP₁, Iic (-)</td>
<td>~↓ P₃; ~↑ vitD₃; ↓ PTH; ↑ Ca²⁺/yes</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>FD/MAS (#174800)</td>
<td>GNAS1 (+)</td>
<td>~↑ P₃; ~vitD₃; ~ PTH; ~ Ca²⁺/yes</td>
<td></td>
</tr>
</tbody>
</table>
FGF23-regulating factors

Except for the crucial post-translational modification by the GALNT3 enzyme, regulation of FGF23 is managed by several other factors.

Calcitriol
Calcitriol may directly stimulate FGF23 transcription by binding to a VDRE in the FGF23 promoter (127, 128). On the contrary, inhibition of VDR signaling in chondrocytes disrupts FGF23 secretion from osteoblasts (129, 130), with the subsequent decrease in serum FGF23 resulting in increased calcitriol levels.

Phosphate and calcium
Increased dietary/serum Pi itself stimulates FGF23 secretion (106, 131, 132) by a yet unknown mechanism and the levels of the two are paralleled in hyperphosphatemia-associated renal disease (133). An increased Pi intake has not always been accompanied by a detectable increase in FGF23 in individuals with healthy kidneys (133-135), possibly due to other acute mechanisms involving PTH or Pi-induced autoregulation at the level of a well-functioning kidney. It is unknown whether FGF23 and PTH, two factors of large importance in Pi and calcitriol homeostasis, share any direct interregulatory communication.

No present data exist on direct, calcitriol-independent, FGF23 involvement in Ca^{2+} regulation, although Ca^{2+} does stimulate the FGF23 secretion independently of VDR signaling (130). An independent positive correlation of unknown biological significance has been reported between the two in hypercalcemic and/or hyperparathyroid disorders (133, 136-139).

Other
Inactivating and XLH-causing mutations in the bone-expressed PHEX^2 gene give rise to increased serum FGF23 levels by a yet unknown mechanism (112, 140). One hypothesis has been that the endopeptidase encoded by PHEX is involved in the degradation of FGF23, but that does not seem to be the case (122). Targeted overexpression of PHEX in the osteoblasts of the *Hyp* (murine version of XLH) mice did not completely correct the mineralization defect or the systemic hypophosphatemia, supporting that PHEX expression alone does not explain the pathogenesis of XLH (114, 141).

The dentin matrix protein-1 (DMP1) gene which is mutated in ARHR is also expressed in bone and TIO tumors (103). The overexpression of DMP1 from CHO cells transplanted into nude mice does not result in hypophos-

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^2 Pi-regulating gene with homologies to endopeptidases on the X-chromosome
phatemia (103) whereas DMP1 null mice have elevated serum FGF23 levels and hypophosphatemia (142). Together with the fact that DMP1 is elevated in TIO tumors, this establishes the consensus theory that DMP1 is not a phosphatonin itself but rather negatively regulates FGF23 levels by an unknown mechanism (143).

The GNAS1 gene encodes the α-subunit of the stimulatory G-protein (Gₐ) that is implicated in the G-protein-coupled receptor signaling pathway. In FD, activating mutations in GNAS1 result in hyperproliferation and incomplete differentiation of marrow stromal cells into osteoblasts (144, 145). The exact underlying mechanism behind the elevated FGF23 levels in FD is not completely understood, however they are associated with low serum Pi levels (97, 146).

FGF23 signaling

Receptors
There are four FGF-receptors, FGFR1–4. All exist in two isoforms, epithelial b- and mesenchymal c-isoform, except the forth, which only exists as FGFR4 and corresponds to the c-isoform of the others. The b-isoforms are alternatively spliced transcripts of the same genes encoding the c-isoforms. The FGFRs belong to a family of transmembrane tyrosine kinase receptors that homodimerize and autophosphorylate to ignite the signaling cascade upon ligand-binding (Figure 4) (147). The downstream cascade of FGF23-binding includes phosphorylation of ERKs (extracellular signal-regulated kinases) of the MAPK signaling pathway, and induction of early growth response genes like Egr-1 (148).

FGF23 signals only via the c-isoforms of FGFR1–3 and FGFR4, initially appearing to require high concentrations of GAGs (149), until its receptor affinity-enhancing co-receptor α-Klotho was discovered (148, 150, 151). The FGFR1c has the highest affinity for FGF23. The tissue distribution of FGFRs is wide and the specificity of FGF23 signaling is mediated by the less distributed expression of the co-receptor.

Type I membrane-bound α-Klotho³

Structure
The two isoforms of the Klotho protein differ in their length and attachment to the cell membrane. The membrane-bound Klotho (mKlotho) is mainly expressed in PTGs, kidney and choroid plexus of the brain (152, 153). The

³ α-Klotho is a gene of 5.2 kbp that carries the name of the daughter of Themis and Zeus who spins the thread of life. The (membrane-bound) α-Klotho is distinct from β-Klotho, a membrane-bound relative, and will herein be referred to as (m)Klotho.
shorter, “half”-length Klotho is the predominate (154), secreted form observed in cerebrospinal fluid, serum and urine (155), whose function stays quite uncertain. Both isoforms have been detected as ~130 kDa bands, suggesting dimerization of the secreted Klotho which can be either released from the mKlotho or alternatively spliced (155).

Functions

FGF23 receptor co-factor

The renal expression of mKlotho is restricted to the DCTs, initially perplexing the picture of the FGF23/Klotho-mediated regulation of P<sub>i</sub> handling in the PCTs. The phosphaturic effect of FGF23 is now proven to be mKlotho-dependent (156) and the FGF23 signaling is confirmed to specifically occur in the DCTs where FGFR1 is co-expressed (157, 158). The mechanism behind the signal transmission from the DCTs to the P<sub>i</sub>-excreting PCTs in the kidney remains unknown.

Figure 4. The FGFR1c/FGF23/mKlotho complex: upon binding of the ligand (FGF23), FGFR1c homodimerizes and autophosphorylates (yellow stars) initiating an intracellular signal cascade. The β-glucuronidase domain of Klotho (light blue) remains intact in the secreted isoform (159).

Renal ion transport regulation

Klotho belongs to a family of β-glucuronidases which cleaves off sugar residues of glycosylated proteins (160). The trafficking towards the cell membrane and the activity of the renal TRPV5 channel depend on its glycosylation level. Klotho-mediated hydrolyzation of the extracellular sugar residues of TRPV5 entraps the Ca<sup>2+</sup> channel on the luminal cell surface and prolongs its Ca<sup>2+</sup> permeability and reabsorption (Figure 1) (159-162). On the contrary,
the apical accumulation of the renal $K^+$ channel (ROMK1) by a similar mechanism of the soluble Klotho isoform seems to produce $K^+$ excretion (163).

**PTH secretion**

Secreted Klotho recruits $Na^+/K^+$ ATPases from the Golgi apparatus to the surface of epithelial cells in choroid plexus and kidney. The electrochemical gradient that develops across the cell membrane is thought to help drive the transcellular $Ca^{2+}$ transport (21). Similarly, the secretion of Klotho and the Klotho-dependent increase in apical $Na^+/K^+$ ATPases of the chief cells in PTGs may account for the rapid release of PTH observed only in response to hypocalcemia (21). This mechanism triggered by alterations in $s[Ca^{2+}]$ is absent in Klotho-ablated mice, although their PTH secretion is not entirely blunted in the absence of Klotho (21). Again, the parathyroid expression of Klotho and its function as the FGF23 co-receptor suggest that FGF23 may have direct effects on the PTGs.

**Animal models**

Overexpression of the Klotho gene in mice prolongs lifespan by two evolutionarily conserved anti-aging mechanisms; inhibition of insulin-like signaling and increasing the resistance to oxidative stress (164, 165).

In contrast, Klotho hypomorph mice have reduced lifespan, elevated serum levels of calcitriol, $P_i$, $Ca^{2+}$ and FGF23, arteriosclerosis, ectopic calcifications, skin atrophy, hypogonadism, osteoporosis; all of which are seen in aging organisms (153). A similar phenotype is seen in a HFTC patient with a homozygous mutation in the Klotho gene (Table 1) (118). Feeding the Klotho null mice with a low-calcitriol rescue diet (166) as well as crossing them with $1\alpha$-hydroxylase null mice (167) (or VDR null mice; unpublished and reviewed in (168, 169)) indicates that the phenotype observed is due to an imbalance in the calcitriol and Ca homeostasis. Except for the elevated FGF23 levels, the Klotho nulls show a phenotype very similar to that of the FGF23 nulls (170). Disruption of the calcitriol production or signaling in the FGF23 null mice similarly rescues the phenotype (171, 172). Similarly to the calcitriol/FGF23 interplay, calcitriol stimulates Klotho expression whereas recombinant Klotho protein suppresses $1\alpha$-hydroxylase expression in kidney cells in vitro (166, 173).

