Review

A feline-focused review of chronic kidney disease-mineral and bone disorders – Part 1: Physiology of calcium handling

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Abstract

Mineral derangements are a common consequence of chronic kidney disease (CKD). Despite the well-established role of phosphorus in the pathophysiology of CKD, the implications of calcium disturbances associated with CKD remain equivocal. Calcium plays an essential role in numerous physiological functions in the body and is a fundamental structural component of bone. An understanding of calcium metabolism is required to understand the potential adverse clinical implications and outcomes secondary to the (mal)adaptation of calcium-regulating hormones in CKD. The first part of this two-part review covers the physiology of calcium homeostasis (kidneys, intestines and bones) and details the intimate relationships between calcium-regulating hormones (parathyroid hormone, calcitriol, fibroblast growth factor 23, α-Klotho and calcitonin) and the role of the calcium-sensing receptor.

Keywords: α-Klotho; Calcium homeostasis; CKD-MBD; FGF23; Hormones

Introduction

The kidneys have a key role in the regulation of phosphate and calcium homeostasis. In chronic kidney disease (CKD), phosphate excretion decreases as a consequence of reduction in glomerular filtration rate (GFR) due to the declining number of functioning nephrons (Slatopolsky et al., 1968a, 1968b). Phosphate retention stimulates the secretion of phosphaturic hormones, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), both directly and indirectly via the inhibition of renal production of calcitriol (a.k.a. 1,25 dihydroxycholecalciferol or 1,25 dihydroxyvitamin D_3) (Liu et al., 2006a; Centeno et al., 2019). In early stage CKD, increased PTH and FGF23 production, as a "trade-off" mechanism, allows maintenance of plasma phosphate concentration within physiological limits by increasing the fractional excretion of phosphorus from the remaining nephrons (Gutierrez et al., 2005). Secondary renal hyperparathyroidism (SRHP) gradually develops as a consequence of phosphorus retention and decreased renal production of calcitriol (Slatopolsky et al., 1971; Szabo et al., 1989). These hormonal alterations, in response to phosphate retention, also have reciprocal influences on calcium regulation. Since calcium and phosphate are integral inorganic components of bone, disturbances in these minerals play a significant role in driving vascular and soft tissue calcification in CKD.

Chronic kidney disease-mineral and bone disorder (CKD-MBD) is a systemic disorder that encompasses a complex interplay between mineral and hormonal metabolism, leading to bone remodelling and extraskeletal calcification, occurring as a result of CKD. CKD-MBD represents a combination of three closely interrelated disease conditions which may be manifested by one or a combination of the following: (1) laboratory abnormalities indicative of disturbed mineral and bone metabolism, including calcium, phosphorus, FGF23, PTH, vitamin D, osteocalcin, runt-related transcription factor 2 (Runx2), and alpha-1 type 1 collagen

concentrations and alkaline phosphatase (ALP) activity; (2) calcification of the vasculature and other soft tissues; (3) abnormalities in skeletal morphology, representing the presence of renal osteodystrophy (Moe et al., 2006). Ever since the introduction of the concept of CKD-MBD in human medicine, its definition and classification have advanced the development of evidence-based clinical practice guidelines and provided a new framework for diagnostic and therapeutic approaches for the management of CKD-MBD. This has also become an increasingly recognised phenomenon in veterinary medicine (Geddes et al., 2013).

This article, the first part of the two-part review of CKD-MBD with a focus on the cat, describes the physiology of calcium homeostasis focusing on the hormonal regulation. Since basic science research is has been more thoroughly explored in humans and rodent studies, information on calcium physiology presented in this article is based on our current knowledge from these species, with specific reference to feline data where they are available (clearly indicated where that is the case). Although it is anticipated that there will be a degree of homology in many of the physiological pathways pertaining to calcium homeostasis amongst mammals particularly at the level of hormonal regulation, inter-species differences exist and extrapolation from one species to another cannot be relied upon. A comprehensive understanding of these pathways can consequentially enable understanding of the pathophysiological mechanisms associated with calcium disorders in feline CKD-MBD, discussed in detail in part two.

Calcium homeostasis

Calcium is a fundamental structural component of bone and is involved in crucial physiological functions, such as cellular signalling, muscle contraction, blood coagulation and neuronal function (Reid et al., 2016). In adult humans, the majority of calcium (99%) is stored

in bone as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), with only 1% present in extracellular fluid (including plasma) and intracellular fluid (Zhou et al., 2013). Plasma total calcium concentrations vary slightly across different species: human, 2.15–2.57 mmol/L (8.6–10.3 mg/dL) (Eknoyan et al., 2003); cat, 2.05–2.95 mmol/ (8.2–11.8 mg/dL) (van den Broek et al., 2017); dog, 2.25–3.00 mmol/L (9–12 mg/dL) (Schenck and Chew, 2005). In plasma, calcium is distributed in three fractions with small variations between species: free ionised (52–56%), protein-bound (34–40%), complexed (8–10%) (Goldstein, 1990; Schenck et al., 1996). This distribution is subject to variation depending on physiological conditions such as acid-base status, plasma protein concentrations, and anion concentrations. In the presence of acidaemia, protein-bound calcium decreases as calcium and hydrogen ions compete for the negatively-charged protein binding sites; concentrations of ionised and complex calcium may therefore be increased (Toffaletti and Abrams, 1989). Approximately 80% of protein-bound calcium is associated with plasma albumin, the remainder being bound to various globulins (Moore, 1971). The remaining calcium is complexed with anions, including bicarbonate, phosphate, lactate and citrate (Takano et al., 2012). Plasma ionised calcium is the biologically active form and subject to rigorous homeostatic control to maintain concentrations in a narrow range; this will be discussed in detail below.

Calcium homeostasis is achieved by a complex interplay between four organ systems: kidney, gastrointestinal tract, parathyroid gland, and bone (Fig. 1). Table 1 provides a summary of the hormonal regulation of calcium.

Renal calcium handling

In the kidney, only ionised and complexed calcium fractions are freely filtered and able to reach the lumen of the renal tubules. In rodents, under normal conditions 98% of filtered

calcium undergoes tubular reabsorption (Peacock and Nordin, 1968), with proximal convoluted tubules (PCTs) absorbing 60–70%, thick ascending limbs (TAL) of Henle's loops absorbing 20–25% and distal convoluted tubules (DCT) 5–10% (Lassiter et al., 1963).

Renal reabsorption of calcium involves two major pathways: paracellular and transcellular (Fig. 2). Movement of calcium through tight junctions between epithelial cells is defined as the paracellular pathway, while transcellular absorption, comprising calcium transport through tubular epithelial cells, is more complicated. At the level of the PCT and TAL calcium movement is primarily paracellular, driven by passive diffusion or solvent drag down an electrochemical gradient. At the level of the DCT calcium movement is primarily transcellular, with passive entry across the apical membrane, intracellular translocation mediated by calcium-binding proteins and buffers, and active extrusion across the basolateral membrane. It is this DCT transcellular pathway that is crucial to the fine-regulation of renal calcium reabsorption, regulated by PTH, calcitonin, calcitriol, the FGF23-Klotho endocrine axis, and extracellular calcium (via the calcium-sensing receptor; CaSR) (Sherwood, 1968; Shimizu et al., 1990; Hoenderop et al., 2001).

