The Klotho proteins in health and disease

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Abstract | The Klotho proteins, αKlotho and βKlotho, are essential components of endocrine fibroblast growth factor (FGF) receptor complexes, as they are required for the high-affinity binding of FGF19, FGF21 and FGF23 to their cognate FGF receptors (FGFRs). Collectively, these proteins form a unique endocrine system that governs multiple metabolic processes in mammals. FGF19 is a satiety hormone that is secreted from the intestine on ingestion of food and binds the βKlotho–FGFR4 complex in hepatocytes to promote metabolic responses to feeding. By contrast, under fasting conditions, the liver secretes the starvation hormone FGF21, which induces metabolic responses to fasting and stress responses through the activation of the hypothalamus–pituitary–adrenal axis and the sympathetic nervous system following binding to the βKlotho–FGFR1c complex in adipocytes and the suprachiasmatic nucleus, respectively. Finally, FGF23 is secreted by osteocytes in response to phosphate intake and binds to αKlotho–FGFR complexes, which are expressed most abundantly in renal tubules, to regulate mineral metabolism. Growing evidence suggests that the FGF–Klotho endocrine system also has a crucial role in the pathophysiology of ageing-related disorders, including diabetes, cancer, arteriosclerosis and chronic kidney disease. Therefore, targeting the FGF–Klotho endocrine axes might have therapeutic benefit in multiple systems; investigation of the crystal structures of FGF–Klotho–FGFR complexes is paving the way for the development of drugs that can regulate these axes.

We serendipitously discovered the *Klotho* gene while producing unrelated transgenic mouse lines. We noticed that the homozygous progeny of a particular transgenic founder had a complex ageing-like phenotype, and we therefore reasoned that integration of the transgene into the founder mouse genome had disrupted a putative 'ageing-suppressor' gene. We named this putative gene *Klotho* after one of the goddesses of destiny in Greek mythology who spins the thread of life. Several years later, in 1997, we identified the *Klotho* gene, which encodes a single-pass transmembrane protein with unknown function that is predominantly expressed in the distal convoluted tubules of the kidneys[1].

Nearly 10 years later, we demonstrated that Klotho functions as the obligate co-receptor for fibroblast growth factor 23 (FGF23)[2], a finding that was later independently confirmed[3]. This breakthrough linking Klotho to FGF23 was driven by the fact that *Fgf23*-knockout mice[4] had a complex ageing-like phenotype that was identical to that observed in Klotho-deficient mice. At that time, FGF23 was known as a bone-derived hormone that promotes urinary phosphate excretion and lowers serum levels of active vitamin D (1,25-dihydroxyvitamin D_3)[5]. However, the identity of the FGF23 receptor was unknown because FGF23 has low affinity for all FGF receptor (FGFR) isoforms[6] and shows minimal binding to FGFRs at physiological concentrations. We now know that Klotho is required for FGF23 to bind to FGFR with high affinity, and it is the binary complex of Klotho and FGFR that functions as the physiological receptor for FGF23 (REF.[2]). This review comprehensively describes the current understanding of the FGF–Klotho endocrine system, including the accumulating evidence of its crucial role in the pathophysiology of common ageing-related disorders. In this context, the resolution of the crystal structure of Klotho–FGFR has provided crucial information to enable structure-based design of agonists and antagonists for the FGF–Klotho endocrine axes[7], which might be of therapeutic benefit in ageing-related disorders.

FGFs and their binding partners

In humans, the FGF family has 22 members, namely FGF1–FGF14 and FGF16–FGF23 (REF.[8]). FGF15 exists only in rodents and is the orthologue of human FGF19 (REF.[9]). The first-discovered members of the FGF family, such as FGF1 and FGF2, function as growth factors and act in a paracrine and/or autocrine manner. By contrast, FGF19 (FGF15 in rodents), FGF21 and FGF23

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Key points

- The Klotho proteins αKlotho and βKlotho are essential components of endocrine fibroblast growth factor (FGF) receptor complexes, as they are required for the high-affinity binding of FGF19, FGF21 and FGF23 to their cognate FGF receptors.
- FGF21 is a starvation hormone that induces stress responses by activating the sympathetic nervous system and the hypothalamus–pituitary–adrenal axis.
- FGF19 is a satiety hormone that promotes metabolic responses to feeding.
- FGF23 is a phosphaturic hormone; increased FGF23 levels in patients with early-stage chronic kidney disease or elderly individuals is indicative of excess phosphate intake relative to the residual nephron number.
- Calciprotein particles are colloids of calcium phosphate adsorbed to fetuin A, which increase in concentration as renal function declines and that can induce innate immune responses and cell death, suggesting that they are mediators of phosphate-induced damage.
- Solving the crystal structure of αKlotho and βKlotho will facilitate the development of agonists and antagonists of endocrine FGFs, which will be potentially useful for the treatment of various disorders, including chronic kidney disease and other ageing-related disorders.

function as endocrine factors that have modest mitogenic activity[10]. The difference in the mode of action between paracrine and endocrine FGFs relates mainly to their affinity for heparin[11–13]. Paracrine FGFs contain heparin-binding domains that enable them to bind with high affinity to heparan sulfate proteoglycans in the extracellular matrix, which tethers these growth factors to the cells that produce them and promotes a paracrine mode of action. Furthermore, heparan sulfate is required for paracrine FGFs to bind to and activate their cognate FGFRs. For example, heparan sulfate binds directly to both FGF2 and FGFR1 to form the heparan sulfate–FGF2–FGFR1 ternary complex, which is required for the dimerization of FGFR1 and activation of its tyrosine kinase activity. This activation triggers the canonical intracellular FGF signalling pathway, which is characterized by phosphorylation of FGFR substrate 2α (FRS2α) and its downstream targets ERK1 and/or ERK2 (REF.[14]).

In contrast to paracrine FGFs, endocrine FGFs lack heparin-binding domains, and thus their low affinity for heparan sulfate[15] allows them to diffuse away from their source and enter the systemic circulation. However, endocrine FGFs still require a co-receptor in order to bind with high affinity to cognate FGFRs in their target organs; instead of heparan sulfate, Klotho proteins function as co-receptors[1,2]. Unlike heparan sulfate, Klotho proteins are not ubiquitously expressed and thus their expression pattern in combination with that of specific FGFRs, determines which organs are targeted by endocrine FGFs. Interestingly, and in contrast to many receptor complexes for which assembly is ligand-dependent, the formation of Klotho–FGFR complexes is constitutive and independent of the presence of FGF ligand[2].

Endocrine FGFs (that is, FGF19, FGF21 and FGF23) are 200–250-residue proteins[15] that interact with FGFRs through their ~120-residue amino-terminal region, which shares sequence homology with paracrine FGFs and is thus known as the FGF core homology domain. By contrast, the carboxy-terminal region of endocrine FGFs interacts with Klotho proteins and is substantially different from that of paracrine FGFs, both in length and primary sequence[15].

Soon after the discovery of Klotho, a homologous protein was identified[16] and was named βKlotho (encoded by the *βKlotho* gene; also known as *KLB*). The Klotho protein that was identified as the FGF23 co-receptor became known as αKlotho (encoded by the *αKlotho* gene; also known as *KL*). βKlotho forms binary complexes with FGFR1c (expressed by adipocytes) and FGFR4 (expressed by hepatocytes), which function as the physiological receptors for FGF21 and FGF19, respectively[17,18]. Similarly to insulin, FGF19 induces metabolic responses to feeding, whereas FGF21 is akin to glucagon and regulates metabolism during fasting[10]. Furthermore, FGF21 was reported to extend lifespan when overexpressed in mice[19], suggesting that the FGF–Klotho endocrine system has an effect on ageing processes.

Structure of Klotho proteins

The crystal structures of αKlotho and βKlotho have been solved. Structural studies of FGF23 co-crystallized with soluble αKlotho (that is, the extracellular domain of αKlotho) and the FGFR1c ligand-binding domain[20] revealed that αKlotho extends a long thread — termed the receptor-binding arm (RBA) — which interacts directly with FGFR1c. FGF23 fits into a groove that is created between αKlotho and FGFR1c, with the FGF23 N terminus oriented towards FGFR1c and the C terminus oriented towards αKlotho (FIG. 1). These structural findings confirm that the two components of the receptor complex interact with distinct regions of the ligand and thereby clarify the function of αKlotho as the obligate co-receptor for FGF23. Interestingly, the ternary complex of FGF23, αKlotho and FGFR1c requires heparan sulfate to dimerize and activate the tyrosine kinase of FGFR1c. Although its affinity for FGF23 is low, heparan sulfate interacts with both FGFR1c and FGF23.

βKlotho has been crystallized both with and without FGF21 (REF.[21]). Similarly to the interaction of αKlotho with FGF23, βKlotho interacts directly with the C terminus of FGF21. However, the structure of some regions of this complex could not be resolved, including the RBA of αKlotho. These findings suggest that the putative RBA of βKlotho may be intrinsically disordered but might become structured following its binding to FGFR1c (BOX 1). Assuming that the RBA of αKlotho is also intrinsically disordered, it is possible that αKlotho has binding partners other than FGFRs, which might explain the multiple functions of soluble αKlotho that are independent of FGF23 (REF.[7]) (discussed later).

Given the role of endocrine FGFs in a range of physiological responses, including mineral and energy metabolism, stress responses and ageing-related disorders, agonists and antagonists of these hormones are potential therapeutic candidates for various disorders[21–23]. The structural data obtained for endocrine FGFs and the Klotho proteins is expected to accelerate the development of these drugs.

The FGF23–αKlotho endocrine axis

FGF23 binds to the αKlotho–FGFR complex and mediates various physiological processes to maintain phosphate and calcium homeostasis, primarily by regulating the function of the kidneys and the parathyroid glands.

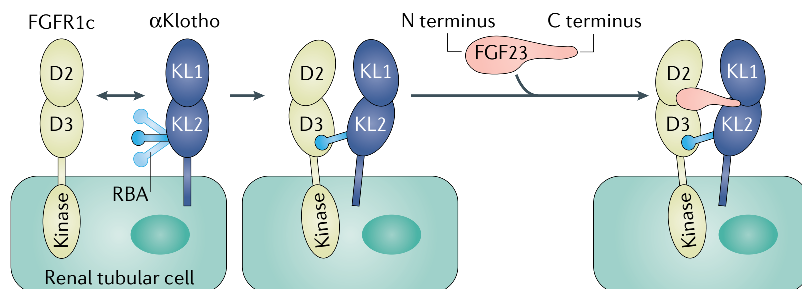

Fig. 1 | **Structural basis of FGF23–αKlotho–FGFR1c complex formation.** The KL2 domain of the αKlotho protein has a protruding loop termed the receptor-binding arm (RBA) that binds to the D3 domain of fibroblast growth factor receptor 1c (FGFR1c). Fibroblast growth factor 23 (FGF23) fits into a groove generated by the binding of αKlotho to FGFR1c and binds to αKlotho via its carboxyl terminus, whereas the amino terminus binds to the D2 domain of FGFR1c.