**Primary hyperparathyroidism**

Primary hyperparathyroidism (pHPT) is a common disease affecting as much as 1–5‰ of the general population (5). It is most frequent in post-menopausal women (1–2%) (174) and appears in a sporadic form, while hereditary forms are rarer. The etiology is most often an autonomous, monoclonal neoplasm in one of the PTGs in the form of adenomas (~85%; which
further on are referred to as ‘pHPT’), followed by multi-glandular hyperplasias (~15%) and carcinomas (< 1%) (5, 175). The sigmoidal curve describing s[Ca\(^2+\)]-dependent PTH release is severely right-shifted in pHPT. The HPT persists despite high sCa, giving rise to the classical symptoms of bone lesions, nephrolithiasis, cardiovascular disease (CVD), fatigue and possibly psychiatric alterations (176, 177). Parathyroidectomy is currently the most appropriate treatment. Surrounding normal rims can accompany removed neoplastic tissue.

The genetic and epigenetic mechanisms behind the parathyroid tumorigenesis are far from entirely mapped. Primary HPT is a multifactorial disease where different proteins gain or lose their functionality, for instance proto-oncogenes required for normal G1/S transition during the cell cycle (cyclin D1 and c-myc (178-180)), Wnt pathway components (179, 181-183), tumor suppressors (MEN1 (184, 185)) and PTH secretion regulators (CaSR, VDR and cyclin-dependent kinase inhibitors (CDKIs) p18/p21/p27 (186, 187)). Parathyroid 1α-hydroxylase and 24-hydroxylase are deregulated in pHPT towards a potential increase in local calcitriol production (61). However, calcitriol has not proven to be clinically effective against the parathyroid autonomy in pHPT, even though calcitriol analogues block the growth of parathyroid tumor cells (52, 188). Notably, serum FGF23 levels have been reported slightly elevated in the face of concomitant hypophosphatemia in pHPT (137, 138).

Chronic kidney disease

Kidney function is most frequently damaged by hypertension, diabetic angiopathy, polycystic kidney disease and various inflammatory and systemic disorders. The prevalence of chronic kidney disease (CKD) has been estimated to 10–15% in Taiwan and USA, making it a global public health concern. The degree of renal deficiency can be divided into five CKD stages according to the GFR level. Stage 1 of GFR ≥90 mL/min/1.73cm\(^2\) includes normal kidney function, whereas 60 is the frequent cutoff value between mild renal deficiency (stages 1–2) and moderate/severe kidney failure (stages 3–5). GFR below 15 describes the most severe CKD stage 5 or end-stage renal disease (ESRD), when urine production ceases and dialysis treatment is initialized.

CKD-MBD

The term ‘chronic kidney disease mineral bone disorder’ covers all the abnormalities in the mineral metabolism, as well as the vascular calcifications and the skeletal pathologies that appear due to chronic renal failure (189, 190).
**Alterations in mineral metabolism**

Failing kidney function leads to severe disturbances in mineral homeostasis, mirrored by several clinical manifestations. The disability of the kidney to filtrate Pi leads to increased retention and eventually elevated serum Pi levels that in turn inhibit the activation of 25(OH)D3 in the renal tubules (191-193). Together with the irreversible decrease in the nephron viability, this leads to reduced serum calcitriol levels with a consequential hypocalcemia. A hyper-secretion of PTH takes place as an early compensatory response preventing the latter (191, 194). This guaranties normocalcemia and helps keeping the serum Pi levels normally regulated during these primary events of the renal decline. These biochemical alterations are an integral part of the complex CKD-MBD.

Like PTH, serum FGF23 levels are elevated in CKD and correlate strongly with the decreasing GFR (195-197). The rise in serum FGF23 starts relatively early (GFR >50) in response to the increased Pi retention. Together with PTH, FGF23 postpones the hyperphosphatemia, until the Pi excretion is overridden by the Pi retention (GFR <30) (194). FGF23 functions thereby as an early and better marker of Pi retention than serum Pi itself (133, 191, 197). However, the hyperphosphatemia alone will later exacerbate the hypocalcemia and the hyperparathyroidism (14, 198-200). FGF23 is also thought to aggravate the latter through additional suppression of calcitriol, despite its phosphaturic effects (the revisited ‘trade-off’ theory) (201). Serum FGF23 levels correlate positively with serum Pi and PTH, and inversely with serum calcitriol levels, while independently predicting the progression of CKD (194, 197).

**Secondary hyperparathyroidism**

The above mentioned hyperparathyroidism is a very common complication in CKD-MBD. During the decline in renal function, PTH levels go from mildly elevated to very high in ESRD, which is mirrored by the transition of the glandular hyperplasia from diffuse to nodular type (5). Sporadic alterations in parathyroid gene expression are common, e.g. the expression of the suppressive VDR, CaSR and CDKIs is decreased due to unknown non-mutational causes (187, 202-205). These rather regulatory changes are to a large part responsible for the parathyroid insensitivity to treatment in ESRD.

Contrarily to the pHPT, all glands get involved in this initially physiological, glandular response referred to as uremic or secondary hyperparathyroidism (sHPT). Uremic sHPT is opposed to the sHPT caused by intestinal malabsorption, inadequate nutrition, or calcitriol deficiency due to mutated renal 1α-hydroxylase (206). A summarized cause of the progressing uremic sHPT discussed above is increased Pi retention together with decreased serum calcitriol and Ca, and possibly high serum FGF23 levels (Figure 5). Serum FGF23 levels are an independent predictor of sHPT pro-
gression in long-term dialysis patients with mild sHPT, and it has therefore been speculated that FGF23 may directly contribute to the development of sHPT (207).

![Diagram](image)

**Figure 5.** Factors that stimulate sHPT.

**Cardiovascular disease and mortality**
Having CKD at young age is thought to increase the risk of death a 1000-fold, reaching that of a healthy 80-year-old (208, 209). Alterations in mineral metabolism related to CKD, such as hyperphosphatemia, HPT, (initial) hypo-/(later) hypercalcemia, low calcitriol and high FGF23 levels are all associated with high morbidity and increased cardiovascular mortality (210-216). More than 50% of CKD patients die due to CV complications like arrhythmia and sudden heart failure.

**Skeletal abnormalities**
The skeletal aberrations can present themselves either as high bone turnover due to the elevated PTH levels, or low, in the (diminishing) form of osteomalacia or iatrogenically induced adynamic bone disease (ABD) (217-219). The latter can e.g. appear in cases where therapy focusing on PTH suppression has been too effective. Either way, disturbances in bone metabolism predispose the patient to a higher risk of bone fracture, complicating the biochemical and cardiovascular profile at the same time.

**Treatment**
Treatment of the mineral disturbances in CKD includes dietary Pi restriction and oral Pi binders, Ca supplementation, VDR activators (VDRAs), calcimimetics, renal replacement therapy (kidney transplantation or dialysis) and parathyroidectomy. Treatment with VDRAs may upregulate the parathyroid expression of VDR, CaSR and p21 in the responsive stages of sHPT (220-222). Parathyroidectomy with or without brachial autotransplantation is otherwise the only solution to the sHPT (223, 224). If surgery is inappropriate in some patients, refractory sHPT can develop in the form of a monoclonal tumor, or tertiary HPT (tHPT), despite kidney transplantation (225, 226).

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4 High bone turnover occurs e.g. in osteoporosis where resorption is excessive, or as accelerated formation of (woven) bone during fracture healing.
Aims of the current investigation

The overall aim of this investigation was to discern a possible direct role of FGF23 and Klotho in parathyroid biology. Our goals were to find out whether parathyroid glands pertain to the extrarenal target organs of FGF23 and investigate the nature of its actions. Also, in a wider perspective, we were interested in comprehending the role of FGF23 in the progression of sHPT, as well as in comparing the possibility of FGF23 to signal in hyperparathyroid disorders by looking at the expression of its co-receptor Klotho.