Gastrointestinal calcium handling

Similar to renal reabsorption of calcium, gastrointestinal absorption occurs across the intestinal mucosa either by a passive, paracellular process or an active, hormonal-dependent, transcellular process (Bronner et al., 1986). In healthy humans, approximately 35% of dietary calcium is absorbed, primarily within the small intestine; however, this is dependent upon intestinal transit time and calcium solubility, which itself is influenced by the presence of various anions (e.g. dietary phosphate) and pH (Shiga et al., 1987; Duflos et al., 1995). Calcium solubility decreases as pH increases, which is seen as chyme moves from the duodenum (pH

6.0) to the distal ileum (pH 7.5) (Duflos et al., 1995). The transcellular pathway accounts for most intestinal calcium absorption when dietary intake is low. This process predominates in the duodenum, where vitamin D receptors (VDR) are highly expressed under the influence of calcitriol (Van Cromphaut et al., 2001; Xue and Fleet, 2009). In contrast, passive paracellular calcium diffusion down a chemical gradient occurs throughout the intestinal tract. This process is in proportion to the transit time of the chyme in each intestinal segment and has been show to occur predominantly in the ileum in rats and humans during conditions of normal dietary calcium intake (Marcus and Lengemann, 1962; Pansu et al., 1993). Tight junction proteins, claudins-2, -12 and -15, mediate intestinal paracellular calcium transport (Fujita et al., 2008). Both paracellular and transcellular transport occur in the caecum and ascending colon, which accounts approximately 10% of dietary calcium uptake (Petith and Schedl, 1976; Barger-Lux et al., 1989). Intriguingly, recent studies demonstrated a linear relationship between dietary calcium intake and faecal calcium excretion in adult cats and dogs (Mack et al., 2015; Paßlack et al., 2016), suggesting a lack of adaptability in intestinal calcium absorption following acute alterations in dietary intake at least. This is in contrast to the situation in humans and rodents, where a non-linear relationship between dietary calcium intake and faecal calcium excretion is seen (Cashman and Flynn, 1996; Brown et al., 2005). This may reflect a comparatively minor role of diet-dependent adaptation in calcium digestibility in contributing to the maintenance of calcium homeostasis in cats.

Bone calcium handling

Calcium and phosphorus are integral components of bone. Bone is a dynamic tissue continually remodelling in adults to maintain skeletal integrity through the opposing activities of osteoblasts (forming new bone) and osteoclasts (resorbing bone), and under the tight control

of calcium-regulating hormones, including PTH, calcitriol, FGF23 and calcitonin (Holtrop et al., 1981; Chambers and Moore, 1983; Wang et al., 2008; Ben-awadh et al., 2014).

Regulation of plasma calcium concentration

Parathyroid hormone (PTH)

Parathyroid hormone is an 84-amino-acid peptide, synthesised and released by parathyroid chief cells in response to ionised hypocalcaemia (Habener et al., 1971; D'Amour et al., 1986). The PTH sequence is conserved among mammalian species, with >83% homology between feline and human PTH (Toribio et al., 2002). Both whole (amino acids 1 to 84) and fragmented (amino acids 7 to 84) PTH molecules circulate in the body (Bringhurst et al., 1988). It is imperative to differentiate these because whole PTH is the biologically active form, whereas fragmented PTH may partially antagonise the classic biological activities of PTH (Nguyen-Yamamoto et al., 2001). Accumulation of PTH fragments, due to reduced clearance, can also occur in kidney disease (Brossard et al., 2000). PTH increases plasma calcium concentration by stimulating calcium reabsorption in the TAL of the loop of Henle and the DCT (Gesek and Friedman, 1992) and activating calcitriol production, which enhances intestinal calcium absorption. Increased PTH concentrations typically occur when the GFR drops below 60 mL/min/1.73m^2 in humans (Levin et al., 2007). PTH secretion is induced by phosphate retention, decreased calcitriol synthesis, and hypocalcaemia resulting from reduced renal function (Yamamoto et al., 1989; Slatopolsky et al., 1996; Martinez et al., 1997). PTH secretion is suppressed by FGF23 and, potentially, α-Klotho (Ben-Dov et al., 2007; Krajisnik et al., 2007; Krajisnik et al., 2010; Fan et al., 2018).

PTH stimulates transcellular calcium reabsorption in the DCT by activation of transient receptor potential vanilloid subtype 5 and 6 (TRPV5 and TRPV6) via protein-kinase A-

mediated phosphorylation, the rate-limiting step in calcium entry as established in rodent models (Groot et al., 2009). PTH also promotes paracellular calcium transport in the TAL by suppressing claudin-14 expression (Sato et al., 2017), a tight-junction protein that acts as calcium barrier between renal tubular epithelial cells (Gong et al., 2012).

PTH enhances intestinal calcium absorption indirectly via upregulation of calcitriol production by stimulating and suppressing renal activities of 1α-hydroxylase and 24-hydroxylase, respectively (Zierold et al., 2003). Direct actions of PTH on intestinal calcium absorption have previously been postulated (Nemere and Norman, 1986), and supported by the localisation of PTH receptor (PTHR1) to the basolateral membranes of intestinal epithelial cells in rats (Gentili et al., 2003). However, the exact mechanism of activity of PTH directly on the intestines and physiological significance remain unclear as does whether species differences are present.

PTH is central to movement of calcium into and out of bone, as well as bone remodelling, in the normal animal. PTH promotes bone resorption, leading to bone calcium efflux, via the indirect activation of osteoclast activity through increased expression of receptor activator of nuclear factor kappa-B ligand (RANKL) on osteoblasts and osteocytes (McSheehy and Chambers, 1986; Ben-awadh et al., 2014). Persistent hyperparathyroidism increases bone calcium efflux and may cause several catabolic alterations in cortical bones, such as reduced bone mineral density and increased cortical porosity, resulting in decreased bone quality and increased fracture susceptibility (Parisien et al., 1990). In contrast, uraemia-induced over-suppression of PTH and skeletal PTH resistance leads to the development of adynamic bone disease (Torres et al., 1995; Ballanti et al., 2001). Deterioration of bone quality, with increased bone resorption, is reported in cats with advanced stages of CKD (Shipov et al., 2014).

However, despite similarities in bone architecture and the pathophysiology of CKD-MBD between cats and humans (Hillier and Bell, 2007), in contrast to human patients, clinically apparent fractures are rare in cats with CKD, even at advanced stages. We speculate that the comparatively shorter lifespan, lower physiological loading (i.e. due to lower body mass), and distinct biomechanical characteristics of quadrupedism in cats contribute to this reduction in fracture risk associated with CKD. Current knowledge on the influence of CKD on bone remodelling in cats is limited and requires further investigation.