Consequently, disruption of the FGF23–αKlotho endocrine axis has a crucial role in the pathophysiology of renal and bone disorders.

The FGF23–αKlotho axis in health

FGF23 as a phosphaturic hormone. FGF23 is secreted by osteocytes[24,25] in response to phosphate intake. Its major target organ is the kidneys, in which αKlotho is most abundantly expressed[1,26]. In fact, the binding of FGF23 to a complex of αKlotho and FGFR1c, FGFR3c or FGFR4, which are all expressed in the kidneys, leads to an increase in urinary phosphate excretion within a few hours[10]. Mice that lack FGF23 or αKlotho show phosphate retention, which suggests that the FGF23–αKlotho endocrine axis is indispensable for maintaining phosphate homeostasis[27].

In the kidneys, FGF23 induces the internalization and degradation of sodium-dependent phosphate transport protein 2A (NPT2A) and downregulates the expression of *NPT2A* (also known as *SLC34A1*). NPT2A is localized to the apical brush border membrane of proximal tubular cells, a major site of transcellular phosphate reabsorption[28]. Loss of NPT2A results in decreased phosphate reabsorption in renal proximal tubules and increased urinary phosphate excretion[5,29]. FGF23-induced phosphaturia is assessed clinically by measuring the fractional excretion of phosphate (FEP)[30].

FGF23 as a counter-regulatory hormone for vitamin D and PTH. The expression of *Fgf23* is increased in cultured osteoblastic cells in vitro and in rodents in vivo by treatment with active vitamin D[31] and parathyroid hormone (PTH)[32]. Binding of active vitamin D to the vitamin D receptor (VDR) results in heterodimerization of VDR with retinoid X receptor (RXR), followed by translocation of the complex to the nucleus, where it binds to vitamin D-responsive elements in 5′-flanking regions of target genes, including *Fgf23*, and transactivates them[31,33,34]. The mechanism by which PTH induces *Fgf23* upregulation is more complex than that of *Fgf23* regulation by vitamin D — the binding of PTH to the PTH receptor activates protein kinase A (PKA), which suppresses the expression of sclerostin, a secreted glycoprotein that antagonizes the WNT signalling pathway and suppresses *Fgf23* expression. Thus, PTH increases *Fgf23* expression in a PKA-dependent and WNT-dependent manner[32,35].

In contrast to the effects of vitamin D and PTH on FGF23, serum levels of both active vitamin D[5] and PTH are lowered by FGF23 (REFS[36,37]). In renal proximal tubules, FGF23 suppresses the expression of *Cyp27b1*, which encodes 25-hydroxyvitamin D_3 1α hydroxylase, the enzyme that converts 25-dihydroxyvitamin D_3 to active vitamin D (1,25-dihydroxyvitamin D_3). In addition, FGF23 increases the expression of *Cyp24a1*, which encodes 24-hydroxylase, the enzyme that converts 25-dihydroxyvitamin D_3 to inactive vitamin D (24,25-dihydroxyvitamin D_3), although the effect of FGF23 on *Cyp24a1* expression might not be direct[38]. By suppressing the synthesis of active vitamin D and promoting the formation of inactive vitamin D, FGF23 lowers the serum levels of active vitamin D.

The parathyroid gland is one of the few tissues in which αKlotho is expressed, suggesting that it is a target organ of FGF23. FGF23 suppresses the expression and secretion of PTH when it is administered to cultured parathyroid glands in vitro or injected into healthy rats[36]. However, parathyroid gland-specific deletion of *αKlotho* does not affect the ability of FGF23 to suppress PTH, suggesting that the local expression of membrane-bound αKlotho is not required for this effect. In fact, in addition to triggering canonical FGF signalling in an αKlotho-dependent manner, FGF23 can also activate a calcineurin-dependent signalling pathway in the absence of αKlotho to suppress PTH[39].

Thus, these three crucial hormones that regulate mineral metabolism — namely, FGF23, vitamin D, and PTH — are interconnected via negative feedback loops that operate coordinately to maintain phosphate homeostasis (FIG. 2).

FGF23 as a sodium-conserving and calcium-conserving hormone. Injection of mice with recombinant FGF23 induces the phosphorylation of ERK1 and ERK2, which is indicative of canonical FGF signalling, in the distal convoluted tubules (DCTs) of the kidneys, in which αKlotho is most abundantly expressed[40]. In addition, FGF23 induces the phosphorylation of serine/threonine-regulated kinase SGK1 and serine/threonine-protein kinase WNK4 in the DCTs in an αKlotho-dependent manner[41]. The activation of SGK1 and WNK4 upregulates the trafficking of several ion channels and transporters, including epithelial sodium channel (ENaC), Na–Cl cotransporter (NCC; also known as SLC12A3) and transient receptor potential cation channel subfamily V member 5 (TRPV5), to the plasma membrane[42–44]. It is therefore conceivable that FGF23 increases the abundance of ENaC, NCC and/or TRPV5 at the apical membrane in the DCTs by effects on SGK1 and WNK4, thereby enhancing sodium and calcium reabsorption. This hypothesis is supported by studies in mice showing that injection of FGF23 increases sodium and calcium reabsorption, which was associated with increased plasma membrane levels of NCC[41] and TRPV5 (REF.[44]) in the DCTs. In addition, mice lacking FGF23 or αKlotho have reduced levels of NCC and TRPV5 in the apical membrane of the DCTs and have higher urinary

Box 1 | **Intrinsically disordered proteins**

Structural biologists often find that specific regions of a protein cannot be modelled owing to insufficient electron density. These regions lack a stable 3D structure and are described as intrinsically disordered or unstructured regions[196]. Based on an analysis of the primary amino acid sequence of proteins in the Swiss Protein Database, about one-third of eukaryotic proteins are predicted to contain intrinsically disordered structures, either globally or locally[197]. The fact that many disordered regions are conserved during evolution[197] suggests that they are crucial for protein function, which argues against the central dogma of structural biology that a solid, folded protein structure is required for its function. Intrinsically disordered regions can fold and undergo transition to a stable ordered structure following binding to their targets[197]. It is possible that the structure of the receptor-binding arm (RBA) in αKlotho was solved[20] because αKlotho was co-crystallized with its binding partner FGFR1c. If so, co-crystallizing βKlotho and FGFR1c might enable the modelling of residues 538–574 in βKlotho to determine whether this region includes an RBA that is comparable to that in αKlotho.

excretion of sodium and calcium compared with wild-type mice, suggesting that the sodium-conserving and calcium-conserving activity of FGF23 might be of physiological relevance.

Active vitamin D and PTH have a positive effect on calcium balance, as they promote calcium absorption from the intestine and calcium mobilization from the bone, respectively[45]. The calcium-conserving activity of FGF23 discussed above may help minimize its potential negative effect on calcium balance owing to its ability to lower serum levels of active vitamin D and PTH, while preserving its primary function as a regulator of phosphate balance. These feedback mechanisms are somewhat comparable to the actions of PTH[46], which minimizes disturbances in phosphate balance by its ability to increase serum levels of active vitamin D, mobilize mineral from bone and promote urinary phosphate excretion, even though the regulation of calcium balance is its primary function. Of note, the sodium-conserving activity of FGF23 might contribute in part to its ability to increase phosphate excretion per nephron, because volume expansion per se (achieved by sodium reabsorption) can suppress phosphate reabsorption in proximal tubules[47,48].

Regulation of FGF23 and αKlotho expression. The mechanism by which osteocytes produce FGF23 in response to phosphate is unknown. One possibility is that osteocytes express a putative ‘phosphate-sensing receptor’ that, when activated, induces FGF23 secretion, analogous to how parathyroid cells express a calcium-sensing receptor that, when activated by increases in serum calcium levels, suppresses PTH secretion[49]. However, serum levels of FGF23 correlate not only with serum phosphate levels but also with serum calcium levels. In fact, in conditions of hypocalcaemia, a rise in serum phosphate levels does not lead to an increase in FGF23 levels. Similarly, FGF23 levels do not increase in response to increases in serum calcium levels in conditions of hypophosphataemia, indicating that both calcium and phosphate are required to stimulate FGF23 secretion by osteocytes[50,51].

In addition to phosphate, calcium, PTH and active vitamin D, the expression of FGF23 is also increased by aldosterone[52]. Treatment of mice with aldosterone results in an increase in serum and bone levels of FGF23. Of note, treatment of cultured osteoblastic cells with aldosterone increased FGF23 expression in a nuclear factor κB (NF-κB)-dependent manner[52]. Given that the promoter region of *Fgf23* contains an NF-κB-responsive element, aldosterone-induced NF-κB activation might be responsible for the increase in FGF23 expression in response to aldosterone[53].

Similarly, pro-inflammatory cytokines, such as IL-1, IL-6, and tumour necrosis factor, might induce FGF23 expression through NF-κB activation, the canonical signalling pathway of inflammation[54]. Pro-inflammatory cytokines also induce the secretion of hepcidin in the liver. Hepcidin is a hormone that lowers circulating iron and causes functional iron deficiency when present at abnormally high levels. In contrast to aldosterone, iron is a negative regulator of FGF23 levels, and iron deficiency increases FGF23 production independently of inflammation, probably by increasing the expression and stability of hypoxia-inducible factor 1α (HIF1α), which binds to hypoxia-responsive elements in the promoter region of *Fgf23* and induces its transcription[55].

Several factors that regulate FGF23 expression can also control αKlotho expression. Active vitamin D can directly induce the expression of αKlotho, as the promoter of *αKlotho*, similarly to *Fgf23*, contains multiple vitamin D-responsive elements[56,57]. In addition to vitamin D, thiazolidinediones, which are ligands for peroxisome proliferator-activated receptor-γ (PPARγ), induce the expression of αKlotho[58]. The *αKlotho* gene contains a non-canonical PPAR-response element in its 5′-flanking region, and the binding of PPARγ to this element transactivates αKlotho expression. In contrast to its effect on FGF23, aldosterone reduces the renal levels of αKlotho mRNA and protein[59]; the vasoconstrictor angiotensin II has the same suppressive effect[60]. Conversely, administration of angiotensin II type 1 receptor (AT1) blockers and angiotensin-converting enzyme (ACE) inhibitors increases the renal levels of αKlotho mRNA and protein in rats, independently of the ability of these renin–angiotensin–aldosterone system (RAAS) blockers to lower blood pressure or reduce proteinuria[60]. These observations suggest that the RAAS is a negative regulator of renal αKlotho levels[60]. In addition to the RAAS, FGF23 is a potent negative regulator of αKlotho expression. In fact, αKlotho mRNA was the most significantly decreased transcript in a microarray analysis of gene expression in the kidneys from FGF23-overexpressing transgenic mice[61]. Therefore, any factors that increase FGF23 expression, including increased phosphate intake, NF-κB activation in inflammation and HIF1α activation, can indirectly downregulate αKlotho expression in vivo. The downregulation of αKlotho expression by FGF23 might be part of a regulatory system whereby upregulation of the ligand induces a decrease in the expression of its receptor, although the precise mechanism of this potential regulation remains to be determined.