Specific aims

- To identify the genetic cause of the clinical and biochemical features of HHS in a patient, and determine the relation between FGF23 and the abnormalities in mineral metabolism (Paper I)

- To investigate whether parathyroid cells are a direct target of FGF23 (Paper II)

- To explore the Klotho expression in parathyroid adenomas from pHPT patients (Paper III)

- To study the regulation of Klotho expression by FGF23, Ca, P_i, PTH and the calcitriol analogue EB1089, or combinations thereof, in isolated normal bovine parathyroid cells (Paper III)

- To study the parathyroid Klotho expression in CKD patients with sHPT (Paper IV)
Materials and methods

Biochemistry (Paper I, III & IV)
The University Hospital of Malmö and the department of Clinical Chemistry at the Uppsala University Hospital assessed the serum biochemical analyses according to their standard clinical procedures. The routine analyses included Pi, Ca, albumin, creatinine, PTH and ALP. Serum 25(OH)D3 and calcitriol in Paper I were analyzed at the Malmö University Hospital, whereas ELISAs measuring the C-terminal (Immutopics Inc., USA) and the intact FGF23 (Kainos Laboratories Int., Japan) were used at the Uppsala University Hospital. Regarding the CKD patients in Paper IV, measurements of PTH and Pi were performed within a period of 6 months to a few days prior to surgery. All except five patients had their serum Ca and albumin levels determined within those few days before surgery. One patient’s Ca value was uncorrected due to lack of albumin data. Other missing values were: Pi (n=2) and PTH (n=2). Total albumin-corrected Ca was calculated by using the following equation: corrected Ca (mmol/L) = Ca (mmol/L) – {0.018 x [(albumin; g/L) – 42]}. The GFR values were estimated by using the Cockcroft-Gault formula: GFR (mL/min/1.73m²) = (140 – age; yrs) x (weight; kg) x (0.85; if female) / (72 x creatinine; mg/dL). Conversion factors are: Ca (mmol/L) x 4.0 = (mg/dL); Pi (mmol/L) x 3.1 = (mg/dL); PTH (ng/L) / 9.5 = (pmol/L); creatinine (mg/dL) x 88.4 = (µmol/L).

Reverse transcriptase – polymerase chain reaction (RT-PCR) (Paper I)
Genomic DNA was extracted from blood using the Wizard Genomic DNA Purification Kit (Promega, USA). All exons including the intron-exon boundaries of the genes FGF23 and GALNT3 were amplified according to a standard PCR protocol using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA).

DNA sequencing (Paper I)
Amplicons were separated by gel electrophoresis, cut out and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany). BigDye v3.1 was used for direct sequencing with forward and reverse primers by the 3130xl Genetic Analyzer (Applied Biosystems, USA). The sequencing primers are listed in Table 2.
**Table 2. Sequencing primers**

<table>
<thead>
<tr>
<th>Gene / Primer</th>
<th>5′-Sequence-3′ / Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF23, exon 1 / Forward</td>
<td>cag agg atg tgg aca gtg ga (472)</td>
</tr>
<tr>
<td>FGF23, exon 1 / Reverse</td>
<td>aga tgg aca aca agg gtg ct</td>
</tr>
<tr>
<td>FGF23, exon 2 / Forward</td>
<td>ttc agg agg tgc tgt aag gt (291)</td>
</tr>
<tr>
<td>FGF23, exon 2 / Reverse</td>
<td>gga aac agg tca cca ggg ta</td>
</tr>
<tr>
<td>FGF23, exon 3 / Forward</td>
<td>gct caa cgc cct aag aac tg (593)</td>
</tr>
<tr>
<td>FGF23, exon 3 / Reverse</td>
<td>gtt aaa gag ggt gcc ctt cc</td>
</tr>
<tr>
<td>GALNT3, exon 1 / Forward</td>
<td>cca tgc atc att tct gtg tat agg (718)</td>
</tr>
<tr>
<td>GALNT3, exon 1 / Reverse</td>
<td>tcc tta gct cac ccc tct ctc</td>
</tr>
<tr>
<td>GALNT3, exon 2 / Forward</td>
<td>ggt gag tga ttt gct tgt aaa aa (469)</td>
</tr>
<tr>
<td>GALNT3, exon 2 / Reverse</td>
<td>cca gct cgg aga tgg cat caa</td>
</tr>
<tr>
<td>GALNT3, exon 3 / Forward</td>
<td>cat ttt gct gga agg aca ca (447)</td>
</tr>
<tr>
<td>GALNT3, exon 3 / Reverse</td>
<td>ctt gaa cta tta gaa cac aa</td>
</tr>
<tr>
<td>GALNT3, exon 4 / Forward</td>
<td>tct gaa caa gaa aat ctc ca</td>
</tr>
<tr>
<td>GALNT3, exon 4 / Reverse</td>
<td>gag ctc act cac tgc tct tt (587)</td>
</tr>
<tr>
<td>GALNT3, exon 5 / Forward</td>
<td>cca tgg gag aag aca cga ag (385)</td>
</tr>
<tr>
<td>GALNT3, exon 5 / Reverse</td>
<td>acc agc cga tta gaa cac aa</td>
</tr>
<tr>
<td>GALNT3, exon 6 / Forward</td>
<td>atg gca ggg gac aga gac ta (416)</td>
</tr>
<tr>
<td>GALNT3, exon 6 / Reverse</td>
<td>atg aat cga cgc aca aag ac</td>
</tr>
<tr>
<td>GALNT3, exons 7&amp;8 / Forward</td>
<td>ggc tgt tga att gcc ctc tg</td>
</tr>
<tr>
<td>GALNT3, exons 7&amp;8 / Reverse</td>
<td>agg caa cat ctc act tgt gct (637)</td>
</tr>
<tr>
<td>GALNT3, exon 9 / Forward</td>
<td>aac cac ctc tgt atg aag gaa</td>
</tr>
<tr>
<td>GALNT3, exon 9 / Reverse</td>
<td>tgt tcc act cat ttt ccc aga</td>
</tr>
<tr>
<td>GALNT3, exon 10 / Forward</td>
<td>tca gac atg gct cac ctt aca a</td>
</tr>
<tr>
<td>GALNT3, exon 10 / Reverse</td>
<td>tgt ctc ttc gtc cta a</td>
</tr>
</tbody>
</table>

**Preparation of bovine parathyroid glands and cell culture (Paper II–IV)**

Parathyroid glands were obtained from healthy cattle within minutes after slaughter. The glands were minced with scissors after removal of the surrounding fat tissue. The cell suspensions were digested on a shaker at 37°C for 2h in F-10 medium (pH 7.4) containing 1 mg/mL collagenase, 0.05 mg/mL DNase I, 1.25 mmol/L CaCl₂ and 1.5% BSA. After filtration through a nylon mesh, the cells were centrifuged through 25% standard isotonic Percoll (GE Healthcare, UK). The cell viability was determined by Trypan blue staining prior to the seeding of 0.5x10⁶ cells/well into 6-well plates. The cells were grown at 37°C and 5% CO₂ in DMEM medium containing 10% fetal calf serum (FCS) and penicillin/fungizone/L-glutamine (PFL). Twenty-four hours after their outgrowth, both control- and treated cells were exposed to serum-free DMEM medium at the initiation of the experiments. The cells were harvested after additional 1–24h or more for RNA extraction.

**Production of recombinant FGF23 protein and conditioned media (Paper II & IV)**

The full-length human FGF23 cDNA with an introduced mutation encoding the FGF23(R176Q) protein was initially cloned into the pcDNA3.1-V5-His-
TOPO plasmid by Larsson, et al (99). The hFGF23(R176Q)-pcDNA3.1-V5-His-TOPO plasmid and an empty mock pcDNA3.1(+) plasmid as control were transfected into COS-7 cells using FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., USA), whereupon the conditioned serum-free DMEM media were collected 3–4 days later. Aliquots were centrifuged and immediately stored at -70°C. The FGF23-containing conditioned media were diluted on the day of usage to appropriate experimental concentrations using the conditioned serum-free mock medium as diluent. The mock medium was also used as control medium until it was completely exchanged for unconditioned serum-free DMEM medium in Paper IV for practical reasons, after a dose-response experiment confirmed no effects on the bovine parathyroid cells.

**In vitro experiments (Paper II–IV)**

All the *in vitro* experiments were performed on primary bovine parathyroid cells. Triplicate or quadruplicate wells were used for all experimental concentrations and time-points in dose-response and time-course experiments, respectively. Throughout all the studies, PFL was included at identical concentrations in all cell media. Treatments have included (single or combinations of): control medium, FGF23(R176Q), Ca, calcitriol analogue EB1089, calcitriol, P₁ and PTH. All media contained the same percentage of ethanol (diluent of EB1089 and calcitriol) and ddH₂O (diluent of Ca and P₁). The experiments were repeated 1–3 times.

**ELISA analyses of conditioned media (Paper II & IV)**

The FGF23 protein concentration of the conditioned media from transfected COS-7 cells was estimated by the Kainos´ intact FGF23 ELISA Kit (Kainos Laboratories Int., Japan). The concentration of the secreted bovine PTH protein was estimated by a bovine intact PTH ELISA Kit (Immutopics, USA). In this case, the bovine parathyroid cells were treated with FGF23(R176Q) for 24h, followed by two 1x PBS washes and addition of fresh serum-free DMEM medium. These conditioned media were collected after 4h, centrifuged before freezing and subsequently analyzed for PTH concentrations. The latter were corrected for total protein content in the wells as determined by the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA).