Calcitriol

The concentration of calcitriol, the biologically active form of vitamin D, is regulated by the relative expression of 1α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1). These two enzymes are expressed predominantly in the kidney and work reciprocally on vitamin D metabolism; 1α-hydroxylase mediates the production of calcitriol, while 24-hydroxylase accelerates the degradation of calcitriol, as well as modifying calcidiol (a.k.a. 25-hydroxycholecalciferol or 25-hydroxyvitamin D_3) to 24,25-dihydroxycholecalciferol (a.k.a. 24,25-dihydroxyvitamin D_3) (Masuda et al., 2006; Urushino et al., 2009; Annalora et al., 2010). 24,25-dihydroxycholecalciferol is relatively inactive and this conversion reduces the pool of calcidiol available for 1α-hydroxylation (Shinki et al., 1992). 1α-hydroxylase activity and expression is enhanced by PTH, calcitonin, and low dietary or extracellular calcium and phosphate, increasing calcitriol production (Ash, 1976; Murayama et al., 1999; Bland et al., 2001). In contrast, FGF23 appears to enhance 24-hydroxylase expression and supresses 1α-hydroxylase activity, reducing calcitriol production (Shimada et al., 2004b, 2004a).

Calcitriol is a fundamental regulator of intestinal calcium absorption (Fig. 3). It promotes various steps in the transcellular transport of calcium across the intestinal epithelium

including: expression of calcium-selective channel proteins TRPV5 and TRPV6 on the apical membrane, which modulate calcium entry into the cell; synthesis of cytosolic calcium-binding protein calbindin-D_{9k}, facilitating intracellular calcium translocation (Bronner et al., 1986); and expression and activity of calcium transporters plasma membrane calcium ATPase (PMCA1b) and sodium-calcium exchanger (NCX1), which are responsible for calcium extrusion across the basolateral membrane (Ghijsen et al., 1983; Freeman et al., 1995). It is important to note that cats have distinctively different vitamin D metabolism compared to humans and rodents. Vitamin D synthesis in cats depends exclusively on dietary intake as photosynthesis is inhibited as demonstrated by the very low 7-dehydrocholesterol (7-DHC) concentrations in the skin (Morris, 1999). In cats, studies have also highlighted potential species-related differences in the response of vitamin D to varying dietary calcium intake and identified a novel circulating vitamin D metabolite, C-3 epimer of calcidiol, in healthy adults (Paßlack et al., 2016; Sprinkle et al., 2018). Further work is required to fully elucidate the significance of C-3 epimer of calcidiol in feline calcium homeostasis.

The majority of calcitriol's biological effects are mediated through its binding to the VDR, with downstream modulation of transcription of vitamin D-related genes such as *PTH* (Jin et al., 1996; Liu et al., 1996). However, in vitro rodent studies have also revealed that calcitriol has transcription-independent (non-genomic) effects by binding to caveolae-associated VDR via the activation of second messenger pathways (Wali et al., 1990). This stimulates exocytosis of secretory vesicles containing calcium channels, increasing their expression on the plasma membrane, and opening of expressed calcium channels, resulting in rapid calcium absorption (Zanello and Norman, 2004). Furthermore, calcitriol enhances paracellular calcium absorption by increasing expression of epithelial tight junction proteins claudin-2, -12 and -15, that form calcium-permeable channels that function through both

passive diffusion and solvent drag mechanisms (Fig. 3) (Fujita et al., 2008; Chatterjee et al., 2019).

Similar to PTH, calcitriol stimulates transcellular reabsorption of calcium in the DCT. This is achieved via upregulated expression of calcium channels (TRPV5 and TRPV6) on the apical surface (Hoenderop et al., 2001), cytosolic proteins calbindin-D_{28k} and -D_{9k}, and PMCA1b and NCX1 on the basolateral surface (Hoenderop et al., 2002).

The actions of calcitriol on bone are poorly understood. In rodent models, it appears to stimulate both formation and resorption of bone, dependent upon the relative concentrations of calcium and calcitriol, the chronicity of these relative concentrations, and the differences in RANKL/osteoprotegerin ratio (Holtrop et al., 1981; Simonet et al., 1997; Li et al., 2000; Harada et al., 2012; Nakamichi et al., 2017). Calcitriol is essential for osteoblast differentiation and promotes bone formation by the calcification of osteoid tissue. Long-term treatment of mice with near-physiological doses of vitamin D (50 ng/kg/day eldecalcitol) increased bone mineral density by suppressing bone resorption (Harada et al., 2012; Nakamichi et al., 2017). In contrast, bolus administration of supra-physiological doses of calcitriol (1.5 μg/kg/day) for 2–4 days enhanced osteoclastic bone resorption through upregulation of RANKL by osteoblast lineage cells (Sato et al., 2007). In addition, during calcium depletion states, calcitriol synergises with high concentrations of PTH to induce calcium mobilisation from bone by stimulating osteoclastogenesis and bone resorption, as well as inhibiting bone matrix mineralisation to prevent calcium incorporation into bone (Lieben et al., 2012).

FGF23-α-Klotho endocrine axis

FGF23 is primarily produced by osteocytes and osteoblasts. Its production is stimulated by calcitriol and PTH, and potentially regulated by chronic dietary phosphate loading, albeit the phosphate-sensing mechanism by which this occurs is still unclear (Bai et al., 2004; Saito et al., 2005; Trautvetter et al., 2016). Increasing evidence from humans and rodent models suggests FGF23 production is also directly stimulated by increased dietary or serum calcium concentrations (Shimada et al., 2005; Rodriguez-ortiz et al., 2012; Di Giuseppe et al., 2015), and potentially by α-Klotho (Smith et al., 2012; Xiao et al., 2019). FGF23 is a potent phosphaturic hormone (Bai et al., 2004), acting via decreased expression of renal sodium phosphate cotransporters (NaPi-2a/-2c) (Shimada et al., 2004c, 2004a). It also exerts powerful regulatory effects on calcium homeostasis in a Klotho-dependent manner (Alexander et al., 2009) which is discussed further below.

In general, FGF23 exerts its biological functions through interacting with the α-Klotho-FGF receptor (FGFR) complex, while membrane-bound α-Klotho protein acts as a co-factor to enhance ligand-receptor affinity (Goetz et al., 2007). α-Klotho binding converts FGFR1(IIIc), a canonical receptor for various fibroblast growth factors, to a receptor with strong affinity for FGF23 specifically (Urakawa et al., 2006). The C-terminal region of FGF23 mediates binding to the FGFR-Klotho complex, which, in turn, induces FGFR dimerisation and initiates the MAPK cascade, including downstream activation of extracellular signal-regulated kinases 1/2 (ERK1/2) and serine/glucocorticoid-regulated kinase-1 (SGK-1) (Kouhara et al., 1997; Andrukhova et al., 2012). In the kidney, SGK-1 subsequently activates with no lysine kinase 4 (WNK4), which is critically involved in the regulation of TRPV5 trafficking from the Golgi apparatus to the DCT apical plasma membrane, and ultimately calcium reabsorption (Fig. 4) (Andrukhova et al., 2014). It is also suggested that activation of WNK4, as a result of FGF23 signalling, could influence plasma membrane expression of other ion transporters in the DCT,

including sodium-chloride co-transporter (NCC) and renal outer medullary potassium channel (ROMK1) (Ring et al., 2007; Andrukhova et al., 2014).