The FGF23–αKlotho axis in disease

Primary disorders of the FGF23–αKlotho endocrine axis. A primary excess of serum FGF23 is observed in patients with hereditary phosphate-wasting syndromes, including autosomal dominant hypophosphataemic

Hereditary phosphate-wasting syndromes
Inherited disorders in which the disease symptoms are related to the depletion of phosphorus in the body owing to increased urinary phosphate excretion and resulting in hypophosphataemia and disrupted bone mineralization.

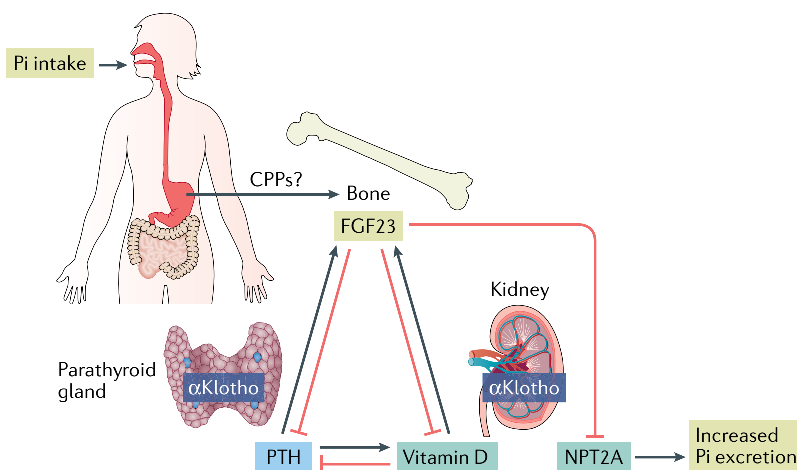

Fig. 2 | **The FGF23–αKlotho endocrine axis.** In response to inorganic phosphate (P_i) intake, osteocytes secrete fibroblast growth factor 23 (FGF23). The mechanisms by which osteocytes detect P_i is unclear but might involve sensing of serum calciprotein particles (CPPs), which are nanoparticles that comprise solid-phase calcium phosphate and the serum protein fetuin A. Increased FGF23 levels result in increased urinary phosphate excretion by downregulating sodium-dependent phosphate co-transporter type 2a (NPT2A) levels in proximal tubules and by reducing calcium entry into the circulation (not shown) by lowering the serum level of parathyroid hormone (PTH) and active vitamin D. Increasing urinary phosphate excretion and suppressing calcium entry into the blood in response to phosphate intake enable the FGF23–αKlotho endocrine axis to maintain phosphate homeostasis and potentially prevent the formation of excess CPPs, which can cause tissue damage.

rickets (ADHR)[62], X-linked hypophosphataemia (XLH) and autosomal recessive hypophosphataemic rickets (ARHR)[63,64]. These patients have abnormally high serum levels of FGF23 that are associated with hypophosphataemia and low (or inappropriately normal) serum levels of active vitamin D, which results in inadequate bone mineralization (that is, rickets or osteomalacia) and leads to bone pain or deformities of the lower extremities.

ADHR is caused by missense mutations in *FGF23* that make the FGF23 protein resistant to inactivation by proteolytic cleavage, resulting in high serum levels of intact FGF23 (REF.[62]). Patients with XLH carry mutations in phosphate-regulating endopeptidase homologue X-linked (*PHEX*)[65] that result in increased FGF23 expression, although the mechanisms are unknown, as PHEX does not cleave FGF23. Patients with ARHR carry mutations in the gene encoding dentin matrix acidic phosphoprotein 1 (DMP1), which is a bone matrix protein that is specifically expressed by osteocytes[25]. However, the mechanism by which mutations in *DMP1* increase FGF23 expression remains elusive. Finally, a sporadic primary excess of serum FGF23 is also observed in patients with tumour-induced osteomalacia, who have FGF23-producing tumours and develop symptoms similar to those observed in patients with hereditary phosphate-wasting syndromes[29].

A primary deficiency of serum FGF23 is observed in patients with familial tumoural calcinosis (FTC) who carry loss-of-function mutations in *GALNT3*, which encodes the glycosyl transferase ppGaNTase T3 (REFS[66,67]). This enzyme adds *N*-acetyl galactosamine by *O*-glycosylation to FGF23 at Thr178, which is adjacent to the proteolytic cleavage site, making FGF23 resistant to inactivation by proteolysis. Loss-of-function mutations in *GALNT3* therefore result in increased FGF23 degradation, which leads to low serum levels of FGF23, resulting in ectopic calcification that is associated with high serum levels of phosphate and active vitamin D.

To date, mutations in or near the *αKlotho* gene have been described in two patients. One of these patients had a homozygous mutation (H193R) in the *αKlotho* gene[68] that seems to be a loss-of-function mutation, as the patient developed symptoms that resemble FTC. The mutation in the other patient was a translocation with a breakpoint near the *αKlotho* gene[69], and this patient presented with hypophosphataemia that was associated with high serum levels of FGF23 and PTH. Furthermore, this patient had high serum levels of soluble αKlotho, which was most likely generated by ectodomain shedding of αKlotho (see below). Interestingly, mice that express soluble αKlotho from an adenoviral vector also develop hypophosphataemia that is associated with high FGF23 levels[70], although the mechanism by which soluble αKlotho increases FGF23 levels remains to be determined.

Secondary excess of serum FGF23. A secondary excess of serum FGF23 is observed in all patients with chronic kidney disease (CKD)[71]. The major causes of CKD include primary renal diseases, such as glomerulonephritis and polycystic kidney disease, as well as renal complications that are secondary to diabetes and hypertension[72]. Regardless of the cause of kidney damage, it is reasonable to assume that the number of functional nephrons decreases as CKD progresses. As nephron number also decreases as a consequence of natural ageing[73], CKD can be viewed as a factor that accelerates kidney ageing.

Unless phosphate intake is reduced, a decrease in nephron number that results from ageing or CKD would require an increase in phosphate excretion per nephron to maintain phosphate balance. This increased phosphate excretion is achieved by increasing FGF23 expression, resulting in an increase in serum FGF23 levels with age and CKD progression[71,74]. This increase in FGF23 lowers serum levels of active vitamin D, which is followed by an increase in PTH levels; these changes that are observed with CKD progression[71] generate a hormone profile that is characteristic of CKD–mineral and bone disorder (CKD–MBD) (FIG. 3a). High PTH levels further increase FGF23 levels, which results in further decreases in active vitamin D levels, and both high FGF23 levels and low active vitamin D levels suppress αKlotho expression. This reduction in αKlotho expression can induce resistance to FGF23 by reducing its activation of FGFR-dependent intracellular signalling. A reduced response to FGF23 in the kidneys potentially enhances and/or prolongs the postprandial increase in the blood phosphate level, leading to a feed-forward loop that exacerbates CKD–MBD (FIG. 3b). Eventually, CKD progresses to the point at which the number of functional nephrons is no longer sufficient to excrete the ingested phosphate, resulting in persistent hyperphosphataemia (FIG. 3a).

Inappropriately normal
Normal protein serum levels when they should be outside of the normal range owing to concurrent levels of other serum proteins. This effect is indicative of impaired physiological responses.

Rickets
A condition characterized by impaired (low) bone mineralization that results in weak, soft bones with increased osteoid (unmineralized bone matrix) in children. In adults, this condition is termed osteomalacia.

Breakpoint
Location in a chromosome where a genomic DNA sequence has been disrupted by deletion, translocation or insertion.

CKD–MBD
Denotes chronic kidney disease (CKD) complications that are associated with and are likely caused by disturbed calcium and phosphate metabolism and by abnormal serum levels of fibroblast growth factor 23 (FGF23), vitamin D, and parathyroid hormone. The term CDK–mineral and bone disorder (CKD–MBD) is used to describe cardiovascular and bone disorders in CKD, including vascular calcification, cardiac hypertrophy and renal osteodystrophy.

Postprandial
The period that follows the ingestion of food.

Sarcopenia
The degenerative decline in skeletal muscle volume and strength with ageing.

Osteopenia
A condition of low bone mineral density. Osteoporosis is a clinical diagnosis of osteopenia that is associated with a decrease in both bone matrix and bone mineral density, as well as altered bone microarchitecture.

As mentioned previously, PTH also has phosphaturic activity; however, the fact that the increase in FGF23 precedes the increase in PTH during CKD progression indicates that FGF23, and not PTH, is primarily responsible for the maintenance of phosphate homeostasis, at least in the early stages of CKD. This notion is supported by findings from a study in which rats were injected with anti-glomerular basement membrane antiserum to model progressive CKD[75]. Treatment of rats that had high FGF23 levels, low active vitamin D levels and high PTH levels, but were not yet hyperphosphataemic, with a neutralizing anti-FGF23 antibody induced a rapid (within 24 h) increase in serum levels of phosphate and active vitamin D and a decrease in the fractional excretion of phosphate. However, the serum levels of PTH and calcium remained unchanged, and it was not until 48 h after the antibody treatment that the serum levels of PTH and calcium started decreasing and increasing, respectively. These findings indicate that FGF23, and not PTH, is primarily responsible for maintaining serum phosphate levels within the physiological range and that the rise in PTH levels occurred in response to low serum levels of active vitamin D and calcium.

As mentioned earlier, the phosphaturic activity of FGF23 stems from its ability to increase phosphate excretion per nephron by suppressing phosphate reabsorption in proximal tubules; however, it has long been known that increased phosphate excretion induces renal tubular damage[76,77]. A study from 1980 used a rat partial nephrectomy model to demonstrate a correlation between the extent of tubular damage in the remnant kidney and phosphate excretion per nephron when the latter exceeded ~1.0 μg/day (physiological phosphate excretion levels are ~0.5 μg/day)[77]. Although FGF23 had not been discovered at the time of this study, serum FGF23 levels probably could have served as a surrogate for phosphate excretion per nephron and thus would correlate with the extent of kidney damage. Also, measuring serum FGF23 levels might make it possible to estimate the nephron number (BOX 2).

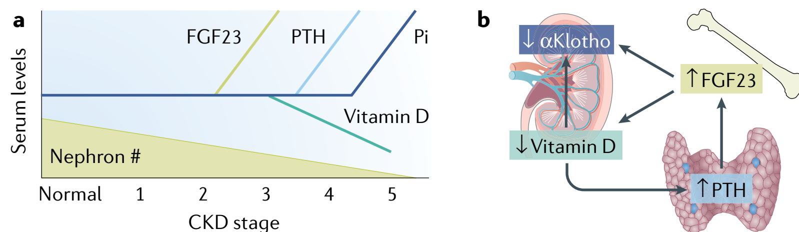

Fig. 3 | **Pathophysiology of CKD progression. a** | Changes in serum biomarker levels during chronic kidney disease (CKD) progression. Regardless of the underlying aetiology, CKD progression is characterized by a progressive decrease in the number of functional nephrons. To maintain phosphate balance, patients with CKD compensate for the decrease in the nephron number with a rise in fibroblast growth factor 23 (FGF23) levels, which increase phosphate excretion per nephron. The rise in FGF23 is followed by a decrease in active vitamin D and an increase in parathyroid hormone (PTH). All these changes, which are triggered by excess phosphate intake relative to residual nephron number, are characteristic of CKD–mineral and bone disorder. Serum inorganic phosphate (P_i) levels are the last to increase (resulting in hyperphosphataemia) and indicate a disruption of phosphate homeostasis owing to insufficient functional nephrons to excrete the ingested phosphate. **b** | The spiral of deterioration in αKlotho expression. The increase in FGF23 levels in patients with renal dysfunction lowers active vitamin D levels, which then elevates PTH levels and further increases FGF23 levels. This positive feedback loop continues to promote an increase in FGF23 levels. Both the increase in FGF23 levels and the decrease in active vitamin D levels can suppress αKlotho expression, potentially resulting in FGF23 resistance and a loss of the renoprotective properties of αKlotho. Part **a** adapted with permission from REF.[202]: Kuro-o, M. Klotho and endocrine fibroblast growth factors: marker of chronic kidney disease progression and cardiovascular complications? *Nephrol. Dial. Transplant.* https://doi.org/10.1093/ndt/gfy126 (2018), by permission of Oxford University Press.