**Flow cytometry analysis (Paper II)**

The bovine parathyroid cells were trypsinized and diluted with 1x PBS and divided into two 5 mL FACS tubes (duplicates). The cells were centrifuged, washed by and resuspended in FACS buffer: 2% FCS, 0.05% NaN₃, 2.5 mmol/L CaCl₂ and 1x PBS. Following a 2nd centrifugation, the cells were resuspended in 100 μL FACS buffer and 5 μL anti-Annexin V-FITC-conjugated antibodies were added. After 20–30 min in dark at room temperature, the cells were washed again and 5 μL propidium iodide (PI) was
added. Non-treated cells were used as FACS controls: a) without FITC or PI, b) only FITC, and c) only PI. Low flow speed and limit of 50,000 cells were set for each measurement. Identical settings were used at later measurements. Total apoptosis was defined as the sum of early (FITC) and late (FITC and PI) apoptosis. Cells detected for only PI represented necrotic cells.

**Tritium-labeled thymidine incorporation (Paper II)**

In order to estimate the proliferation of the bovine parathyroid cells, 1 μCi \[^3\text{H}\]-thymidine/well was added 12h before harvesting. After incubation, medium was removed and unincorporated \[^3\text{H}\]-thymidine was precipitated with 500 μL cold trichloroacetic acid for 20 min. This procedure was repeated twice. The cells were lysed in 1 mL 10% NaOH and 0.5% Triton X. The radioactivity was measured in a β-counter.

**Patients and tumor tissue specimens (Paper III & IV)**

Informed consent was obtained from all the patients and the studies were approved by the institutional ethics committee at Uppsala University. Parathyroid adenomas (n=40) and secondary parathyroid hyperplasias (n=31) were acquired from pHPT and CKD patients, respectively, and their weight was documented. These patients were diagnosed and surgically treated at the Uppsala University Hospital between 1998 and 2008.

For pHPT patients, the inclusion criteria were abnormally high PTH levels in relation to serum Ca, normal creatinine levels and no history of familial HPT. They were not treated with VDRAs or calcimimetics. Four normal parathyroid tissue samples were obtained from glands unintentionally removed during thyroid surgery where autotransplantation was not required, or as normal parathyroid gland biopsies in patients subjected to parathyroidectomy. The tissues were snap-frozen or embedded in paraffin.

For CKD (or sHPT) patients, the inclusion criteria were access to at least 1 frozen parathyroid tissue sample and a GFR <90 mL/min/1.73m². The etiologies of CKD were: glomerulonephritis (n=11), polycystic kidney disease (n=5), diabetic nephropathy (n=4), hypertonic nephrosclerosis (n=2), pyelonephritis (n=2), vasculitis (n=1) and other (n=6). Twenty-one patients had undergone kidney transplantation. In total, 4/3/2/1 (n) hyperplastic glands were removed from 10/10/7/4 (n) patients, respectively (total n=88). The sHPT patients were divided according to CKD stages 1/2/3/4/5 (n=0/4/8/11/8). Fifteen patients were on Pi binders, fourteen were treated with VDRAs and two patients received daily calcimimetics prior to surgery.

**RNA isolation and cDNA synthesis (Paper II–IV)**

Bovine samples: Paper II–IV: According to the E.Z.N.A. Total RNA Isolation Protocol (Omega Biotek, USA), total RNA was extracted and on-membrane DNase I treatment performed. NanoDrop ND-1000 Spectropho-
tometer (Thermo Scientific, USA) was used to estimate the concentration as well as obtain the purity $A_{260}/A_{280}$ ratio (>1.99). Electrophoresis on 1% agarose gel was used to check for RNA degradation. A representative group of samples (Paper II) was further analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to confirm adequate RNA integrity. All RNA samples were kept at -70°C until cDNA synthesis was performed using SuperScript II Reverse Transcriptase and Oligo-(dT) primers (Invitrogen, USA).

Human samples: Paper III: Total RNA was extracted with Trizol Reagent (Life Technologies Inc., USA) and the DNA-free RNA was prepared using the NucleoSpin RNA II Kit (Macherey-Nagel GmbH & Co. KG, Germany). Successful DNase treatments were verified by PCR analysis of all RNA preparations. Paper IV: Total RNA was extracted and directly subjected to DNase digestion using the above mentioned NucleoSpin RNA II Kit. All RNA samples were kept at -70°C until reverse transcription with hexamer random primers using the First-Strand cDNA Synthesis Kit (GE Healthcare, UK) was performed according to the manufacturer’s instructions. All cDNA samples were kept at -20°C.

Real-time quantitative RT-PCR (Paper II–IV)

Bovine samples: Paper II–IV: A total volume of 25 μL per reaction consisted of 12.5 μL (2x) iQ SYBR Green Supermix containing fluorescein (Bio-Rad Laboratories Inc., USA); 2.5 μL (2 μmol/L) Fwd primer; 2.5 μL (2 μmol/L) Rev primer; 5.5 μL sterile H$_2$O; and 2 μL cDNA. The iCycler, MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) was used at the following cycling conditions: (a) 95°C, 3 min; (b) 40 times: 95°C, 15 sec; 54–60°C (annealing temperature depending on the primers), 1 min; (c) 95°C, 1 min; (d) 55°C, 1 min; and (e) 80 times: +0.5°C, starting from 55°C, 10 sec, for melt-curve obtainment. All samples, including non-template negative controls, were amplified in duplicates. All assays were run 3–4 times. Exon-specific primers were designed and their specificity was confirmed by sequencing analysis. Temperature-dependent dissociation curves for all the PCR products revealed one single and identical top for each corresponding transcript and primer pair. The product lengths were verified by gel electrophoresis. The sequences of the bovine 1α-hydroxylase (1α(OH)ase), GAPDH, mKlotho and PTH primer pairs are listed in Table 3 below. Relative gene expression was calculated according to the comparative $C_t$ method ($2^{-\Delta\Delta C_t}$) described by Livak & Schmittgen (227).

Human samples: Paper III–IV: The reaction conditions were as described above. The specificity of the human primer pairs was also confirmed by sequencing analysis and the sequences are listed in Table 3. Standard curves

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5 This primer pair was designed according to the human GAPDH sequence and differed from the bovine counterpart by a few bases. The sequence of its amplicons was identical to the bovine GAPDH (transcript) sequence, as verified by sequencing analysis.
based on serial dilutions of PCR products were co-amplified together with the patient material, and each sample is presented as a ratio between the relative start quantity means of mKlotho and GAPDH (internal control) representing each parathyroid gland (Paper III; n=44 & Paper IV; n=88). In Paper IV, an average of all the analyzed glands of each patient representing her/his GAPDH-standardized mean Klotho level was used in the correlation analyses (n=31).

Table 3. Real-time PCR primers

<table>
<thead>
<tr>
<th>Species / Gene / Primer</th>
<th>5’-Sequence-3’ / Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. Taurus</strong> / 1a(OH)ase / Forward*</td>
<td>atc ccc aaa aat acg ctc ggt</td>
</tr>
<tr>
<td><strong>B. Taurus</strong> / 1a(OH)ase / Reverse*</td>
<td>gct gga cga aaa gaa ttt gg / (89)</td>
</tr>
<tr>
<td><strong>B. Taurus</strong> / GAPDH / Forward</td>
<td>ggt cat cat ctc tgc acc ttc</td>
</tr>
<tr>
<td><strong>B. Taurus</strong> / GAPDH / Reverse</td>
<td>ctt ctc ggg agg aat ggc</td>
</tr>
<tr>
<td><strong>B. Taurus</strong> / mKlotho / Forward</td>
<td>aac tgg ctg aag gcc aag ta</td>
</tr>
<tr>
<td><strong>B. Taurus</strong> / mKlotho / Reverse</td>
<td>ctc gtc ggt ctc taa</td>
</tr>
<tr>
<td><strong>B. Taurus</strong> / PTH / Forward</td>
<td>gac atg gct aat gtt atg</td>
</tr>
<tr>
<td><strong>B. Taurus</strong> / PTH / Reverse</td>
<td>cag ctc acc cag cca ttc tac / (162)</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / GAPDH / Forward (Paper III)*</td>
<td>cca cca tgg aga agg ctc ggg cta a</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / GAPDH / Reverse (Paper III)*</td>
<td>atc acg cca cag ttc ccc gga ggg g / (287)</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / GAPDH / Forward (Paper IV)</td>
<td>gaa ggt gaa ggt ctt ctc ggt</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / GAPDH / Reverse (Paper IV)</td>
<td>gaa gat ggt gat ggg att tc / (226)</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / mKlotho / Forward (Paper III)*</td>
<td>tac gga gac ctc ccc atg ta</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / mKlotho / Reverse (Paper III)*</td>
<td>cca tcc agt atg tgg gct tt / (131)</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / mKlotho / Forward (Paper IV)</td>
<td>atc caa tgg aat cga tga cg</td>
</tr>
<tr>
<td><strong>H. Sapiens</strong> / mKlotho / Reverse (Paper IV)</td>
<td>aag caa agt atc cgc aaa ga / (131)</td>
</tr>
</tbody>
</table>

*Specific for exon-exon boundaries

Immunohistochemistry (Paper III & IV)

Frozen sections of parathyroid glands were used for all histological analyses of the Klotho protein levels. A polyclonal goat anti-Klotho antibody (Santa Cruz Biotechnology Inc., USA; catalog no. sc-22220) was used as primary antibody at a dilution of 1:5–1:10. Standard immunohistochemical procedures were followed. Consecutive sections were used as negative controls (without sc-22220). In Paper III, paraffin sections were used for detection of proliferating cell nuclear antigen (PCNA), VDR and FGFR1c. All sections, except those stained for PCNA, were counterstained with Mayer’s hematoxylin (nuclear staining). All antibodies were purchased from Santa Cruz Biotechnology Inc.: sc-56 for PCNA, sc-1008 for VDR and sc-7945 for FGFR1c (Flg).