FGF23 decreases intestinal absorption of calcium, both directly, via an unknown mechanism, and indirectly by suppressing calcitriol production by the kidney via simultaneous downregulation of 1α-hydroxylase and upregulation of 24-hydroxylase (Shimada et al., 2004b, 2004a; Gutierrez et al., 2005; Khuituan et al., 2012). Using mouse models of hypoparathyroidism and hypophosphataemic rickets, studies have shown that calcitriol is a potent stimulator of FGF23 synthesis in bone (Liu et al., 2006b), thereby maintaining a negative feedback loop. Apart from its fundamental role in acting as a FGFR co-factor promoting trafficking of TRPV5, secreted α-Klotho is involved in TRPV5 stabilisation and maintenance of renal calcium permeability and reuptake in an FGF23-independent manner (Chang et al., 2005; Cha et al., 2008). This is mediated via the removal of the sialic acid moiety from N-glycan of TRPV5, exposing the underlying disaccharide N-acetyl-lactosamine that then binds to galectin-1 at the extracellular surface, enhancing retention of TRPV5 at this position (Cha et al., 2008). In α-Klotho knock-out mice renal calcium reabsorption is impaired (Alexander et al., 2009).

α-Klotho is also important in intestinal calcium absorption. Similar to its effects on renal TRPV5, α-Klotho enhances intestinal calcium absorption by increasing the activity of TRPV6. (Lu et al., 2008). Similar to FGF23, α-Klotho inhibits calcitriol synthesis by suppressing 1α-hydroxylase activity (Woudenberg-Vrenken et al., 2012). Absence of α-Klotho in mice results in increased serum calcitriol concentrations through upregulation in 1α-hydroxylase, together with increased TRPV6 and calbindin-D_{9K} mRNA expression, and increased serum calcium concentrations (Yoshida et al., 2002; Tsujikawa et al., 2003;

Alexander et al., 2009). In mice lacking both α-Klotho and 1α-hydroxylase duodenal TRPV6 and calbindin-D_{9K} mRNA expression and serum calcium concentration were significantly reduced, suggesting calcitriol is responsible for the phenotype (hypercalcaemia, hyperphosphataemia, soft tissue calcification and bone abnormalities) observed in α-Klotho-knockout mice and that the effects of α-Klotho on calcium homeostasis is highly dependent on calcitriol (Woudenberg-Vrenken et al., 2012).

Similar to the effects of FGF23, in mouse and human renal cells mRNA expression of both membrane-bound and secreted isoforms of α-Klotho was stimulated by calcitriol (Forster et al., 2011). Whilst in cow parathyroid and mouse kidney cells, α-Klotho mRNA expression was downregulated by FGF23 in a concentration-dependent manner (Marsell et al., 2008; Krajisnik et al., 2010); suggesting a counter-regulatory mechanism to attenuate the physiological and pathological activities of FGF23. Cow parathyroid α-Klotho mRNA expression was downregulated in response to increasing calcium concentrations in the culture medium, supporting the observation of an inverse relationship between α-Klotho mRNA expression and serum calcium concentrations in human patients with primary hyperparathyroidism (Björklund et al., 2008). Although it is apparent from the literature that α-Klotho is critically involved in calcium homeostasis, the way in which α-Klotho secretion is regulated and integrated into the calcium homeostatic system remains to be delineated.

Increasing evidence has shown that FGF23 may play a role in regulating bone mineralisation in osteocytes in a paracrine/autocrine manner (Sitara et al., 2008; Lu and Feng, 2011). Pyrophosphate is a key inhibitor of the mineralisation process; FGF23 was shown to increase pyrophosphate concentrations via the suppression of tissue nonspecific alkaline phosphate (TNAP) activity in an α-Klotho-independent manner, leading to the development of

mineralisation defect in mice (Murali et al., 2016a). α-Klotho-knockout mice display osteopenia and osteoporosis, a skeletal disease prevented by α-Klotho overexpression (Kuro-o et al., 1997; Xiao et al., 2019). α-Klotho is expressed in osteocytes, albeit at approximately 500 times lower than in the kidneys (Rhee et al., 2011). Osteocytic α-Klotho has a negative influence on bone formation, potentially in cooperation with FGF23 (Smith et al., 2012; Komaba et al., 2017). Recently, it is suggested that the low-turnover osteoporotic phenotype seen in α-Klotho-knockout mice was not a direct result of α-Klotho deficiency in osteocytes, but a consequence of overproduction of calcitriol and inhibition of PTH secondary to the disrupted mineral metabolism (Murali et al., 2016b; Komaba et al., 2017). Additional investigations are required to better elucidate the functional role of α-Klotho on bone metabolism.

Calcitonin

Calcitonin is a 32 amino acid peptide hormone, secreted from the parafollicular cells of the thyroid gland in response to increased blood calcium concentration (Potts, 1992). Calcitonin antagonises PTH and protects against development of acute hypercalcaemia (Rodriguez et al., 1991). Its primary effect is to inhibit osteoclastic bone resorption, thereby preventing calcium efflux from bone (Chambers and Moore, 1983). A reciprocal relationship exists between calcitonin and calcitriol; calcitonin stimulates calcitriol synthesis, which enhances intestinal calcium absorption, while calcitriol suppresses calcitonin secretion (Kawashima et al., 1981; Jaeger et al., 1986).

In contrast, calcitonin was shown to stimulate renal calcium reabsorption in rodents, via an unknown mechanism, independent of TRPV5/6 (Carney and Thompson, 1981; Elalouf et al., 1983; Hsu et al., 2010). However, with discordant published findings, particularly in

non-rodent species, calcitonin's hypocalciuric effect remains controversial (Clark and Kenny, 1969; Cochran et al., 1970; Quamme, 1981; Shimizu et al., 1990). There is also no consensus as to the physiological significance of calcitonin in adult mammals. This may be attributed, in part, to species variation (Marx and Aurbach, 1975). Indeed, various veterinary literature suggests that calcitonin plays a relatively minor role in calcium homeostasis in adult cats (Pineda et al., 2013; van den Broek et al., 2018).

Calcium-sensing receptor (CaSR)

The CaSR is a Class C G protein-coupled receptor that has ionised calcium as its primary ligand (Brown et al., 1993). CaSR are ubiquitously expressed in multiple organs, but are most abundant in parathyroid glands and kidneys (Kantham et al., 2009; Gal et al., 2010). The CaSR appears to play a vital role in maintaining serum calcium at physiological concentrations. In the thyroid gland, activation of CaSR, in response to increased extracellular ionised calcium concentration, suppresses PTH synthesis and secretion, whilst simultaneously stimulating calcitonin secretion (Garrett et al., 1995; Motoyama and Friedman, 2002). In rodent models at least, CaSR activation potentiates the inhibitory effects of calcitriol on PTH mRNA expression by upregulating VDR expression (Garfia et al., 2002).