Phosphate and ageing
Mice lacking αKlotho or FGF23 not only have a phosphate imbalance and hyperphosphataemia owing to impaired urinary phosphate excretion but also develop complex phenotypes that are characteristic of premature ageing, including poor growth, atrophy of multiple organs (for example, the gonads, thymus and skin), vascular calcification, sarcopenia, cardiac hypertrophy and fibrosis[78,79], osteopenia[80], emphysematous lung[81], hearing disturbances[82], cognitive impairment[83] and shortened lifespan[1,4]. Most of these symptoms of premature ageing are alleviated by feeding these mice a low phosphate diet to restore phosphate balance[84]. Of note, mice lacking αKlotho or FGF23 also have high serum levels of active vitamin D[4,56], which are further increased by a low phosphate diet[84,85]. These results indicate that the phosphate imbalance, rather than the increased serum vitamin D levels, is primarily responsible for the development of ageing-like symptoms in mice lacking functional FGF23 or αKlotho.

In humans, phosphate retention associated with low αKlotho expression, high serum FGF23 levels and symptoms of premature ageing have been observed in patients with end-stage renal disease, and phosphate restriction can be an effective therapy to improve ageing-related symptoms. The similarities between mice lacking αKlotho and patients with CKD have prompted the notion that CKD can be viewed as a clinical model of premature ageing[86] and that any animal models that are susceptible or resistant to CKD or ageing might provide insights into novel treatment strategies for CKD[87].

Calciprotein particles as mediators of phosphate-induced damage. Calciprotein particles (CPPs) are nanoparticles comprising solid-phase calcium phosphate and the serum protein fetuin A and are dispersed as colloids in the blood[88,89]. Because blood is supersaturated in terms of calcium and phosphate ions, even a slight, transient increase in calcium and/or phosphate concentration can trigger the precipitation of calcium phosphate. A transient increase in the levels of serum phosphate, but not calcium, occurs after each meal (postprandial hyperphosphataemia)[90], which can trigger the precipitation of calcium phosphate. However, adsorption by fetuin A, which can bind large amounts of calcium phosphate precipitates[89], prevents calcium phosphate precipitates from growing into large crystals. In fact, CPP levels in the blood transiently increase postprandially[91]; these nanoparticles are thought to function as a defense mechanism that prevents the growth of calcium phosphate crystals in the extracellular space (BOX 3).

However, CPPs are not necessarily harmless; both calcium phosphate nanocrystals and CPPs can activate

Emphysematous lung
Lung tissue that is affected by pulmonary emphysema, which is characterized by enlarged alveolar spaces and damaged alveolar walls.

cultured macrophages[92] and could act as pathogenic stimuli that trigger innate immune responses. In addition, an increase in the phosphate concentration of cell culture medium induces apoptosis of cultured vascular endothelial cells and calcification in smooth muscle cells in vitro[93–96]. Interestingly, this 'phosphate toxicity' is not observed in the presence of inhibitors that prevent the precipitation of calcium phosphate[97], suggesting that it is not phosphate per se but calcium phosphate or CPPs that are pathogenic.

In addition to the presence of CPPs in the blood, it is possible that these particles are also formed in the renal tubular fluid, because FGF23 suppresses the reabsorption of phosphate but not water, which probably leads to an increase in phosphate concentration in the proximal tubular fluid. In fact, micropuncture studies in rats that were fed a high phosphate diet or were subjected to subtotal nephrectomy show that phosphate concentrations are substantially higher in the tubular fluid of the late proximal tubules than in the blood; in tubular fluid, phosphate concentrations exceeded the level at which calcium phosphate usually precipitates[98]. As calcium phosphate nanocrystals can cause renal epithelial injury by inducing oxidative stress[99], the CKD that is associated with high FGF23 levels might be due, at least in part, to damage caused by calcium phosphate or CPPs that are generated in the proximal tubular fluid. Whether calcium phosphate precipitates are present in the proximal tubular lumen and whether they contribute to nephron loss or kidney ageing remains to be determined.

Colloids disperse insoluble materials and are used to transport these materials between organs through the bloodstream. For example, lipids are adsorbed onto apoproteins to form colloids termed lipoproteins, which are dispersed in the blood and are transported to adipose tissue for storage[100]. However, if mistargeted to arteries, skeletal muscles or the liver, lipids induce atherosclerosis, insulin resistance and fatty liver, respectively. These adverse effects caused by the mistargeting of lipids are collectively termed lipotoxicity[101]. Similarly, CPPs can be used to disperse calcium phosphate precipitates in the blood and transport them to bone for storage. However, if mistargeted to arteries or immune cells, calcium phosphate precipitates could potentially induce arteriosclerosis (that is, vascular calcification and stiffness) as well as chronic non-infectious inflammation (TABLE 1).

Clinical studies have identified several parameters that correlate with increased levels of CPPs in the blood, including age[102], increased serum phosphate levels[102,103] and declining estimated glomerular filtration rate (eGFR)[103,104]. Furthermore, serum CPP levels positively correlate with parameters of vascular calcification[105], vascular stiffness[103] and inflammation[106]. Of note, acute dietary phosphate loading induces endothelial dysfunction (as determined by a decrease in flow-mediated dilatation) that is associated with the transient increase in serum phosphate levels[90]. The fact that calcium phosphate nanocrystals and CPPs can induce vascular endothelial cell damage, smooth muscle cell calcification and innate immune responses in vitro raises the possibility that plasma and/or serum CPP levels might be causally linked to arteriosclerosis and inflammation in vivo. Importantly, chronic non-infectious inflammation is a possible mechanism underlying the ageing process; thus, CPPs might trigger inflammation and thereby link phosphate dysregulation and the development of ageing-related symptoms[107,108] (FIG. 4).

Box 2 | **Estimation of nephron number**

Assuming that serum fibroblast growth factor 23 (FGF23) levels correlate with phosphate excretion per nephron, nephron number can be estimated by measuring FGF23 levels and 24-h urinary phosphate excretion[198] using the following equations:

$$\frac{\text{Phosphate excretion}}{\text{per nephron}} = \frac{\text{Urinary phosphate excretion}}{\text{Nephron number}} \propto [FGF23] \quad (1)$$

$$\therefore \text{Nephron number} \propto \frac{\text{Urinary phosphate excretion}}{[FGF23]} \equiv \text{Nephron index} \cdots (1) \quad (2)$$

The ratio of urinary phosphate excretion (in mg/day) to serum FGF23 levels should correlate with nephron number and is defined as the nephron index. In fact, a decrease in the nephron index was observed in patients with early-stage renal dysfunction and was identified as an independent risk factor for macroangiopathy in patients with diabetes mellitus[198].

As FGF23 increases phosphate excretion per nephron, it should increase the urinary fractional excretion of phosphate (FEP), which is defined as the ratio of phosphate clearance $\left(=\frac{Up \times V}{Pp}\right)$ (3) to creatinine clearance $\left(=\frac{Ucr \times V}{Pcr}\right)$ (4).

$$FEP = \frac{\frac{Up \times V}{Pp}}{\frac{Ucr \times V}{Pcr}} = \frac{Up \times V}{Ccr \times Pp} \cdots (2) \quad (5)$$

Serum FGF23 levels correlate with FEP in patients with early to mid-stage chronic kidney disease (CKD)[30]; however, the correlation between serum FGF23 levels and FEP is not as strong as would be expected, indicating that the ratio of FEP to serum FGF23 levels is quite variable and depends on the renal sensitivity to FGF23. From equation 1,

$$Up \times V = \text{Nephron index} \times [FGF23] \quad (6)$$

Substitute $\text{Nephron index} \times [FGF23]$ (7) for $Up \times V$ (8) in equation 2.

$$FEP = \frac{\text{Nephron index} \times [FGF23]}{Ccr \times Pp} \quad (9)$$

$$\therefore \frac{FEP}{[FGF23]} = \text{Nephron index} \times \frac{1}{Ccr \times Pp} \cdots (3) \quad (10)$$

Equation 3 indicates that the renal sensitivity to FGF23 (that is, the ratio of FEP to FGF23) correlates with the nephron number among patients with CKD who have comparable renal function (that is, Ccr) and serum phosphate levels (that is, Pp). Therefore, patients with reduced renal sensitivity to FGF23 (that is, a low FEP/FGF23 ratio) might have poor clinical outcomes[30] because they have fewer nephrons. In addition, assuming that Ccr correlates with estimated glomerular filtration rate (eGFR), equation 3 can be converted to:

$$\text{Nephron index} \propto \frac{FEP \times eGFR \times Pp}{[FGF23]} \quad (11)$$

Thus, by assuming that FGF23 and Ccr correlate with FEP and eGFR, respectively, the nephron index can be estimated by measuring phosphate and creatinine levels in the blood and spot urine collections, together with serum FGF23 levels[199]. Further investigation is necessary to determine whether the nephron index reflects the functional nephron number and is a useful parameter in the management of patients with CKD.

Ccr, creatinine clearance; Pcr, serum creatinine concentration; Pp, serum phosphate concentration; Ucr, urinary creatinine concentration; Up, urinary phosphate concentration; V, 24-hour urinary volume.

Box 3 | **Bony fish and the evolution of the *Klotho* genes**

Consistent with the notion that life arose in the oceans, the composition of the human body resembles that of sea water. In fact, nine of the ten most abundant elements in the human body are present in sea water[200]; the exception is phosphorus[87], implying that this element was selectively acquired at some point during evolution. This change occurred 400 million years ago in the Devonian period, when bony fish evolved and accumulated phosphorus in their bones as calcium phosphate[87,201]. Until that time, animal skeletons were made of calcium carbonate or cartilage. Calcium phosphate bones, especially hydroxyapatite, are harder and stronger than calcium carbonate bones or cartilage and are considered to have been a prerequisite for the evolution of terrestrial vertebrates, as they were required to support their body on land without the help of buoyancy. However, the evolution of calcium phosphate bone must have been accompanied by the development of mechanisms to prevent 'phosphate toxicity', which results from the growth of calcium phosphate crystals in extraosseous tissues. The formation of calciprotein particles might be regarded as one of these mechanisms. Of note, orthologues of *Klotho* genes exist only in animals that have calcium phosphate bones[201], suggesting that the FGF23–αKlotho endocrine axis also evolved to prevent phosphate toxicity by maintaining strict phosphate homeostasis.