Statistical analyses (Paper II–IV)

GraphPad Prism versions 3 and 5 (GraphPad Software Inc., USA) as well as Statistica 8 (StatSoft, USA) were used for the statistical data analyses. A p-value of less than 0.05 was considered statistically significant. Paper II–IV: Student’s unpaired t-tests were performed and the results are shown as
arithmetic means ± SEM, unless otherwise stated. *Paper III & IV*: Patients’ biochemistries are presented as means ± SD. *Paper III*: Spearman’s non-parametric univariate correlation and multivariate linear regression analyses were applied where applicable. The adenomas with undetectable Klotho mRNA levels were arbitrarily assigned a relative Klotho value of 0.5. *Paper IV*: Normality-testing of patients’ biochemistry data showed a normal distribution of mean Klotho values, sCa and age. All other parameters were logged and Pearson’s parametric univariate correlation analysis was applied where applicable.
Results and discussion

Paper I

*A novel missense point mutation in a highly conserved region of GALNT3 causes hyperostosis-hyperphosphatemia syndrome*

The medical history of a 19-year-old patient of Columbian origin included painful cortical lesions in the left tibia at the age of five, diaphyseal hyperostosis with periosteal apposition accompanied by oedema in the surrounding tissue of the right tibia at the age of 11, and additional cortical thickening and progressing sclerosis at the age of 13. Throughout the years, the patient had suffered from recurring skeletal pain and continuously marked hyperphosphatemia. In addition, elevated serum Ca and ALP, normal calcitriol and creatinine, and PTH levels below the reference range constituted her biochemical profile.

The hyperostotic bone together with the severe and irrepressible hyperphosphatemia suggested that this HHS-like disorder could be due to poor FGF23 profile. To determine the role of FGF23 in this patient, we analyzed both the intact and the C-terminal (fragmented) FGF23 by two different ELISA techniques. The iFGF23 level was subnormal (15 pg/mL) compared to the reference range (28.9±11.2 pg/mL; mean±SD (94)) whereas the C-terminal FGF23 was markedly elevated (839 RU/mL vs. normal range of 55±50 RU/mL), suggesting increased processing and degradation of iFGF23. In order to exclude genetic aberrations that could be causative of the differential processing of the protein, we sequenced the entire coding region along with the exon-intron boundaries of the genes FGF23 and GALNT3, the latter being responsible for proper post-translational glycosylation of FGF23.

We discovered a novel homozygous 1584 G>A substitution in exon 6 of GALNT3, corresponding to an Arg>His amino acid change at residue 438 (R438H; Figure 6).
R438H did not affect any functional motif of the protein, as predicted by the PROSITE prediction database. However, the expectedly detrimental change was predicted by the NNPREDICT Protein Secondary Structure Prediction program to disrupt a C-terminal β-strand in GALNT3. Accordingly, a dysfunctional GALNT3 due to structural changes would lead to the enhanced degradation of iFGF23 and consequently to the observed hyperphosphatemia (228). The 1584 G>A substitution has not been reported previously as a single-nucleotide polymorphism in the NCBI SNP database, the Japanese SNP database nor was it present in 200 Caucasian, 240 Chinese or 232 Nigerian control alleles (HapMap). Although we were unable to establish a family
pedigree due to adoption and unknown family history, our findings suggest that this novel homozygous missense mutation is causative of the recessive disorder HHS in our patient of South American origin.

Our findings confirm that iFGF23 is required for normal P_i homeostasis, and that failing local expression of FGF23 can lead to different bone phenotypes (119, 120).

Paper II

*FGF23 exerts direct effects on cultured bovine parathyroid cells by suppressing the PTH mRNA and protein levels, while increasing the expression of the 25(OH)D_3-activating enzyme, 1α-hydroxylase*

The high serum levels of iFGF23 in CKD patients (136, 229) have led to the question of whether FGF23 contributes to the development of sHPT (207, 230). This enticed us to investigate the existence and nature of direct effects of FGF23 on the PTH production. For this purpose, we cultured primary bovine parathyroid cells and incubated them with the stable mutant form of FGF23, FGF23(R176Q). The presence of the FGF23 receptor co-factor Klotho and the increase in the mRNA expression of the early response gene Egr-1 after 1h treatment supported the possibility of direct signaling and impact of FGF23 on the parathyroid cells in vitro. Both the mRNA and the protein levels secreted into conditioned media were dose-dependently decreased by the treatment. The maximal effect and the highest cell viability coincided at 24h which determined the time-point of the dose-response experiment. A concentration of 400 pg/mL FGF23(R176Q) (286 nmol/L) was the lowest having effect on the PTH transcript levels. The suppression of PTH was first detected at 12h of treatment and lasted for at least 48h.

Previous reports on FGF23 suppression of the renal 1α-hydroxylase expression (103, 106) inspired us to study its effects on the parathyroid counterpart. Surprisingly, we found an increase in the mRNA levels of the parathyroid 1α-hydroxylase within 3h which sustained for 24h. Again, FGF23(R176Q) showed to be increasingly effective at the concentrations 400–2000 pg/mL.

Since the viability of primary cells in culture naturally declines with time, we needed to exclude the possibility of additional treatment-induced cell growth effects influencing our findings. Therefore, we examined the cell number, viability, apoptosis and proliferation. The highest concentration of FGF23(R176Q) used in the experiments (2000 pg/mL) did not affect cell number or viability at 24h, as compared to the control-treated cells. Similarly, no significant effects were observed on apoptosis, whereas a small increase in proliferation in terms of increased DNA synthesis was detected.
The proportion of PTH-expressing cells was ~90–95% during the first 48h regardless of treatment.

Although an increase in Egr-1 gene expression was detected as a marker of FGF23(R176Q) signaling in the cells (148), we cannot exclude the possibility of FGF23(R176Q) stimulating the release of locally produced factor(s) which could be the actual inducer(s) of the observed effects. Either way, our study ensures direct negative effect(s) of FGF23 on the hormone production of normal parathyroid cells that are independent of systemic changes in mineral metabolism. Our findings demonstrate that FGF23 does not alter the growth of parathyroid cells at short-term in vitro conditions. The possibility however remains that its actions are important in pathophysiological states or even that its chance to interfere in the hyperactivity of the parathyroid cells may be altered in the long perspective, as in disorders of mineral metabolism. The concentrations of FGF23(R176Q) used in our study are similar to those detected at later CKD stages (136, 229).

The increased expression of 1α-hydroxylase could be explained by a potential attempt of the PTGs to locally compensate for the loss of circulatory calcitriol provoked by FGF23, which of course would be a systemic response and only hold as a theory in in vivo settings. Nonetheless, no substrate (25(OH)D₃) was added to our isolated cell system and could not have been present in the serum-free treatment media. This means that although the increase in 1α-hydroxylase mRNA levels was detected hours before the decrease in PTH mRNA, it is not probable that FGF23 suppressed PTH in a VDR-dependent manner. This supports that FGF23 has two ways of inhibiting PTH, one of them including local 25(OH)D₃ activation. The discrepancy between FGF23 effects on the parathyroid and the renal 1α-hydroxylase expression could be explained by differential tissue-specific effects of FGF23. Further functional studies are needed to clarify the FGF23 effect on the parathyroid 24-hydroxylase and whether an actual increase in local calcitriol occurs in the presence of a substrate. Calcitriol treatment however may postpone the development of sHPT in CKD not only by direct parathyroid signaling, but also by stimulating FGF23 secretion (127, 231, 232).