In the kidney, CaSR activation leads to reduced renal calcium reabsorption, in both a PTH-dependent and independent manner (Motoyama and Friedman, 2002; Kantham et al., 2009; Loupy et al., 2012). CaSR expression is upregulated by calcitriol whilst CaSR activation increases VDR expression; hence further potentiate the action of calcitriol, suggesting a synergistic relationship between CaSR and VDR (Maiti and Beckman, 2007). There is a widespread distribution of CaSR along the nephron, with the highest expression within the TAL of human, mouse and rat (Graca et al., 2016). However, the localisation and cellular

polarisation of the CaSR varies in a similar way between different nephron segments in the mouse, rat, and human (Graca et al., 2016). In the PCT and collecting duct the CaSR is expressed predominantly on the apical surface, whereas in the TAL the CaSR is only expressed on the basolateral membrane. In the DCT, the CaSR is expressed on both apical and basolateral membranes, allowing the detection of calcium in both urine and the interstitial space (Sands et al., 1997; Riccardi et al., 1998). Activation of PCT CaSR dampens the phosphaturic actions of PTH and the responses to calcitriol (Egbuna et al., 2009). In vitro, 1α-hydroxylase is also shown to be downregulated in the presence of high calcium, suggesting a direct effect of calcium on calcitriol production (Bland et al., 1999). However, renal PCT CaSR expression is suppressed following acute PTH infusion and dietary phosphate loading (Riccardi et al., 2000) and upregulated by calcitriol (Canaff and Hendy, 2002), suggesting an independent, local negative feedback loop for phosphate regulation and calcium movement in this nephron segment. In the TAL, CaSR activation by hypercalcaemia disrupts the process of generating a lumen-positive transepithelial potential difference by inhibiting the activities of ROMK2 and sodium-potassium-chloride cotransporter (NKCC2) (Wang et al., 1996), which abrogates calcium paracellular transport and reduces the rate of calcium reabsorption (Vargas-Poussou et al., 2002). In the human DCT, CaSR and TRPV5 co-localise on the apical membrane and in intracellular vesicles (Topala et al., 2009); an increase in urinary calcium activates luminal CaSRs and enhances TRPV5 activity, resulting in an increase in apical entry of calcium (Topala et al., 2009). In vitro, PMCA1b activity, which mediates basolateral efflux of calcium in the DCT, is inhibited upon activation of the basolateral CaSR, limiting the transcellular reabsorption of calcium (Blankenship et al., 2001). Instead of regulating calcium reabsorption, the primary role of apical CaSR in the collecting duct is in the modification of urinary acidification and water reabsorption by promoting proton-transporting ATPase (H^+-ATPase) activity and inhibiting aquaporin 2 expression, respectively (Sands et al., 1997; Bustamante et

al., 2008); it has been suggested that these mechanisms may have evolved to protect against nephrolithiasis (Riccardi et al., 1996; Renkema et al., 2009).

CaSRs are expressed widely in the epithelial cells throughout the gastrointestinal tract in rats (Gama et al., 1997; Chattopadhyay et al., 1998; Cheng et al., 2002), humans (Gama et al., 1997; Rutten et al., 1999; Sheinin et al., 2000), rabbits (Butters et al., 1997) and chickens (Hui et al., 2021). In addition to its essential role in modulating calcium transport across the enterocytes (Chattopadhyay et al., 1998; Lee et al., 2019), the CaSR is fundamental in modulating normal gut physiology, including gastric acid secretion, neuronal responses, epithelial transportation, intestinal barrier function and immune responses (Oda et al., 2000; Geibel et al., 2001; Cheng et al., 2004; Dufner et al., 2005; MacLeod et al., 2007; Kelly et al., 2011). Activation of gastric CaSRs stimulates acid production which promotes the dissolution of dietary calcium; this enhanced solubility of calcium in the acidic aqueous phase facilitates it intestinal absorption (Geibel et al., 2001). Furthermore, the increase in acid secretion promotes protein digestion and the release of L-amino acids that could act as CaSR agonists to synergistically activate the CaSR alongside calcium (Conigrave et al., 2002). By contrast, in an ex vivo study using small intestinal-like Caco-2 cells, activation of apical and basolateral CaSR increased local production of FGF23 by enterocytes, resulting in suppression of calcitriol-induced intestinal calcium transport, possibly preventing excess calcium absorption (Rodrat et al., 2018). Direct apical CaSR activation has been suggested to enhance colonic calcium absorption in caecectomised rats, potentially via the transcellular pathway involving TRPV6 and calbindin-D_{9k} (Jongwattanapisan et al., 2012). In contrast, in a mouse study basolateral CaSR activation attenuates transcellular intestinal calcium transport by modulating TRPV6 function (Lee et al., 2019). Therefore, it is plausible that the opposing effects of CaSR on local calcium absorption may depend on the polarisation of CaSR; further investigations are

required to better elucidate the mechanism underlying the regulation of intestinal calcium absorption by CaSR.

CaSRs are also expressed in osteoblasts and osteoclasts, where they sense calcium changes in local bone microenvironment and act as critical regulators for bone remodelling (Kameda et al., 1998; Kanatani et al., 1999; Yamaguchi et al., 2001). In vitro, stimulation of the CaSR in osteoblastic cells promotes chemotaxis, proliferation and differentiation of osteoblasts, augments bone matrix mineralisation and reciprocally reduces expression of RANKL and enhances expression of osteoprotegerin, to prevent osteoclastogenesis (Kanatani et al., 1999; Brennan et al., 2009; Takaoka et al., 2010). Additionally, exposure to high extracellular calcium at the resorptive site activates osteoclasts CaSR, resulting in the downregulation of osteoclast activity, inhibits formation of osteoclasts and reduces bone resorption (Datta et al., 1989; Moonga et al., 1990; Mentaverri et al., 2006).

Conclusions

Calcium homeostasis is a rigorous process that is dependent on four major organ systems (kidney, gastrointestinal tract, parathyroid gland and bone) with a complex interplay between various hormone regulators (PTH, calcitriol, FGF23, α-Klotho and calcitonin). Understanding the physiology of calcium homeostasis is a prerequisite for defining the pathophysiology of calcium and hormonal dysregulation in CKD-MBD.

Conflict of interest statement

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Zierold, C., Mings, J.A., Deluca, H.F., 2003. Regulation of 25-hydroxyvitamin D3-24-hydroxylase mRNA by 1,25-dihydroxyvitamin D3 and parathyroid hormone. Journal of Cellular Biochemistry 88, 234–237.

1354 **Table 1.** A summary of the effects of hormones and factors involved in calcium homeostasis.

Hormones/ factors	Normal actions on kidney	Normal actions on intestine	Normal actions on bone	Effects on other hormones/ factors	Overall change seen with CKD	References
PTH	↑ calcium reabsorption (via upregulation of TRPV5 and calbindin-D_{28k} and indirectly via ↑ calcitriol)	↑ calcium absorption (indirectly via ↑ calcitriol)	↑ bone formation and bone resorption; actions depend on the chronicity of exposure (continuous hyperparathyroidism inhibits osteoblast differentiation and leads to a fall in bone mass)	↑ calcitriol ↑ calcitonin ↑ FGF23 ↓ CaSR	↑	McSheehy and Chambers, 1986; Nemere and Norman, 1986; Parisien et al., 1990; Gesek and Friedman, 1992; Torres et al., 1995; Kifor et al., 1996; Zierold et al., 2003; Levin et al., 2007; Groot et al., 2009; Lavi-Moshayoff et al., 2010; Ben-awadh et al., 2014
Calcitriol	↑ calcium reabsorption (via upregulation of TRPV5 and calbindin-D_{28k})	↑ calcium absorption (via upregulation of TRPV6, calbindin-D_{9k}, PMCA1b and NCX1; and increased expressions of Claudin-2, -12 and -15)	↑ bone formation ↑ / ↓ bone resorption depends on calcium and RANKL/osteoprotegerin ratio	↓ PTH ↓ calcitonin ↑ FGF23 ↑ α-Klotho ↑ CaSR	↓	Hoenderop et al., 2001, 2002; Tsujikawa et al., 2003; Liu et al., 2006; Forster et al., 2011; Harada et al., 2012; Nakamichi et al., 2017