FGF23-independent activity of αKlotho

Production of soluble αKlotho

αKlotho is a type I single-pass transmembrane protein that comprises a very short intracellular domain and a large extracellular domain (~950 amino acids) that contains two tandem internal repeats with 21% amino acid identity, termed the KL1 (~450 amino acids) and KL2 (~430 amino acids) domains[1]. The full length αKlotho protein (~140 kD) can be cleaved by membrane-anchored α-secretases, β-secretases and γ-secretases to release the entire extracellular domain (~130 kD)[109,110], a process termed ectodomain shedding. This soluble αKlotho fragment that is produced by ectodomain shedding can circulate in the blood, urine and cerebrospinal fluid[111,112]. Although the mRNA of a splice variant encoding a putative truncated αKlotho protein (comprising only the KL1 domain) has been detected[113,114], membrane αKlotho and soluble αKlotho are the only two αKlotho protein isoforms that have been convincingly detected in vivo (FIG. 5a). As the ectodomain shedding of αKlotho seems to occur constitutively[112], the serum and/or urine level of soluble αKlotho can serve as a surrogate marker of renal αKlotho expression and possibly of functional nephron number. In fact, serum levels of soluble αKlotho decline with CKD progression, starting as early as stage 2 CKD[115].

Although soluble αKlotho can form complexes with FGFRs, which FGF23 binds to with high affinity, the level of activation of the canonical intracellular FGF signalling pathway by soluble αKlotho is substantially lower than by membrane-bound αKlotho[2]. This difference in signal strength suggests that the short intracellular domain of αKlotho, which is absent in ternary complexes containing soluble αKlotho, might contribute to signal transduction via the tyrosine kinase domain of FGFRs and FRS2α. The serum concentration of FGF23 and soluble αKlotho is ~40 pg/ml (~1.3 pM) and ~500 pg/ml (~4 pM), respectively, in healthy humans[116]. As the activation of FGFRs in cultured HEK293 cells requires a large dose of FGF23 (0.5–10 nM) and soluble αKlotho (10 nM)[20], it is unlikely that soluble αKlotho can promiscuously mediate FGF23-dependent signalling in organs that do not already express membrane αKlotho; however, all tissues that have been examined express either FGFR1c, FGFR3c or FGFR4 (REF.[26]). Instead, it is plausible that the physiological functions of soluble αKlotho, if any, might be independent of FGF23.

Activity of soluble αKlotho

The KL1 and KL2 domains in αKlotho are homologous to family 1 glycosidases[117] and share the highest homology with mammalian lactase-phlorizin hydrolase (LPH)[1]. LPH is a transmembrane protein that is present in the brush border membrane of the small intestine, where it hydrolyses lactose in milk to produce galactose and glucose; a deficiency in this enzyme can cause lactose intolerance[118]. All family 1 glycosidases have two conserved glutamate residues in their active site, which function as a pair of acid–base catalysts and are indispensable for their enzymatic activity. However, one of these two glutamate residues is not conserved in KL1 and KL2 (REF.[119]), raising the possibility that soluble αKlotho might not have glycosidase activity but instead might function as a lectin (that is, a sugar-binding protein).

Soluble αKlotho is thought to regulate the activity of several ion channels and transporters, including the calcium-selective channel TRPV5 and the ATP-sensitive inward rectifier potassium channel 1 (ROMK1). This regulation was originally thought to occur through the removal of terminal sialic acids from the *N*-linked glycans in these proteins[120,121], mediated by a putative sialidase activity of soluble αKlotho, as the use of a sialidase inhibitor blocked this effect of soluble αKlotho. This process was thought to create ligands for galectin 1 by exposing the underlying disaccharide galactose-*N*-acetylglucosamine (GalNAc) on both channels. Galectin 1 is an abundant lectin in the extracellular matrix, which tethers receptors and transporters to the cell surface, including cytokine receptors and glucose transporters, and thereby increases their cell-surface abundance. Soluble αKlotho was thought to increase the interaction of these transporters with galectins, thereby increasing the cell-surface abundance of TRPV5 and ROMK1 and leading to an increase in calcium reabsorption and potassium secretion, respectively[122,123]. However, the solved crystal structure of the αKlotho–FGF23–FGFR ternary complex excludes the possibility that αKlotho binds to and hydrolyses glucosylceramide[20], suggesting that αKlotho might lack sialidase activity.

Interestingly, another group modelled the structure of soluble αKlotho in the absence of FGF23 and FGFR and identified a sugar-binding site that might mediate binding to α2,3-sialyllactose[124], suggesting that αKlotho might function as a lectin rather than as an enzyme. If soluble αKlotho contains galectin 1 ligands, it might regulate TRPV5 and ROMK1 abundance by binding to α2,3-sialyllactose in the glycans of these ion channels, thereby indirectly tethering them to the cell surface via galectin 1. The possibility that soluble αKlotho may interact with specific sugars needs to be carefully evaluated. Of note, FGF23 is also reported to activate TRPV5 in a membrane αKlotho-dependent manner in the distal tubules[44], and soluble αKlotho was reported to activate Na/K ATPase and contribute to PTH secretion in isolated parathyroid glands, independently of FGF23[125].

Colloids
Uniform mixtures of small particles (dispersoids) in the dispersion medium. Dispersoids are not dissolved but are evenly distributed in the dispersion medium.

Arteriosclerosis
A condition that includes two distinct pathologies, atherosclerosis and vascular calcification. Atherosclerosis is characterized by the accumulation of foam cells (macrophages laden with lipids) in the tunica intima, potentially causing obstruction of blood flow. By contrast, vascular calcification occurs in the tunica media and minimally obstructs the blood flow but increases vascular stiffness.

Flow-mediated dilatation
Clinical test in which the expansion rate of the brachial artery is calculated to evaluate vascular endothelial function.

A renoprotective activity of αKlotho has been reported in various animal models of acute kidney injury (AKI) and CKD. A pioneering study showed that adeno-associated virus (AAV)-mediated gene transfer of membrane αKlotho prevented AKI in a rat ischaemia–reperfusion injury (IRI) model of AKI[126]. Similar AAV approaches have been used to treat CKD in spontaneously hypertensive rats[127], in mice with genetically induced glomerulonephritis[128] and in mice with uninephrectomy and dietary phosphate-induced interstitial fibrosis[128]. Although all of these models were successfully treated by introducing a transgenic allele that overexpressed membrane αKlotho, it is unclear whether membrane αKlotho, soluble αKlotho or both isoforms were required for the therapeutic effects, as serum levels of soluble αKlotho are also increased when membrane αKlotho is overexpressed[129]. More recent studies have demonstrated that recombinant soluble αKlotho protein can improve outcomes in models of AKI induced by IRI[130], reduce renal fibrosis induced by unilateral ureteral obstruction[131] and slow the transition of IRI-induced AKI to CKD[132].

The protective effect of soluble αKlotho in models of kidney injury is probably related to its ability to regulate the activity of several growth factors and ion channels and/or transporters in an FGF23-independent manner[10] (FIG. 5b). For example, TGFβ1 is a potent inducer of epithelial–mesenchymal transition, which is an important feature of organ fibrosis and cancer metastasis[131], but soluble αKlotho can inhibit TGFβ1 by binding to TGFβ receptor type 2 (TGFBR2). Soluble αKlotho also suppresses the activity of WNT, which is another potent inducer of epithelial–mesenchymal transition, by directly binding to WNT3 and WNT4 (REF.[133]). In addition, soluble αKlotho binds to lipid rafts in the plasma membrane and can inhibit PI3K activation, which is induced by various growth factors, such as insulin-like growth factor I (IGF1)[129]. Specifically, soluble αKlotho binds to raft-associated, clustered α2,3-sialyllactose in the glycan of monosialogangliosides on the cell surface. The ability of soluble αKlotho to inhibit raft-dependent PI3K signalling contributes to suppression of the activity of the calcium channel short transient receptor potential channel 6 (TRPC6) in podocytes[134], which is activated by its PI3K-dependent translocation to the cell surface. Because increased abundance and/or activation of TRPC6 causes glomerular dysfunction and proteinuria[135,136], soluble αKlotho might also prevent proteinuria.

Importantly, injection of recombinant soluble αKlotho protein increases urinary phosphate excretion and decreases serum phosphate, not only in wild-type mice but also in FGF23-deficient mice[137], which indicates that soluble αKlotho functions as a phosphaturic factor independently of FGF23. In fact, in the absence of FGF23, soluble αKlotho promotes the endocytosis and degradation of NPT2A in cultured proximal tubular cells; downregulation of NPT2A levels was associated with modification of its glycans and was blocked by a β-glucuronidase inhibitor[137], suggesting that soluble αKlotho might function as a β-glucuronidase. This finding is in contrast to those from studies of TRPV5, which showed that modification of TRPV5 glycans by soluble αKlotho[138] was blocked by a sialidase inhibitor[120]. Soluble αKlotho also suppresses the expression of housekeeping sodium-phosphate co-transporters NPT3 (also known as SLC17A2), phosphate transporter 1 (PiT1; also known as SLC20A1) and PiT2 (also known as SLC20A2), which are involved in cellular phosphate uptake[128].

The in vivo phosphaturic activity of soluble αKlotho might be attributable not only to its ability to modify NPT2A glycans but also to its capacity to induce FGF23 expression in bone. Marked elevations of FGF23 levels were observed in a patient with increased plasma soluble αKlotho levels due to a translocation involving the *αKlotho* locus[69], as well as in mice treated with an AAV-producing soluble αKlotho[140]. Furthermore, although the physiological significance of FGF23 regulation by soluble αKlotho remains to be determined, soluble αKlotho may be a useful therapy for vascular calcification, as it is recognized to have not only a renoprotective but also vasoprotective effects. For example, αKlotho deficiency causes a reduction in nitric oxide production and in resistance to acetylcholine-induced vasodilation[141]. In addition, AAV-mediated stable delivery of soluble αKlotho increased serum FGF23 levels, lowered serum phosphate levels and ameliorated vascular calcification in αKlotho-deficient mice and in CKD–MBD mice that lack the leptin receptor and endothelial nitric oxide synthase [140].

αKlotho deficiency also compromises endothelial integrity and leads to increased vascular permeability that is associated with a rise in the expression of several factors that are also relevant to cell senescence, including plasminogen activator inhibitor 1 (PAI1), $p21^{Cip1}$, $p16^{Ink4a}$ and senescence-associated β-galactosidase[142]. Several genetic and pharmacological interventions, including administration of inhibitors of μ-calpain[143] or cyclooxygenase 2 (COX2, also known as PGHS2) and ablation of *Cox2*, were reported to improve vascular calcification in αKlotho-deficient mice without substantially reducing serum phosphate levels[144]. Whether μ-calpain, COX2 and/or cellular senescence contribute to the vascular calcification observed in the vast majority of elderly individuals or patients with CKD remains to be determined.