Paper III

The expression of the type I membrane-bound FGF23 receptor co-factor Klotho is markedly decreased or undetectable while inversely correlated to sCa in patients with primary hyperparathyroidism

As determined by real-time PCR and immunohistochemistry, Klotho mRNA and protein levels were markedly reduced (n=22) or undetectable (n=17) in pHPT adenomas as compared to normal control parathyroid tissues (30.4±4.2 (n=40) vs. 95.9±7.0 (n=4), respectively; mean±SEM; Figure 7).
The remaining 40th adenoma expressed Klotho mRNA and protein at the level of the 4 control tissues.

Figure 7. Left – Klotho mRNA levels in normal parathyroid tissue samples (n=4) and pHPT adenomas (17 out of 40 had undetectable levels (not shown)). Note the logarithmic scale. Right – Klotho protein levels were reduced accordingly in pHPT adenomas.

All patients with undetectable tumor Klotho mRNA levels had sCa above 2.95 mmol/L (11.8 mg/dL), ranging from 3.06–3.60 mmol/L (12.2–14.4 mg/dL; Figure 8). Patients with undetectable adenomal Klotho expression had higher serum Ca and PTH levels in comparison with patients with a Klotho-expressing tumor (Table 4; normal ranges 2.15–2.5 mmol/L (Ca) and 12–72 pg/mL (PTH)). No significant difference in adenoma weight was found between the two groups of tumors.

Table 4. Serum Ca and PTH levels in pHPT patients

<table>
<thead>
<tr>
<th>Klotho</th>
<th>[Ca(^{2+})] (mmol/L)</th>
<th>PTH (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>SEM</td>
<td>n</td>
</tr>
<tr>
<td>Absent</td>
<td>3.12</td>
<td>0.048</td>
</tr>
<tr>
<td>Present</td>
<td>2.69</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Moreover, previous reports showing that low extracellular [Ca\(^{2+}\)] stimulates Klotho-mediated PTH secretion (21) triggered us to explore the relation between Klotho mRNA expression and serum Ca or PTH levels in this cross-sectional study. We observed a strong inverse correlation between Klotho and sCa (r=-0.97), whereas no such was found with adenoma weight, or serum PTH (Figure 8) and P\(_i\) levels. A further analysis of the Klotho/sCa association in a multivariate regression model where PTH, P\(_i\) and adenoma weight were additional independent covariates, sCa remained the only variable independently associated with Klotho (β=-0.97).
Figure 8. A strong correlation was observed between Klotho expression and sCa levels (left), but not with serum PTH (right).

We further investigated whether the decreased Klotho expression could be due to the prevailing hypercalcemia. As expected, our in vitro experiments on normal bovine parathyroid cells showed a dose-dependent decrease in Klotho mRNA levels after Ca treatment. This is in concert with the observed association and confirms the possibility that a critical upper limit in s[Ca\textsuperscript{2+}] is required for complete suppression of parathyroid Klotho mRNA expression, as observed in some of our pHPT patients.

We also studied the expression of the proliferation marker PCNA in order to exclude the possibility that the downregulation of Klotho is due to proliferation of the adenoma cells. There was an equally high expression of PCNA in the cells regardless of their sCa background, indicating no relevant association between Klotho and cellular proliferation. The decrease in Klotho was similarly independent of the parathyroid FGFR1c, or VDR protein levels (typically suppressed in the proliferating adenoma cells (202, 233)), since none of these varied significantly with the sCa status as did Klotho.

The recent report on Klotho involvement in Ca\textsuperscript{2+}-dependent Na\textsuperscript{+}/K\textsuperscript{+} ATPase recruitment and subsequent PTH secretion at hypocalcemic settings (21), together with our current findings of Klotho-non-expressing, highly functional pHPT adenomas in hypercalcemic environment, suggests an existence of at least two separate mechanisms for PTH secretion. These seem to differ in Klotho dependence and calcemic background.

The diminished Klotho expression in pHPT could abrogate the protective effect of FGF23 on PTH secretion described in Paper II. For instance, mild elevations in serum FGF23 have been reported in pHPT patients and a pHPT mouse model, despite co-existing hypophosphatemia (137, 234). Also, the higher expression of PTH observed in the Klotho-non-expressing tumors could be explained by the inability of FGF23 to exert its inhibitory effects. Unfortunately, serum FGF23 levels of our patients remain undetermined due to lack of samples.

It remains uncertain to what extent the reduced parathyroid Klotho expression in pHPT is due to altered Ca metabolism, and whether the suppression is partly a product of intrinsic pathological changes in the affected
glands. In support of the latter, a potential role of the secreted Klotho in tu-
morigenesis is based on its involvement in the Wnt signaling pathway as a
decoy receptor and antagonist of several Wnt ligands (235). Aberrations in
the same pathway have been implicated in the pathogenesis of many tumors
and cancers, including pHPT adenomas (179, 181, 182, 236).

Paper IV

Parathyroid expression of mKlotho parallels the declining renal function in
hyperparathyroid patients with CKD, and is regulated in an isolated cell
system by FGF23, Ca and VDRAs, but not by Pi and PTH

In this study, we investigated the expression of Klotho in 88 hyperplastic
PTGs corresponding to 31 CKD patients. A large variation in Klotho mRNA
expression levels was found among the glands of some patients. Similarly,
diverse intraglandular distribution and intensity of the Klotho protein levels
was obvious in the immunohistochemically analyzed sections of frozen tis-
sues, which is typical for heterogeneous secondary hyperplasias. No relation
was evident between Klotho mRNA levels and glandular weight, nor did the
former differ due to the calcitriol treatment of the patients. A slightly higher
expression was measured in men as compared to women, although the dif-
ference did not reach the level of statistical significance.

Twenty-seven patients suffered from hypercalcemia (2.76±0.16 mmol/L;
cutoff >2.50) and fifteen from hyperphosphatemia (2.06±0.76 mmol/L; cut-
off >1.50). An inverse correlation was found between mean Klotho mRNA
and log serum Pi levels (r=-0.53; n=29). No associations existed between
mean Klotho mRNA and sCa (n=31) or log PTH (n=29) levels. The positive
correlation between mean Klotho mRNA levels and log GFR (r=0.42; n=31)
was supported by the significant decrease in mean Klotho levels across the
CKD stages (stage (2) n=4; (3) n=8; (4) n=11 and (5) n=8; Figure 9).

Figure 9. **Left** – Correlation between Klotho mRNA levels and log GFR; each gray
dot represents the only gland removed from a patient (n=4), whereas each black dot
is a mean Klotho value derived from 2–4 glands of a patient (n=27). **Right** – Mean
Klotho mRNA expression decreases across the CKD stages.
We further explored the effects of several biochemical parameters of relevance in uremic settings (Figure 10). Klotho regulation experiments performed on isolated bovine parathyroid cells demonstrated a dose-dependent increase in Klotho mRNA levels after 24h of treatment with EB1089, a calcitriol analogue. This was also observed with calcitriol. On the contrary, increasing concentrations of FGF23(R176Q) induced a dose-dependent decrease in Klotho transcript levels, similar to Ca in *Paper II*. Co-treatment with FGF23(R176Q) and EB1089 resulted in decreased Klotho expression in comparison with the control-treated cells (Figure 10). Addition of Ca induced an even further suppression of Klotho. Moreover, co-treatment of FGF23(R176Q) and Ca caused a more profound suppression of Klotho than FGF23(R176Q) treatment alone. However, this combination was not more effective than Ca treatment alone, indicating that Ca is the ultimate inhibitor of parathyroid Klotho expression at the indicated concentrations (all of which are supraphysiological). We observed no effect on Klotho transcript levels after treatment with Pi, or PTH (data not shown).

**Figure 10.** Co-treatment of bovine parathyroid cells with Ca, calcitriol analogue EB1089 and FGF23 for 24h.

Despite the large variation of Klotho expression among and within individual glands and the heterogeneity typical of secondary hyperplastic glands of sHPT, we found a positive correlation between mean Klotho expression levels and renal function. The finding that the mean parathyroid Klotho mRNA levels decline with progressing kidney disease was obvious even when the patients were divided according to CKD stages.

There are several possible explanations to our findings. The declining Klotho expression may reflect a glandular defect that is likely to become more pronounced with the increasing nodularity of the hyperplasia towards late CKD, similarly to the reduced expression of VDR and CaSR (202, 237, 238). The reduced responsiveness of hyperplastic glands to the suppressive effects of calcitriol and Ca could be analogous to a reduced sensitivity to the inhibitory effects of FGF23 due to decreased Klotho expression. One should not forget that the Ca/CaSR and the calcitriol/VDR pathways are the most
important mechanisms behind PTH inhibition in normal physiology, however the possibility exists that additional loss of FGF23/Klotho signaling might be of importance in pathology.