FGF23	↑ calcium reabsorption (together with α-Klotho via upregulation of TRPV5)	↑ calcium absorption (via upregulation of TRPV6) ↓ calcium absorption (indirectly via ↓ calcitriol and ↓ PTH)	↓ bone formation (via the regulation of bone mineralisation inhibitors e.g. Sfrp1, Dkk1 and osteopontin)	↓ PTH ↓ calcitriol ↓ α-Klotho	↑	Shimada et al., 2004b; Ben-Dov et al., 2007; Wang et al., 2008; Marsell et al., 2008; Shalhoub et al., 2011; Khuituan et al., 2012; Olauson et al., 2013; Andrukhova et al., 2014; Carrillo-López et al., 2016; Han et al., 2016; Murali et al., 2016a, 2016b
α-Klotho	↑ calcium reabsorption (via upregulation of TRPV5, either independently or together with FGF23)	↑ calcium absorption (via upregulation of TRPV6) ↓ calcium absorption (indirectly via ↓ calcitriol)	↓ bone formation (discordant evidence on bone resorption)	↓ PTH ↓ calcitriol ↑ FGF23	↓	Yoshida et al., 2002; Tsujikawa et al., 2003; Lu et al., 2008; Alexander et al., 2009; Shalhoub et al., 2011; Woudenberg-Vrenken et al., 2012; Komaba et al., 2017; Fan et al., 2018;

						Xiao et al., 2019
Calcitonin[a]	Discordant evidence on calcium reabsorption	↑ calcium absorption (via ↑ calcitriol)	↓ bone resorption	↑ calcitriol ↓ PTH	↑	Clark and Kenny, 1969; Cochran et al., 1970; Nielsen et al., 1979; Carney and Thompson, 1981; Quamme, 1981; Kawashima et al., 1981; Chambers and Moore, 1983; Jaeger et al., 1986; Rodriguez et al., 1991; Monkawa et al., 1999; Kantham et al., 2009; Hsu et al., 2010
CaSR	↓ calcium reabsorption (via decreased response to PTH and calcitriol)	Apical activation ↑ calcium absorption	↓ bone resorption	↓ PTH	↓ expression in parathyroid glands and arteries	Datta et al., 1989; Moonga et al., 1990; Kanatani et al., 1999; Motoyama and Friedman, 2002;

	Basolateral activation ↓ calcium absorption (via downregulation of TRPV6) Modulation of gastrointestinal physiology (extend beyond calcium metabolism)	↓ calcitriol ↑ calcitonin	Dufner et al., 2005; Molostvov et al., 2007; Jongwattanapisan et al., 2012; Lee et al., 2019; Uchiyama et al., 2020

Abbreviations: CKD, chronic kidney disease; CaSR, calcium-sensing receptor; FGF23, fibroblast growth factor 23; MAPK, mitogen-activated protein kinase; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor kappa-B ligand; Sfrp1, secreted frizzled-related protein 1; Dkk1, Dickkopf-related protein 1, TRPV5, transient receptor potential vanilloid subtype 5; TRPV6, transient receptor potential vanilloid subtype 6; PMCA1b, plasma membrane calcium ATPase; and; NCX1, sodium-calcium exchanger.

[a] *Calcitonin plays a relatively minor role in calcium homeostasis in adult cats (Pineda et al., 2013; van den Broek et al., 2018).*

Figure Legends

Fig. 1. Schematic illustration of the major regulatory mechanisms of calcium homeostasis in the body. Calcium is tightly regulated by the complex interplay of the four organs depicted above: kidney, gastrointestinal tract, parathyroid gland and bone. Parathyroid hormone (PTH) is synthesised and released from the parathyroid glands. PTH acts on the kidney to stimulate calcium reabsorption and calcitriol synthesis. It also enhances the mobilisation of calcium and stimulates fibroblast growth factor 23 (FGF23) production from the bone. FGF23, in turn, inhibits the synthesis and secretion of PTH, forming a negative feedback loop. In the kidney, FGF23 stimulates tubular calcium reabsorption and inhibits calcitriol production; calcitriol, on the other hand, stimulates the production of FGF23, forming a second feedback loop. Calcitriol also inhibits PTH synthesis, maintaining a third negative feedback loop. In addition to the effects on calcium handling in the kidney and the bone, the three counterregulatory feedback circuits work reciprocally to modulate the absorption of calcium by the gastrointestinal tract.

Fig. 2. Schematic illustration of calcium reabsorption in the nephron.

(A) At the level of the **proximal convoluted tubule** (PCT): Calcium is reabsorbed paracellularly via passive diffusion or solvent drag down an electrochemical gradient, and this is partially driven by transcellular movement of sodium via sodium phosphate cotransporter (NaPi-2a/-2c), sodium-hydrogen exchanger (NHE3), sodium-glucose cotransporter (SGLT1/2) and various types of sodium-coupled amino acid cotransporters to allow entry of sodium across the apical membrane. In turn, the sodium-potassium adenosine triphosphatase (Na^+K^+ATPase) actively pumps sodium out of the cell at the basolateral membrane. Claudin-2 is the main tight junction protein involving in calcium reabsorption in the PCT. Chloride is reabsorbed via both

paracellular and transcellular pathways, which contributes to the development of an electropositive transepithelial voltage at this level.

(B) At the level of the **thick ascending limb** (TAL): Calcium is reabsorbed via the paracellular pathway involving Claudin-16 and -19, and Paracellin-1. The main driving force is associated with the lumen-positive transepithelial electrical potential, generated by the transcellular movement of sodium, chloride, and potassium. Sodium-potassium-chloride cotransporter (NKCC2; the target site for the loop diuretic drugs, furosemide and torasemide) is responsible for the apical entry of sodium, chloride and potassium; basolateral effluxes of sodium and chloride are facilitated by Na^+K^+ATPase and the chloride channel, respectively. However, potassium is mostly recycled apically via the renal outer medullary potassium channel (ROMK2).