Table 1 | **Colloids in the blood**

Colloid	Insoluble material	Protein	Storage organ	Pathology caused by ectopic storage and/or accumulation
Lipoprotein	Lipid	Apoprotein	Fat	Atherosclerosis, lipotoxicity
Calciprotein particle	Calcium and inorganic phosphate	Fetuin A	Bone	Calcification of blood vessels, symptoms of premature ageing

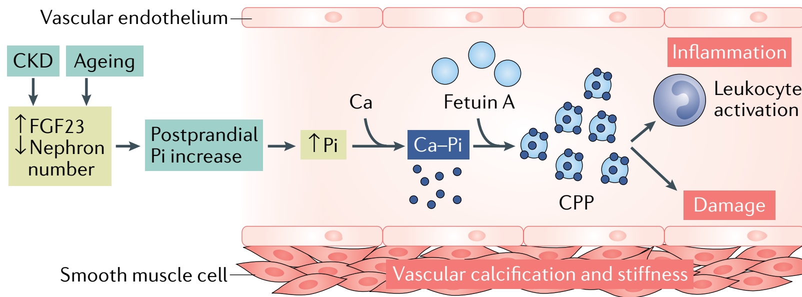

Fig. 4 | **CPP-mediated inflammation and vascular damage.** When nephron number is decreased and fibroblast growth factor 23 (FGF23) levels are increased in the context of chronic kidney disease (CKD) and ageing, the postprandial increase in serum inorganic phosphate (P_i) levels is likely to be enhanced and prolonged. This rise in serum P_i can potentially lead to the formation of calciprotein particles (CPPs), which are colloids that comprise deposits of calcium and P_i (Ca–P_i) adsorbed onto fetuin A. CPPs can induce immune cell activation and trigger an inflammatory response, as well as contribute to arteriosclerosis by inducing vascular damage. In the vasculature, endothelial cell death and dysfunction is accompanied by osteoblastic transformation and calcification of smooth muscle cells. Adapted with permission from REF.[202]: Kuro-o, M. Klotho and endocrine fibroblast growth factors: marker of chronic kidney disease progression and cardiovascular complications? *Nephrol. Dial. Transplant.* https://doi.org/10.1093/ndt/gfy126 (2018), by permission of Oxford University Press.

The FGF21–βKlotho endocrine axis

Various types of physiological stress, including starvation and inflammation, lead to an increase in FGF21 levels in the blood. FGF21 binds to the βKlotho–FGFR1c complex and induces a shift to catabolic metabolism. In addition, FGF21 activates the sympathetic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis. Consequently, FGF21 can be considered to be a stress hormone[10].

The FGF21–βKlotho axis in health

FGF21 was originally identified in research efforts to discover novel therapeutic agents for the treatment of diabetes mellitus, by screening endogenous secreted factors that promote glucose uptake in cultured fibroblasts and adipocytes[145]. In mouse models of diabetes in which this metabolic disorder results from defective leptin signalling (*ob/ob* and *db/db* mice), injection of recombinant FGF21 lowered blood glucose and triglyceride levels and improved insulin sensitivity[145]. Transgenic mice that overexpress FGF21 in the liver under the control of an apolipoprotein E promoter exhibited a metabolic state that resembled fasting, even in the fed state, and that was characterized by lipolysis in white adipose tissue (WAT), ketogenesis, gluconeogenesis, fatty acid oxidation in the liver and torpor[146]. These transgenic mice were also resistant to growth hormone (GH) and had reduced expression of IGF1 in the liver compared with that in wild-type mice[147]. Although βKlotho is expressed in both WAT and the liver, injection of FGF21 into mice does not result in activation of the canonical FGF signalling pathway in the liver[18], as FGFR4 (the receptor for FGF19) is expressed in the liver, whereas FGFR1c is barely detectable. Thus, FGF21 is unlikely to act directly on the liver in an autocrine manner and, instead, its effects on the liver might be partly explained by the increased inflow of fatty acids from WAT[148]. In the liver, fatty acids are metabolized through the β-oxidation pathway to generate acetyl CoA that then enters the ketogenic pathway, thereby explaining the effect of FGF21 on fatty acid β-oxidation and ketogenesis[149]. Of note, FGF21-induced changes in hepatic metabolism in vivo are not recapitulated in isolated perfused livers[145]. Furthermore, deletion of *Fgfr1* in adipose tissue abolishes most of the hepatic effects of FGF21 (REF.[150]). These findings support the notion that FGF21 indirectly regulates liver function. However, some contradictory findings have been reported, including the observation that the injection of FGF21 into mice induces the phosphorylation of hepatic ERK1 and ERK2 within 5 minutes[151]. Further studies are necessary to determine whether FGF21 directly acts on the liver in physiological or pathological conditions.

All of the ‘energy conserving’ effects of FGF21 on metabolism and growth are advantageous under conditions of nutritional deficiency. In fact, transgenic mice that overexpress FGF21 resemble not only calorie-restricted mice in terms of their metabolic state, but also dwarf mice in terms of the paucity of GH action. As calorie restriction and attenuation of the GH–IGF1 endocrine axis have been identified as interventions that favour longevity in various experimental animals[152], the finding that FGF21-overexpressing transgenic mice have a 36% longer median survival time compared with non-transgenic control mice[19] was anticipated and indicates that FGF21 has anti-ageing effects. Provocatively, calorie restriction has been identified as an intervention that suppresses ageing and extends lifespan in all organisms tested so far[152], and therefore it is possible that FGF21, as a starvation hormone, has a role in this process.

FGF21 can cross the blood–brain barrier[153] to act on the central nervous system[154]. In the brain, βKlotho and FGFR1c are expressed in both the suprachiasmatic nucleus (SCN) and the nucleus of the solitary tract (NTS), which are considered direct targets of FGF21. In the SCN, FGF21 increases the expression of corticotropin-releasing factor (CRF) in the paraventricular nucleus, which activates the HPA axis and leads to an increase in serum corticosterone levels. CRF also activates the sympathetic nervous system to induce lipolysis[154]. Because an increase in sympathetic activity is associated with increased energy expenditure, weight loss, improved insulin sensitivity, increased gluconeogenesis and a reciprocal decrease in liver glycogenesis, the ability of FGF21 to activate the sympathetic nervous system might contribute to its complex effects on the liver. These effects include the acute induction of PPARγ co-activator 1α (*Pgc1a*) expression in the liver within 15 minutes of injecting FGF21 into mice, which induces the expression of several genes that are important in fatty acid oxidation, the tricarboxylic acid cycle, mitochondrial oxidative phosphorylation and gluconeogenesis[148]. Although its effects on the NTS remain to be determined, FGF21 seems to not only act as a metabolic regulator of fasting, as initially thought, but as its effects include the activation of the sympathetic nervous system and the elevation of blood corticosterone levels, it

Torpor
Short-term hibernation-like state that is associated with a low body temperature and inactivity.

Suprachiasmatic nucleus
(SCN). Cluster of neurons in the hypothalamus that function as the master circadian pacemaker. Some of these neurons have a direct projection to corticotropin-releasing factor-producing neurons in the paraventricular nucleus.

Nucleus of the solitary tract
(NTS). Cluster of sensory neurons in the medulla oblongata that are innervated from some cranial nerves, including vagus nerves, and project to various nuclei in the brainstem and parasympathetic neurons. It is also known as the central relay for the baroreflex that maintains blood pressure.

Progeroid syndrome
Hereditary disorder in which there are multiple signs and symptoms of ageing in individuals in the early stages of life. Examples include Werner syndrome, Hutchinson–Gilford syndrome, xeroderma pigmentosum and Cockayne syndrome. Patients with progeroid syndromes have defects in DNA repair systems.

should also be considered an autonomic and endocrine regulator of stress responses in general (FIG. 6).

The FGF21–βKlotho axis in disease

Serum levels of FGF21 increase in patients with CKD, as early as stage 2, and continue to rise with declining renal function[155]. Given the anti-ageing effects of FGF21, high levels of circulating FGF21 might be expected to extend lifespan but, in fact, all-cause mortality is high among patients with CKD. A similar phenomenon is observed in mouse models of human premature ageing syndromes[156]. Mice lacking *Ercc1* have a defect in DNA repair (nuclear excision repair) and develop a progeroid syndrome that resembles the human genetic disorder xeroderma pigmentosum. ERCC1-deficient mice also have defects in GH–IGF1 signalling, and their metabolic state is characteristic of calorie restriction[157]; these responses can be regarded as 'longevity assurance programmes' that extend lifespan in mice with an intact DNA repair system. Activation of these longevity assurance programmes in mice with premature ageing syndromes is interpreted as a survival response against genotoxic stress that is caused by an accumulation of somatic DNA damage[158]. Similarly, analogous longevity assurance programmes might be activated in patients with CKD, for example, by promoting an increase in levels of the anti-ageing hormone FGF21, in an attempt to increase survival.

Although an increase in FGF21 levels may be advantageous for surviving CKD, increased FGF21 may also have adverse effects. For example, FGF21 overexpression in transgenic mice has several detrimental effects, including growth retardation, osteopenia, disturbed circadian behaviour, increased sympathetic activity and high serum corticosterone levels[154]. Growth retardation is caused by attenuation of the GH–IGF1 endocrine axis[147] and might underlie the growth retardation and GH resistance observed in children with CKD. The osteopenia in FGF21-overexpressing transgenic mice is associated with decreased osteoblastogenesis and increased adipogenesis in the bone marrow[159] and potentially contributes to the pathophysiology of CKD–MBD. Disturbed circadian behaviour is caused by the actions of FGF21 in the SCN, the centre of circadian rhythm regulation, as SCN-specific ablation of *βKlotho* rescues this phenotype in FGF21-overexpressing trangenic mice[154]. We can also speculate that the circadian rhythm disturbance caused by high FGF21 levels may contribute to the abnormal sleep–wake cycles and daily blood pressure fluctuations that are observed in patients with CKD, which increases their risk of cardiovascular events[160]. In addition to disturbed sleep–wake cycles, high FGF21 levels can cause a chronic increase in serum corticosterone levels; both these effects are known to cause depression[161], which is associated with a poor prognosis in patients with CKD[162]. Consistent with this notion, high FGF21 levels are associated with a poor prognosis in patients with CKD who require dialysis[163]. Although it is still necessary to determine which CKD complications are attributable to the high FGF21 levels that are present in patients with CKD, the available insights suggest that the FGF21–βKlotho endocrine axis is a potential new therapeutic target in CKD.