On the other hand, the observed decrease in parathyroid Klotho expression could rather be a consequence of several biochemical changes that occur in CKD. This is in accordance with our cell experiments showing a suppression of Klotho by FGF23 and Ca, and an increase by calcitriol analogue treatment. Unfortunately, we were unable to estimate the FGF23 levels and the calcitriol status of our cohort due to lack of serum, however strong evidence indicate that CKD patients suffer from rising FGF23 and falling calcitriol levels in parallel with the progressing kidney failure (133, 194, 197). In addition, a majority of our patients were hypercalcemic, many probably due to medical or renal replacement therapy. Our in vitro experiments support the possibility that calcitriol therapy might not suffice in keeping the Klotho expression at a sufficient level in a uremic milieu of high FGF23 and Ca levels, at least not without requiring detrimentally high doses. Our cell experiments did not support hyperphosphatemia having any effects on the parathyroid Klotho expression, yet we observed a negative in vivo correlation between the two. The reason for this discrepancy is unclear but serum Pi could merely be a marker of high FGF23 levels (197). Also, maintenance of the parathyroid tissue architecture may be required for Pi sensing and regulation of parathyroid gene expression (239, 240). The strong Klotho/Ca correlation observed in pHPT (Paper III) was not found in sHPT, indicating that the regulation of Klotho differs between the two disorders which involve different biochemical settings.

The co-occurrence of PTH hypersecretion and declining or undetectable parathyroid Klotho levels as in CKD or pHPT, respectively, indicates that PTH secretion can occur independently of Klotho and despite hypercalcemia. The recent report on the Klotho-dependent fine-tuning of the PTH secretion occurring only at low extracellular Ca levels, also showed the existence of a basal Klotho-independent and Ca-insensitive mechanism for PTH secretion (21). Again, this suggests that the apparent loss of the FGF23 inhibitory influence on PTH does not necessarily commence at late stages of CKD or when greater loss of Klotho has occurred, but could early be ancillary to a yet undefined, Klotho-independent mechanism. Hypothetically, this autonomous mechanism could partly be implicated in the reduced influence of CaSR and VDR signaling as well.

In summary, parathyroid Klotho expression declines with decreasing renal function partly due to the biochemical changes accompanying CKD development. Our data offer a novel plausible mechanism for the apparent failure of FGF23 inhibition of PTH hypersecretion in later CKD stages.
Concluding remarks

- FGF23 is abnormally degraded in a patient with HHS due to a novel homozygous missense point mutation (1584 G>A) in the gene GALNT3, whose encoded protein is responsible for proper glycosylation and processing of FGF23. The substitution translates into Histidine instead of an Arginine amino acid (R438H) and is predicted to have a negative impact on the structure of the enzyme, rather than a direct alteration of its functional domain necessary for glycosylation. It remains to be clarified why certain mutations in GALNT3 cause HHS and others HFTC, however, normal FGF23 activity is required for maintaining Pi homeostasis and bone health.

- FGF23 directly suppresses the PTH mRNA and protein levels in an *in vitro* model of normal bovine parathyroid cells.

- Klotho expression is markedly reduced or absent in adenomas of pHPT. The Klotho suppression could partly be a glandular alteration due to tumorigenesis however our findings suggest hypercalcemia to be at least a strong additive factor. We conclude that a Klotho-independent and Ca-insensitive mechanism of PTH release predominates in pHPT.

- Parathyroid Klotho expression declines in parallel with the renal function in CKD patients. An intrinsic defect of the secondary hyperplastic parathyroid glands cannot be excluded at this stage however Klotho expression levels may be a function of the altered mineral metabolism related to CKD including hypercalcemia, low serum calcitriol and high FGF23 levels. This parathyroid resistance to FGF23 is a possible explanation to the concurrently elevated serum FGF23 and PTH levels, and also to the inadequate protection of FGF23 against severe sHPT in late CKD.
General discussion and future perspectives

The FGF23/Klotho pathway has shown to be of impressively growing importance and interest in the field of bone and mineral research for the past few years. FGF23 is mainly produced by osteoblasts/osteocytes and secreted into the circulation to function in an endocrine manner with the help of its receptor co-factor Klotho. The inclusion of FGF23 in the complex network of endocrine regulation has provided new insights into the pathology of genetic and acquired disorders of P\textsubscript{i} and calcitriol metabolism. The findings in Paper I are in line with other reports on the importance of adequate FGF23 levels in bone health as demonstrated by patients with HHS, HFTC / ADHR, XLH, TIO, and FGF23 null/TG mice (95, 99, 100, 107, 112, 119-121, 172).

We and others before us have reported that Klotho is expressed in the parathyroid glands besides the kidney (152). Our current investigation directs revolutionizing spotlights on the corner of the endocrine axis schema that focuses on the interrelationship between two major phosphatonins, PTH and FGF23. We demonstrate that FGF23 exerts direct suppressive effects on the parathyroid hormone production and secretion, confirming the existence of a parathyroid-skeletal axis. Our findings in Paper II have been confirmed by a coincident in vivo study (241). We also found an FGF23-induced increase in the parathyroid mRNA expression of 1\alpha-hydroxylase, suggesting possible involvement of locally produced calcitriol and an additional mechanism for FGF23 to inhibit PTH synthesis. This hypothesis however needs to be rebutted.

On the contrary, the direct effects of PTH on skeletal FGF23 secretion are not as explored and would be highly interesting to pursue. The possibility remains that PTH may affect the FGF23 release, either by controlled endocrine/locally paracrine signaling or via mechanistic dissolving of bone. For instance, a two-way endocrine axis linking bone with PTGs is accordant with the facts that FGF23-producing osteoblasts express PTHr1, and that pHPT patients and pHPT model mice have elevated serum FGF23 (despite low P\textsubscript{i} levels) which decline after parathyroidectomy (despite a rise in serum P\textsubscript{i}) (137, 234). In addition, small increments in PTH levels have been reported in a number of CKD patients before a rise in FGF23 was detected (195), supporting the possibility that PTH and FGF23 have an interregulatory relationship and perhaps not only rise in order to prevent hyperphos-
phatemia. Opposing the revisited ‘trade-off’ theory, the time-dependent decline in the preoperatively augmented FGF23 levels after total parathyroidectomy is simultaneous with falling serum $P_i$ levels in sHPT patients (242) and suggests that FGF23 may be abnormally high due to both the hyperphosphatemia and the hyperactive PTGs in ESRD.

In Paper III, we describe a reduction or complete absence of parathyroid Klotho expression in adenomas of pHPT. Tumorigenic changes in the parathyroid cell metabolism or epigenetic regulation could in part be causative of this finding, but strong evidence support a parathyroid feedback mechanism involving sCa. These include: (i) direct negative effects of high Ca on parathyroid Klotho expression in vitro, (ii) all tumors with undetectable Klotho expression coming from a setting of sCa above an apparently critical upper limit of 2.95 mmol/L (11.8 mg/dL), and significantly higher serum PTH levels than those with remaining Klotho expression, and (iii) a strong inverse correlation between sCa and Klotho levels. Based on these data, high PTH levels secreted from adenomas would lead to hypercalcemia, which in turn may suppress the parathyroid Klotho expression in a dose-dependent fashion until complete absence, whereby the possibility of FGF23 to signal and intervene against the autonomous PTH hypersecretion is altered. The resistance of hyperplastic glands to FGF23 would be analogous to their resistance to serum Ca and calcitriol due to reduction of CaSR and VDR. The latter occurs in both pHPT and sHPT due to unclear reasons, and does not seem to involve mutations (204, 205).

Similar indications of FGF23 resistance appeared in sHPT with the observation that decreasing Klotho expression in the hyperplastic PTGs parallels the declining renal function of the patients (Paper IV). As for the decreased renal Klotho expression (243), it is not clear whether the suppression of parathyroid Klotho is secondary to the arising biochemical aberrations or to what extent an intrinsic defect of the tissue may be of relevance. Nevertheless, the rising circulatory FGF23 levels could partly be a surrogate marker for the declining Klotho levels. A reduction in both parathyroid and renal Klotho expression indicates that FGF23 signaling is deficient and could explain the additional increase in FGF23 that is observed in ESRD (196). The early rise in FGF23 secretion in developing CKD could merely be a physiological response to the increased renal $P_i$ retention, concomitantly aggravating the loss of calcitriol in the failing kidney and thereby contributing to the development of sHPT. In accordance with this are studies suggesting that the impact of FGF23 on 25(OH)D$_3$ activation is more severe than the loss of renal mass itself (194), and that PTH presence allows maximal phosphaturic effectiveness of FGF23 in the kidney (231). Hence, our findings do not oppose the revisited ‘trade-off’ theory. The inhibitory effects of FGF23 on

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6 The postponement of hyperphosphatemia by the elevated FGF23 levels in CKD occurs at the expense of low calcitriol levels and sHPT.
PTH could however be highly important in order to maintain the biochemical balance in late CKD, but may be undermined by the decreased Klotho expression.