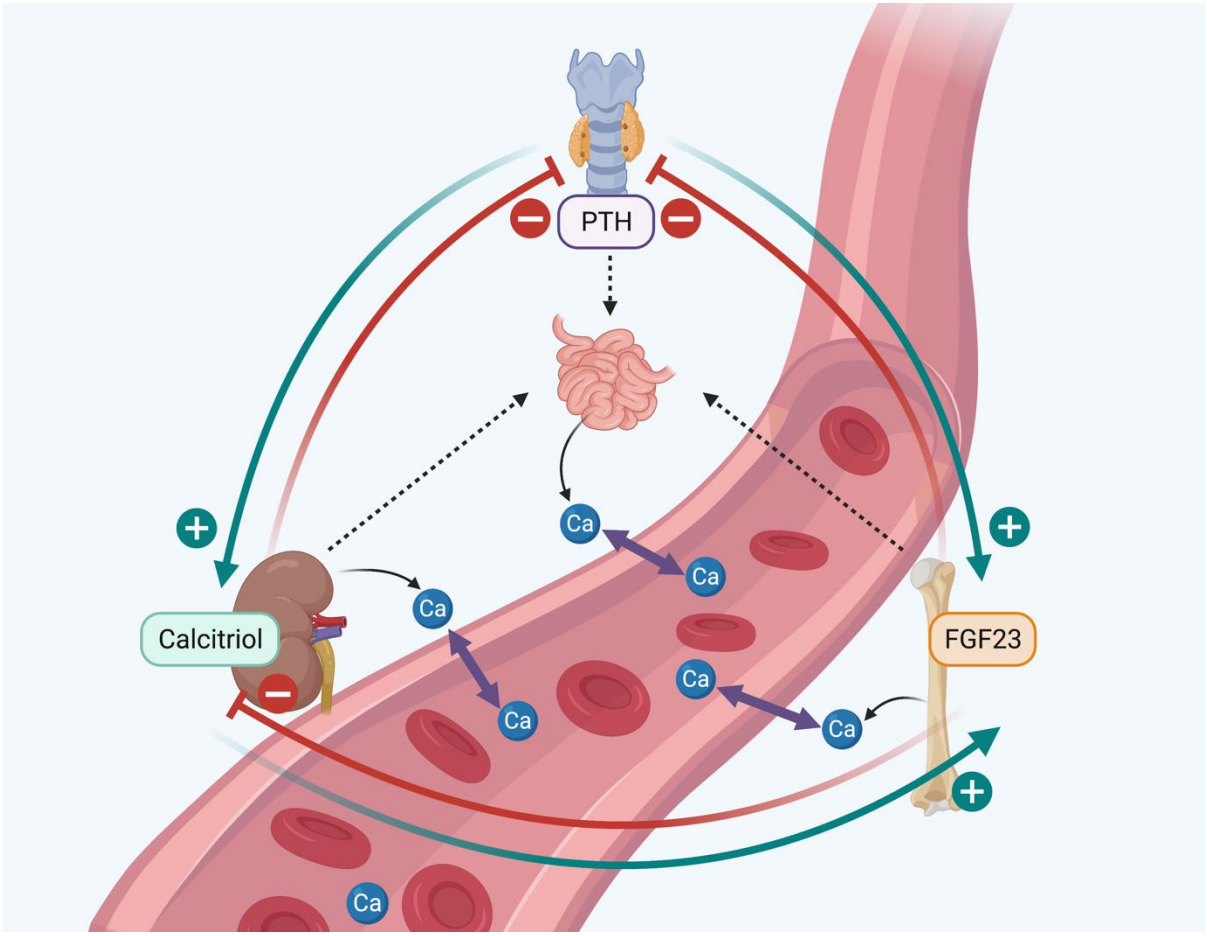
(C) At the level of the **distal convoluted tubule** (DCT): Calcium is reabsorbed via a transcellular pathway in a three-step process: (1) calcium enters the cell at the apical surface via transient receptor potential vanilloid subtypes 5 and 6 (TRPV5 and TRPV6); (2) calcium binds to calbindin-D_{28k} or -D_{9k} for intracellular translocation; and (3) calcium exits at the basolateral membrane through either the plasma membrane calcium ATPase (PMCA1b) or the sodium-calcium exchanger (NCX1).

Fig. 3. Schematic illustration of calcium absorption in the intestine. Calcitriol (1,25-dihydroxycholecalciferol) stimulates absorption of calcium via both transcellular and paracellular pathways. The effects of calcitriol are mediated through binding to the vitamin-D receptor (VDR). These initiate transcription of various genes to enhance the apical expression of transient receptor potential vanilloid subtype 6 (TRPV6), induce synthesis of intracellular calbindin-D_{9K}, and increase the expression of plasma membrane calcium ATPase (PMCA1b) and sodium-calcium exchanger (NCX1) on the basolateral membrane. Calcitriol also increases

the expression of claudin-2, -12 and -15 to facilitate the paracellular calcium transport between enterocytes.

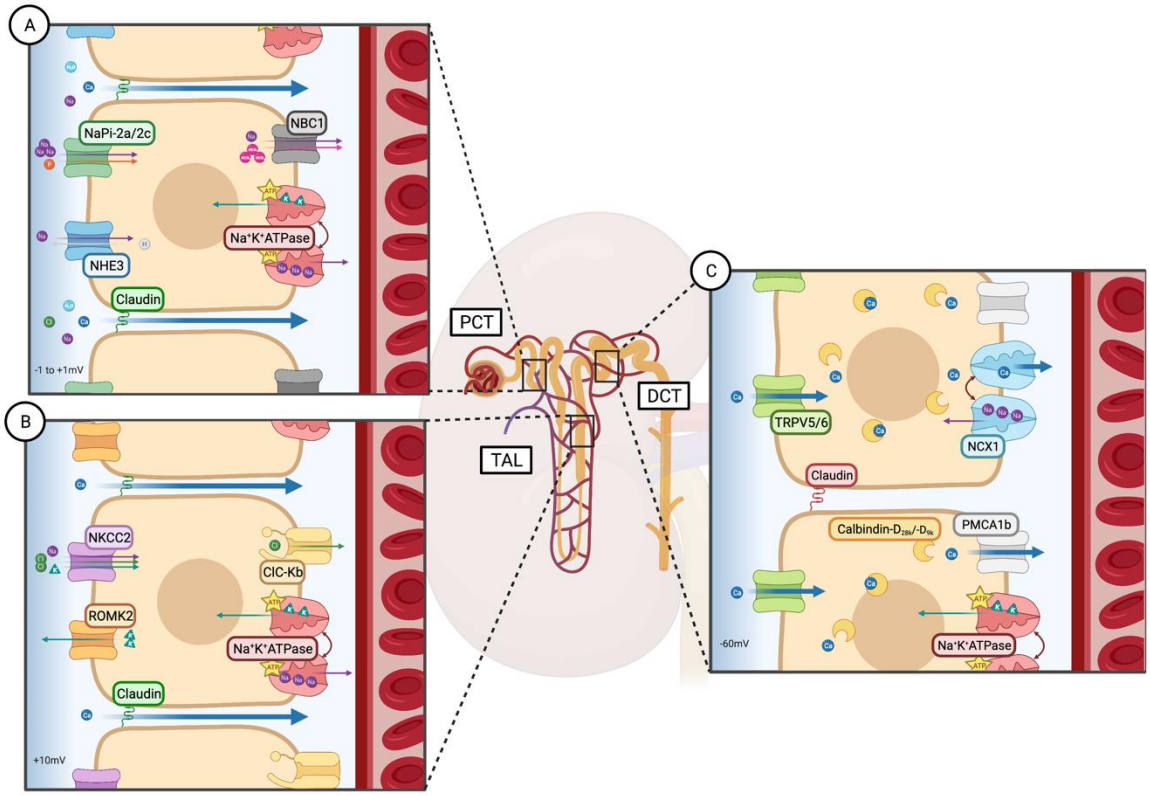
Fig. 4. Schematic illustration of the effects of FGF23-Klotho signalling on the promotion of calcium reabsorption, achieved by the upregulation of the apical expression of transient receptor potential vanilloid subtype 5 (TRPV5) in the renal distal convoluted tubules (DCT). FGF23 binds to FGFR-Klotho complex to induce FGFR dimerization and initiates the MAPK cascade involving the activation of extracellular signal-regulated kinases 1/2 (ERK1/2), serine/glucocorticoid-regulated kinase-1 (SGK-1) and no lysine kinase 4 (WNK4). This stimulates forward trafficking of TRPV5 and promotes tubular calcium reabsorption.