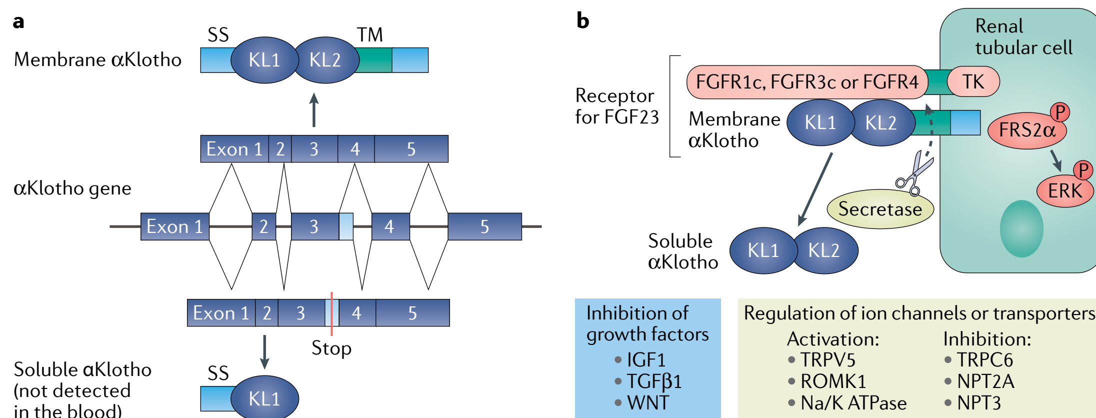

Fig. 5 | **Membrane αKlotho and soluble αKlotho. a** | The *αKlotho* gene and its splice variants. The *αKlotho* gene comprises 5 exons. Alternative splice donor sites are located at the carboxyl terminus of the third exon, which give rise to mRNAs that encode membrane αKlotho and putative secreted αKlotho. The extracellular domain of membrane αKlotho contains two domains, termed KL1 and KL2, which share sequence homology with family 1 glycosidases. The splice variant that encodes the secreted isoform contains a stop codon upstream of exons 4 and 5, which encode the transmembrane and cytoplasmic domains. However, this truncated protein isoform has thus far not been detected in blood. **b** | Membrane αKlotho functions as the obligate co-receptor for fibroblast growth factor 23 (FGF23) to activate the canonical FGF signalling pathway that is transduced through the tyrosine kinase (TK) domain of FGF receptor (FGFR) and leads to the phosphorylation of FGFR substrate 2α (FRS2α) and ERK1 and ERK2. In addition, membrane-bound αKlotho can also be cleaved by membrane-anchored secretases, which releases the extracellular domain of membrane αKlotho into the extracellular space by ectodomain shedding. This soluble αKlotho protein can regulate several ion channels and transporters and inhibit growth factors, including insulin-like growth factor 1 (IGF1), transforming growth factor β1 (TGFβ1) and WNT. The ion channels and transporters activated by soluble αKlotho include transient receptor potential cation channel subfamily V member 5 (TRPV5), renal outer medullary potassium channel 1 (ROMK1), and Na/K ATPase, whereas short transient receptor potential channel 6 (TRPC6), sodium-dependent phosphate transport protein 2A (NPT2A) and NPT3 are inhibited. SS, signal sequence; TM, transmembrane.

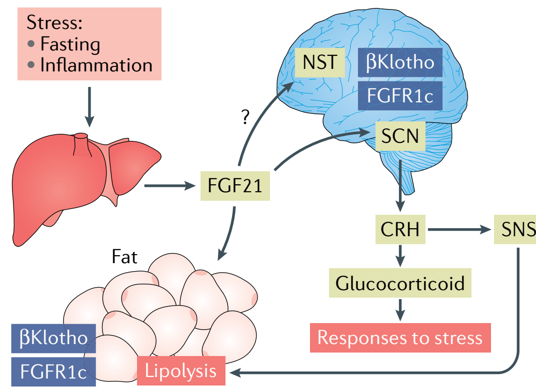

Fig. 6 | **The FGF21–βKlotho endocrine axis.** Fibroblast growth factor 21 (FGF21) is secreted by the liver in response to various types of stress, including fasting and inflammation. FGF21 acts on white adipose tissue, in which βKlotho and FGF receptor 1c (FGFR1c) are co-expressed, to induce lipolysis. Furthermore, FGF21 crosses the blood–brain barrier and acts on the suprachiasmatic nucleus (SCN) and perhaps on the nucleus of the solitary tract (NTS), in which βKlotho and FGFR1c are also co-expressed. FGF21 induces the expression of corticotropin-releasing hormone (CRH), resulting in an increase in serum glucocorticoid levels. CRH also activates the sympathetic nervous system (SNS) and this contributes to lipolysis. Thus, FGF21 induces responses to stress by activating the hypothalamic–pituitary–adrenal axis and the SNS.

The FGF19–βKlotho endocrine axis

The expression of FGF19 (FGF15 in rodents) is induced by bile acids. Bile acids that are released into the intestinal lumen enter intestinal epithelial cells and bind to the bile acid receptor. The bile acid receptor bound to bile acid forms a heterodimer with RXR that directly transactivates expression of *FGF19* (REF.[164]). This regulation is similar to the mechanisms by which induced by PPARα[146,165] and VDR[31] heterodimerize with RXR to control the expression of *FGF21* and *FGF23*, respectively. Thus, expression of endocrine FGFs, including FGF19, can be regulated by lipophilic ligands and their nuclear receptors[166].

The FGF19–βKlotho axis in health

FGF19 is secreted by intestinal epithelial cells upon feeding and enters the portal circulation to be delivered to hepatocytes, which express both βKlotho and FGFR4 (REF.[10]). FGF19 signalling through the βKlotho–FGFR4 complex suppresses the expression of the cholesterol 7α-hydroxylase gene (*CYP7A1*)[164] (FIG. 7), which encodes a rate-limiting enzyme in bile acid synthesis, and thereby downregulates bile acid synthesis. This negative feedback loop on bile acid synthesis mediated by the FGF19–βKlotho endocrine axis is indispensable for maintaining bile acid homeostasis. In fact, mice lacking either FGF15 (REF.[164]), βKlotho[167] or FGFR4 (REF.[168]) have defects in postprandial suppression of bile acid synthesis, which results in an increased bile acid pool size and faecal bile acid excretion. In addition, FGF19 exerts an insulin-like activity that increases protein and glycogen synthesis independently of insulin[169]. Thus, FGF19 functions as a regulator of postprandial metabolism in the liver.

Bile acid pool
Amount of bile acids held in the intestine, portal circulation, liver and gall bladder. Bile acids secreted into the intestine are mostly reabsorbed and returned to the liver to be secreted into the intestine again (enterohepatic circulation). The liver synthesizes the same amount of bile acids that are lost in the faeces to maintain the bile acid pool size.

In contrast to FGF21, which activates βKlotho–FGFR1c but not βKlotho–FGFR4, FGF19 can activate both βKlotho–FGFR4 and βKlotho–FGFR1c[18]. Therefore, FGF19 is expected to exert FGF21-like activity. In fact, transgenic overexpression of FGF19 (REF.[170]) or administration of FGF19 (REF.[171]) in mice increases energy expenditure, decreases body weight and improves insulin sensitivity. However, mice lacking FGF21 are incapable of inducing metabolic responses to fasting[146], which indicates that FGF15 cannot compensate for a loss of FGF21 under physiological conditions. Thus, the FGF21-like activity of FGF19 should be regarded as a pharmacological function that is observed only when exogenous FGF19 is administered. A possible explanation for the inability of FGF19 to compensate for the loss of FGF21 is that the liver might clear FGF19 to prevent it from leaking into the systemic circulation. However, whether this hepatic clearance occurs is uncertain, as one clinical study found that FGF19 levels in portal and systemic blood were similar[172]. Why FGF19 is unable to act systemically under physiological conditions remains to be determined.

The FGF19–βKlotho axis in disease

Transgenic mice that overexpress FGF19 in skeletal muscle develop hepatocellular carcinoma, and accumulating evidence indicates that increased FGF19 levels promotes not only liver cancer but also several other cancer types, including breast, prostate and colon cancer[173–175]. Conversely, decreased FGF19 levels are associated with bile acid diarrhoea, which accounts for 30% of patients with chronic diarrhoea[176]. Although the connection between the FGF19–βKlotho endocrine axis and CKD is poorly understood, patients with CKD frequently show dysbiosis, which may be indicative of FGF19 dysregulation[177]. Ligands of the host bile acid receptor include not only primary bile acids (for example, chenodeoxycholic acid) but also secondary bile acids, such as deoxycholic acid, which are metabolites of primary bile acids that are produced by intestinal bacteria (FIG. 7). Thus, the type and number of enteric bacteria might influence FGF19 expression levels. Conversely, FGF19 expression levels might indirectly influence the enteric microbiota through their regulation of bile acid synthesis, as the type and amount of bile acids in the enteric lumen affects bacterial flora[178]. Interestingly, patients with CKD have an attenuated postprandial FGF19 response[179]. Further studies are needed to evaluate the potential effect of the FGF19–βKlotho endocrine axis on the pathophysiology of CKD.

Questions about the FGF–Klotho axes

Limitations of research tools

The identification of genetic and pharmacological interventions that can modify the phenotype of αKlotho-deficient mice (*kl/kl* mice and mice lacking *αKlotho*) is an important strategy for the elucidation of disease mechanisms. For example, the finding that lowering serum phosphate levels through diet[180] or ablation of *Npt2a*[181] prevented vascular calcification in αKlotho-deficient mice without lowering serum levels of calcium and 1,25-dihydroxyvitamin D_3 suggested

that vascular calcification is not attributable to hypercalcaemia or hypervitaminosis D but is primarily due to hyperphosphataemia. Of note, however, the *kl* allele is not a null allele but is a severe hypomorphic allele[1]. The *kl* allele has an insertion mutation in the 5′ flanking region of the *αKlotho* gene that results in extensive DNA methylation across the insertion site and the first exon of the *αKlotho* gene, abolishing αKlotho expression[182]. Therefore, the open reading frame is intact in the *kl* allele and thus αKlotho expression could potentially be reactivated. In fact, αKlotho expression in *kl/kl* mice was partially restored by a low phosphate diet[180], which raises the possibility that an induction of endogenous αKlotho expression might have contributed to the prevention of vascular calcification. The absence of vascular calcification in mice deficient for both αKlotho and NPT2A[183] supports a central role of hyperphosphataemia in vascular calcification but it will be important to determine whether endogenous αKlotho expression was also restored after other interventions that rescue *kl/kl* phenotypes.

The lack of specificity of anti-αKlotho antibodies is another pitfall in studying αKlotho. We have tested several commercially available anti-αKlotho monoclonal antibodies for their specificity for mouse αKlotho protein, using kidney lysates and paraffin sections from wild-type mice and *kl/kl* mice (as a negative control). Only two antibody clones, KM2076 (TransGenic Inc.) and BAF1819 (R&D Systems), were specific for mouse αKlotho by immunoblotting and immunohistochemistry, respectively (M. Kuro-o, unpublished observations). The use of potentially nonspecific anti-αKlotho antibodies could explain the current controversy about the expression of endogenous αKlotho in the vasculature[184]. Although low levels of αKlotho mRNA are detectable in the aorta of mice and humans, to date no compelling evidence exists that functional endogenous αKlotho protein is present in healthy vasculature[185].