The Klotho-independent and Ca-insensitive mechanism of PTH release, that seems to be a common denominator of pHPT and sHPT, indicates that regardless the cause of the Klotho suppression, the PTH secretion is more or less autonomous in both disorders. This suggests either that (i) Klotho is insignificant in an environment of such parathyroid autonomy, or that (ii) adequate Klotho expression is necessary for allowing a more fine-tuned and Ca-sensitive regulation of PTH (21), besides enabling FGF23 inhibition of the latter.

Finally, elevated FGF23 levels are associated with adverse outcomes such as faster CKD progression, future treatment-refractory sHPT, cardiovascular risk factors and increased mortality in hemodialysis patients (197, 207, 216, 230, 244-246). However, it remains unknown whether FGF23 is a uremic toxin that contributes to the high morbidity and mortality in CKD, or if it is rather beneficial in terms of protection against P$_i$-related toxicity and CVD. Our current belief is that FGF23 may be a protective agent in mild to moderate CKD, counteracting hyperphosphatemia and uncontrolled PTH secretion. Possible detrimental effects of the supraphysiological concentrations of FGF23 in ESRD however remain uncertain. In the mean time, since the serum FGF23 levels start rising early in the process of CKD, perhaps even before the PTH levels, FGF23 may serve as an early biomarker of P$_i$ retention, and allow identification of those individuals who are at the greatest need for early treatment.
Fibroblast tillväxtfaktor-23 och Klotho & deras roll i hyperparathyroidism och tillstånd av störda ben- och mineralmetabolism

Fibroblast tillväxtfaktor-23 (FGF23) är ett relativt nyupptäckt, benproducent, cirkulerande hormon som spelar en central roll i ben- och mineralmetabolismen. FGF23 hämmar återupptaget av inorganiskt fosfat (Pi) i njurarna, vilket leder till sänkta Pi-koncentrationer i serum. Vidare minskar FGF23 serumnivåerna av aktivt vitamin D₃ (1α,25(OH)₂D₃; kalcitriol) genom påverkan på nyckelenzymer i vitamin D₃-metabolismen. För att FGF23 ska kunna signalera i målcellen och utöva sin effekt krävs en FGF-receptor samt ytterligare en membranbunden ko-faktor, Klotho.

Ett flertal nedärvda sjukdomstillstånd med ökad FGF23-produktion karaktäriseras av låga serumnivåer av Pi och kalcitriol, samt hämmad benmineralisering (osteomalaci). Omvänt existerar ärftliga sjukdomar med låg FGF23-aktivitet, vilket istället leder till höga serumnivåer av Pi och kalcitriol, samt omfattande kalcifikationer i blodkärl och mjukdelar, eller lokal överproduktion av ben (hyperostos).

Patienter med kronisk njursvikt (CKD) är mycket sjuka p.g.a. störningar i deras mineralmetabolism. Serumnivåerna av Pi stiger beroende på en minskad förmåga att eliminera Pi via njurarna samtidigt som aktivering av vitamin D₃ minskar. Detta leder i sin tur till förhöjda serumnivåer av bisköldkörtelhormonet⁷ (PTH) och tillväxt av parathyroidea-körtlarna, så kallad sekundär hyperparathyroidism (sHPT). Sekundär HPT ökar risken för kardiovaskulära sjukdom och bidrar till försämrad kvalitet på skelettet. Vid utebliven effekt av medicinsk behandling måste därför i regel de hyperplastiska parathyroidea-körtlarna opereras bort. FGF23-nivåerna är i regel kompensatoriskt kraftigt ökade vid senare stadien av kronisk njursvikt (CKD) beroende på en manifest hyperfosfatemi. Höga serumnivåer av FGF23 är associerade till ett flertal kardiovaskulära riskfaktorer samt ökad mortalitet hos dialyspatienter.

Huvudmålsättningen med detta arbete har varit att studera eventuella direkta effekter av FGF23 på parathyroidea samt förstå kopplingen mellan FGF23 och Klotho och patofysiologiska mekanismer vid HPT. I ett vidare

⁷ Parathyroidea hormon, parathormon
perspektiv ville vi förstå FGF23´s roll i utvecklingen av sHPT i relation till CKD. Avhandlingen bygger på fyra delarbeten.

En tidigare gängse teori var att FGF23 orsakade sHPT. Detta baserades på två observationer. Den ena var att FGF23 och PTH ökar parallellt med att njurfunktionen avtar vid CKD, och den andra att FGF23 minskar produktionen av calcitriol, som används i behandlingen mot sHPT. Vi fann tvärtom att FGF23 hämmade både PTH-syntes och -sekretion, utan att påverka tillväxten hos de isolerade bovina parathyroidea-cellerna in vitro (Studie II). Dessa fynd styrkte att skelettet har en direkt hormonell påverkan på parathyroidea, och indikerade att FGF23-okningen i CKD skulle kunna vara en adaptiv respons, snarare än en direkt orsak, till sHPT. Vi fann även att FGF23-behandlingen ökade uttrycket av det vitamin D3-aktiverande enzymet 1α-hydroxylas i samma cellsystem. Spekulativt skulle detta kunna möjliggöra ökad lokal produktion av calcitriol i parathyroidea och därmed utgöra ytterligare en mekanism för att hämma sHPT.

I nästa steg undersökte vi uttrycket av FGF23´s receptor ko-faktor Klotho i hyperparathyroidea tillstånd. Det har tidigare visats att FGF23 inte kan signalera utan Klotho, d.v.s. ett förändrat Klotho-uttryck medför en förändrad effekt av FGF23 i den aktuella vävnaden. Vi fann att Klotho-uttrycket var ofintligt eller markant reducerat i parathyroidea-adenom hos patienter med primär HPT (pHPT) jämfört med kontrollvävnad, samt korrelerade starkt negativt med serumnivåerna av kalcium (Ca) (Studie III). Detta överensstämde med våra in vitro studier på bovina parathyroidea-celler där Ca dosberoende hämmade Klotho-uttrycket, samt med observationen att de patienter som hade odetekterbart Klotho hade högre serumnivåer av PTH och Ca jämfört med de som hade mätbart Klotho. FGF23´s inhibitionsmöjligheter på PTH-sekretionen vid pHPT är alltså begränsade eller ofintliga. Serum Ca verkar vara den mest avgörande faktorn för Klotho-nedregleringen i pHPT-adenomer.

Vidare studerade vi Klotho-uttrycket hos uremiska sHPT-patienter. Vi fann att Klotho-mRNA-nivåer korrelerade med den glomerulära filtrationshastigheten och avtog med sjunkande grad av njurfunktion (Studie IV). Det minskade Klotho-uttrycket i sHPT kan bero på på en bakomliggande körteldefekt, men våra in vitro data styrker snarare en direkt reglering av Klotho med ett flertal biokemiska parametrar. Ca och FGF23 hämmar Klotho-uttrycket in vitro medan calcitriol-analogen EB1089 stimulerar Klotho-uttrycket. En kombination av högt Ca och FGF23 men lågt calcitriol är ofta den rådande situationen i CKD-patienter med sHPT, vilket i samtliga fall skulle bidra till reducerade Klotho-nivåer och minskad hämmande effekt av FGF23 på sHPT.

Det bör påpekas att både pHPT- och sHPT-patienterna som undersöktes var hyperkalcemiska, vilket talar för att det existerar en Klotho-oberoende och Ca-okändlig mekanism bakom PTH-hypersekretionen i de båda tillständen som kan bidra till de okontrollerade PTH-nivåerna.

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Sammanfattningsvis har vi påvisat ett helt nytt endokrint samspel mellan skelettet och parathyroidea-körtlarna, och bevisat att FGF23 har en direkt negativ effekt på parathyroidea-körtlarnas hormonproduktion och -sekretion. Förändrat uttryck av Klotho och FGF23 förefaller vara viktiga patofysiologiska mekanismer vid både pHPT och CKD-relaterad sHPT. Vi bekräftar att ett normalt FGF23-uttryck krävs för att upprätthålla en adekvat mineralmetabolism och skelettfunktion.

8 ‘Märker’ färdiga proteiner med sockermolekyler
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References


29. Brown EM. Extracellular Ca\(^{2+}\) sensing, regulation of parathyroid cell function, and role of Ca\(^{2+}\) and other ions as extracellular (first) messengers. *Physiol Rev* 1991; **71**: 371-411.


32. Mosekilde L, Sogaard CH, Danielsen CC, et al. The anabolic effects of human parathyroid hormone (hPTH) on rat vertebral body mass are also reflected in the quality of bone, assessed by biomechanical testing: a comparison study between hPTH-(1-34) and hPTH-(1-84). *Endocrinology* 1991; **129**: 421-428.


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