1419 Figure 1



1420

1421 Figure 2



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1423 Figure 3

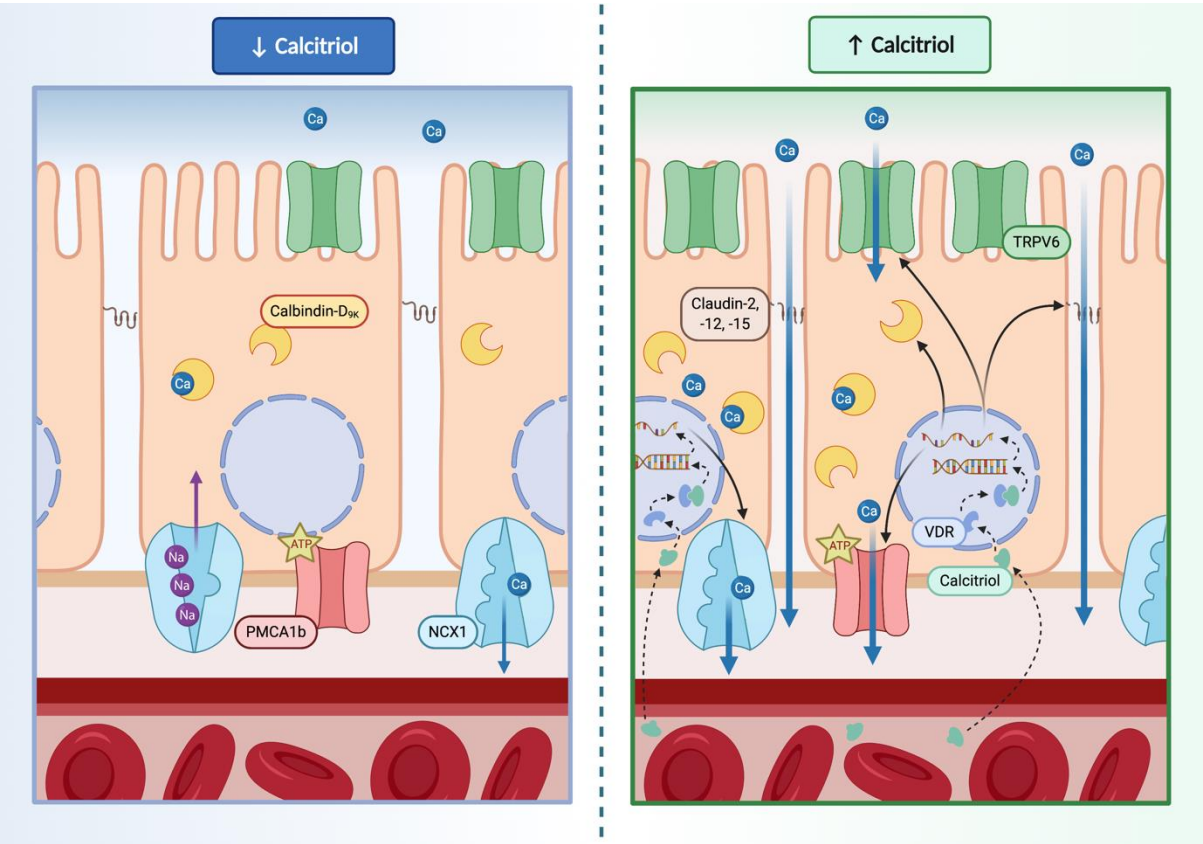

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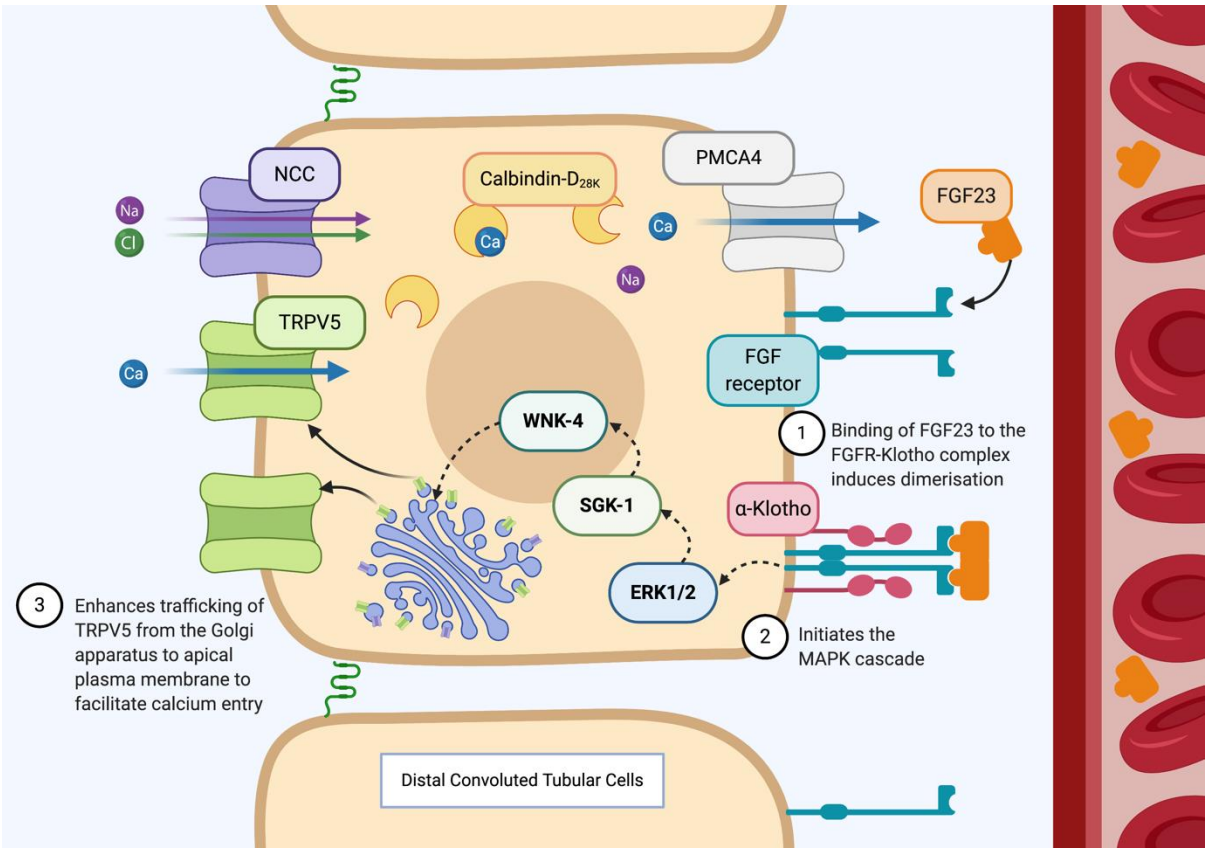
Figure 4


NCC
Na
Cl
Calbindin-D28K
Ca
PMCA4
Ca
FGF23
Na
TRPV5
Ca
FGF receptor
WNK-4
1
Binding of FGF23 to the FGFR-Klotho complex induces dimerisation
SGK-1
α-Klotho
ERK1/2
2
Initiates the MAPK cascade
3
Enhances trafficking of TRPV5 from the Golgi apparatus to apical plasma membrane to facilitate calcium entry
Distal Convoluted Tubular Cells