A similar problem may exist in clinical studies that have measured levels of circulating αKlotho during CKD progression, for which conflicting reports exist. Some studies reported a decrease in soluble αKlotho, whereas others showed no change or an increase[115]. Several enzyme-linked immunosorbent assay (ELISA) kits for serum αKlotho are commercially available, but one of the most widely used assays (IBL International) is specific for the human αKlotho protein[116] and therefore no negative control is available. A different assay involves immunoprecipitation (IP) of αKlotho with a synthetic anti-αKlotho antibody and detection of the protein with KM2076 by immunoblotting (IB)[115]. Although far from high-throughput, this IP–IB assay has been validated for use in both humans and mice, and its specificity has been confirmed by showing that αKlotho is undetectable in the serum of *kl/kl* mice[115]. However, measurement of αKlotho levels in the same serum samples using both the ELISA kit and the IP–IB assay failed to show a consistent correlation[115]. The ELISA kit gave higher values in fresh samples and lower values in freeze-thawed samples than the IP–IB assay. Furthermore, even though the kidney is known to be the major source of circulating αKlotho in rodents and humans[112], the ELISA kit detected a considerable amount of αKlotho in the serum of patients on dialysis, despite negligible αKlotho expression in the kidneys[186]. By contrast, with the IP–IB assay, αKlotho was barely detectable in the serum or urine from patients on dialysis[115], suggesting that the ELISA kit might detect a crossreacting protein that is degraded after repeated freeze-thaw cycles.

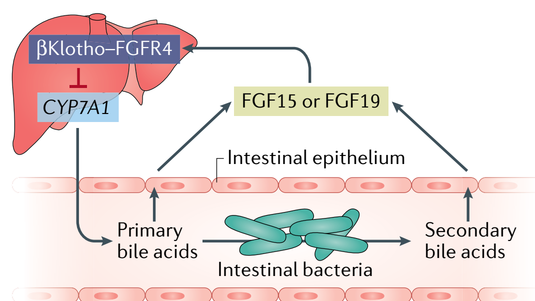

Fig. 7 | **The FGF15/FGF19–βKlotho endocrine axis.** Fibroblast growth factor 19 (FGF19; FGF15 in rodents) is secreted by intestinal epithelial cells in response to primary bile acids (that is, bile acids that are released from the liver) and secondary bile acids (that is, primary bile acid metabolites that are produced by intestinal bacteria). FGF19 binds to the βKlotho–FGF receptor 4 (FGFR4) complex that is present on hepatocytes to suppress the expression of *CYP7A1*, which encodes the rate-limiting enzyme of bile acid synthesis, cholesterol 7α-hydroxylase. Thus, the FGF19–βKlotho endocrine axis potentially affects the composition of the microbiome and vice versa.

Endocrine FGFs and Klotho controversies

Although many questions about the biology of endocrine FGFs and Klothos remain unanswered, two crucial questions are discussed in this section. First, what is the mode of action of FGF23? As the expression of αKlotho is most abundant in the DCTs, FGF23 might be assumed to act directly on this region of the nephron. However, FGF23 functions to suppress the reabsorption of phosphate and the synthesis of active vitamin D, both of which occur in the proximal tubules. To explain this discrepancy, two non-mutually exclusive hypotheses have been proposed. In the first hypothesis, FGF23 actually acts on the proximal tubules, which also express αKlotho, albeit at much lower levels than in the DCTs[187]. If correct, then the role of αKlotho in the DCTs will need to be determined. In the second hypothesis, FGF23 induces the secretion of a paracrine factor in the DCTs that acts on proximal tubules[188]. This putative paracrine factor could be soluble αKlotho itself for several reasons — soluble αKlotho induces phosphaturia independently of FGF23 (REF.[138]); soluble αKlotho is transported from the basal to the apical side of the proximal tubules by transcytosis[112]; and soluble αKlotho modifies glycans on NPT2A on the apical brush border membrane of the proximal tubules to promote the internalization and degradation of NPT2A[138]. In this scenario, FGF23 functions as a soluble-αKlotho-releasing hormone. To test whether αKlotho expression in both the proximal tubules and the DCTs is required for FGF23 action, mice lacking *αKlotho* specifically in the proximal tubules or the DCTs have been generated. Ablation of the *αKlotho*

gene in the DCTs resulted in a substantial increase in serum FGF23 and phosphate levels, which were associated with an increased level of NPT2A on the brush border membrane of proximal tubules[189]. By contrast, ablation of the *αKlotho* gene in the proximal tubules resulted in a modest or no increase in serum FGF23 and phosphate levels[190]. These studies argue against the first hypothesis; however, whether FGF23 functions as a soluble αKlotho-releasing hormone remains to be determined.

Second, what mechanism controls FGF23 secretion by osteocytes? As discussed earlier[50], both phosphate and calcium are required for the increase in serum FGF23 levels in response to a rise in circulating phosphate. Interestingly, ionized calcium levels in serum must be above a threshold (>1 mmol/l or >4.0 mg/dl) in order for serum FGF23 levels to increase in response to a high phosphate diet in rats[51]. A possible explanation for these findings is that osteocytes sense CPPs but not phosphate or calcium alone and respond to them by secreting FGF23. To test this hypothesis, it would be necessary to determine whether CPPs can leave the blood vessels through bone marrow sinusoids to act directly on osteoblasts and/or move through bone canaliculi to act on osteocytes, as well as which properties of the CPP colloid might be responsible for stimulating FGF23 secretion.

New paradigm for phosphate restriction

Insights into the FGF23–αKlotho endocrine axis in particular demonstrate that in the absence of a reduction in dietary phosphate intake the decrease in nephron number as a result of ageing and/or CKD induces an increase in FGF23 to maintain phosphate homeostasis by increasing phosphate excretion per nephron. As mentioned earlier, renal tubular damage and interstitial fibrosis ensue when phosphate excretion per nephron exceeds ~1.0 μg/day[77], which further reduce nephron number. This spiral of deterioration in nephron number can be predicted to start when the nephron number is decreased by ~50%, assuming that healthy adults excrete ~1 g/day of phosphate into the urine and have ~1 million nephrons per kidney. A ~50% reduction in nephron number is likely to occur in patients with early stage CKD, as a reduction of the same magnitude occurs in elderly individuals as a consequence of natural ageing[73].

Although uninephrectomy in healthy kidney donors also results in a 50% reduction in nephron number, this loss might not be equivalent to the 50% loss of nephrons that occurs in ageing or CKD; after uninephrectomy in healthy kidney donors, FGF23 levels increase and eGFR decreases[191] but both variables are partially restored during long-term follow up[192]. By contrast, spontaneous improvement of eGFR is not usually expected in the elderly or patients with CKD, possibly because, unlike kidney donors, they have limited renal reserve capacity or have a primary illness that is causing kidney damage. Therefore, in my opinion, an increase in FGF23 levels should be a warning of excess phosphate levels relative to residual nephron number and should prompt consideration of dietary phosphate restriction or the initiation of treatment with phosphate binders in normophosphataemic patients with CKD.

Exercise therapy to prevent bone loss might also be considered a form of 'phosphate restriction', as it could reduce the net efflux of phosphate that is stored in the bone into the systemic circulation. Of note, a 2018 clinical study indicated that adopting a sedentary behaviour (sitting, reclining or lying postures that consume <1.5 metabolic equivalents of energy) for longer than 6–8 h/day was associated with an increased risk of all-cause mortality and cardiovascular mortality[193]. However, it remains to be determined whether sedentary behaviour is associated with high serum FGF23 levels and/or bone loss.

The aim of combining drug therapy, diet and perhaps exercise to achieve phosphate restriction is not to alleviate vascular calcification but to reduce phosphate excretion per nephron, thereby preventing renal tubular damage and fibrosis, and preserve the remaining nephrons. This new paradigm of phosphate restriction challenges existing approaches, which aim to lower serum phosphate levels in patients with hyperphosphataemia to prevent vascular calcification and cardiovascular events.

Table 2 | **The FGF–Klotho endocrine systems in disease**

Hormone	Source	Target organ or tissue	Receptor		Function	Disorder	
			Klotho	FGFR		High levels	Low levels
FGF19	Intestine	Liver	βKlotho	FGFR4	• Metabolic responses to feeding • ↓Bile acid synthesis	Cancer of the liver, colon, breast and prostate	BAD
FGF21	Liver	• Fat • Brain (SCN)	βKlotho	FGFR1c	• Metabolic responses to fasting • ↑SNS activity • ↑HPA axis activity	CKD, T2DM, obesity, NAFLD and CVD	Anorexia nervosa
FGF23	Bone	• Kidneys • Parathyroid gland	αKlotho	FGFR1c, FGFR3c, FGFR4	• Phosphaturia • ↓Active vitamin D • ↓PTH	CKD, ADHR, ARHR, XLH and TIO	FTC

ADHR, autosomal dominant hypophosphataemic rickets; ARHR, autosomal recessive hypophosphataemic rickets; BAD, bile acid diarrhoea; CKD, chronic kidney disease; CVD, cardiovascular disease; FGF, fibroblast growth factor; FGFR, FGF receptor; FTC, familial tumoural calcinosis; HPA, hypothalamic–pituitary–adrenal; NAFLD, nonalcoholic fatty liver disease; PTH, parathyroid hormone; SCN, suprachiasmatic nucleus; SNS, sympathetic nervous system; T2DM, type 2 diabetes mellitus; TIO, tumour-induced osteomalacia; XLH, X-linked hypophosphataemia.

Concluding remarks

A serendipitous discovery of an obscure mouse mutant with ageing-like symptoms has now evolved into a greater understanding of how the FGF–Klotho endocrine system regulates various aspects of mineral metabolism, glucose and fatty acid metabolism, bile acid metabolism, energy expenditure, circadian rhythms and sympathetic activity, responses to stress and ageing. Specifically, FGF19 maintains bile acid homeostasis and induces postprandial metabolic changes in the liver. FGF21 stimulates metabolic responses to fasting and induces stress responses by activating the sympathetic nervous system and the HPA axis. FGF23, together with PTH and active vitamin D, maintains mineral homeostasis. αKlotho, the obligate co-receptor for FGF23, is subject to ectodomain shedding to release soluble αKlotho, which can function as an endocrine or paracrine factor that regulates the activity of several ion channels, transporters and growth factors, independently of FGF23 and FGFRs. This FGF–Klotho system potentially contributes to the pathophysiology of multiple disorders in humans, including CKD, arteriosclerosis, cardiac hypertrophy, diabetes and obesity, and various types of cancer (TABLE 2). Besides CKD, FGF21 levels were reported to increase in patients with type 2 diabetes, obesity, nonalcoholic fatty liver disease and cardiovascular diseases, among others[194]. Furthermore, the fact that overexpression of αKlotho[130] or FGF21 (REF.[19]) extends lifespan in mice and that a specific polymorphism in *αKlotho* correlates with prolonged longevity in humans[195] raises the possibility that αKlotho and FGF21 might counteract ageing processes. Therefore, interventions in the FGF–Klotho endocrine system represent a new approach for the treatment of ageing and ageing-related disorders. Developments in this field will likely be facilitated by structure-based drug design of agonists and antagonists for endocrine FGFs.

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