

FGF23 and Klotho

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1. Introduction

Fibroblast Growth Factor 23 (FGF23) is a protein synthesized by osteocytes and osteoblasts that has been described to have a key role in the „bone-kidney/parathyroid“ axis and the regulation of phosphate/calcium metabolism [1 – 3]. FGF23 acts mainly as a phosphaturic factor and a suppressor of 1α hydroxylase activity in the kidney: it inhibits the expression of type IIa and IIc sodium-phosphate cotransporters on the apical membrane of proximal tubular cells, leading to an inhibition of phosphate reabsorption [4]. Moreover, it also inhibits 1α hydroxylase activity whereas it stimulates the 24 hydroxylase activity, thus leading to a decreased 1,25 dihydroxyvitamin D serum level [5]. These two pathways account together for the hypophosphatemic effect of FGF23. FGF23 was also recently described as an inhibiting factor of parathyroid hormone (PTH) synthesis [6]. Moreover, it is interesting to note that, in contrast to its renal effects, FGF23 can stimulate the local expression of 1α hydroxylase in the parathyroid, suggesting that it can also indirectly down regulate PTH synthesis through an increased local production of calcitriol [7]. The single-pass transmembrane Klotho protein, an anti-aging protein, seems required in vivo for FGF23-mediated receptor activation [8, 9].

2. FGF23: structure and biochemical properties

FGF23 is a 251 amino-acid protein (molecular weight = 30 kDa) with a 24 amino-acid signal peptide in the N-terminal portion; its chromosomal location is 12p13 in humans. It belongs to the FGF family, in the sub-group of the ‘endocrine FGFs’ with FGF19 [10]: indeed, it shares with all the FGF a highly conserved sequence but it also has a unique C-terminal structure as well as a specific three-dimensional configuration (i.e., disulfide bound and β sheet), both accounting for its systemic action [10, 11]. When it was initially described, FGF23 was thought to be cleaved by a specific metalloproteinase called PHEX (phosphate-regulating gene with homologies to endopeptidases on X chromosome) whose role has been highlighted in hypophosphatemic rickets; however, these initial findings have never been reproduced and this part of FGF23 metabolism remains to be fully understood [10, 11]. However, FGF23 can be proteolytically cleaved between Arg¹⁷⁹ and Ser¹⁸⁰, in the Arg¹⁷⁶-X-X-Arg¹⁷⁹ region [4]. The active form of FGF23 corresponds to the protein before cleavage, from the 25th to the 251st amino acid; in contrast, the inactive form of FGF23 is obtained after cleavage [11].

3. Animal models and FGF23

Mice knock-out for FGF23 present with a decreased longevity, in association with growth retardation, skin atrophy, decreased bone density and ectopic as well as vascular calcifications. In addition, they present with hyperphosphatemia, hypercalcemia and increased serum levels of 1,25 dihydroxy vitamin D [12]. These mice also have a trend toward an increased sensitivity to insulin and are therefore at increased risk of hypoglycaemia. In these animals, a diet with low phosphate intake can improve the clinical phenotype while correcting serum phosphate levels (but without modifying serum calcium and 1,25 dihydroxy vitamin D levels). Similarly, a diet with low native vitamin D can correct serum calcium and 1,25 dihydroxy vitamin D levels (but without modifying serum phosphate levels) as well as improve life expectancy [13].

In contrast, mice over-expressing FGF23 will have a clinical phenotype of hypophosphatemic rickets, with hypophosphatemia, increased phosphaturia and hyperparathyroidism; in these animals serum calcium, 1,25 dihydroxy vitamin D circulating levels and renal function are usually normal [14]. However, the renal expression of some of the regulators of phosphate reabsorption is modified, with decreased Klotho and Npt2a for example [14].

4. The role of Klotho inside and outside FGF23 metabolism

Klotho is a single-pass trans-membrane anti-aging protein (1014 amino-acids, 130 kDa, chromosomal location in 13q12 in humans) that has been recently shown to have wide and important biological effects [15]. Its expression occurs mainly in the kidney and in the parathyroid. Its extra-cellular domain is wide whereas its intra-cellular part is very short; of note, it also exists as a soluble protein. In humans, Klotho polymorphisms have been associated both in a positive and negative way to bone mineral density, life expectancy, cardiovascular events (e.g., ischemic stroke, carotid atherosclerosis), biomarkers of metabolic syndrome (e.g., uric acid levels, lipid and glucose metabolisms), and even to cognitive ability [16 – 21].

Mice over-expressing Klotho have an increased life expectancy [15]. In contrast, mice lacking Klotho expression present with a decreased life span, with skin atrophy, decreased bone density, ectopic calcifications and infertility. In addition, they present with hyperphosphatemia, hypercalcemia and increased serum levels of 1,25 dihydroxy vitamin D [22]. A diet with low native vitamin D can improve the whole phenotype [22]. These similarities between mice knock-out for Klotho and FGF23 have led to the description of the fundamental interplay between these two new cornerstones of phosphate/calcium metabolism.

First, Klotho is an essential player for FGF23 biological activity. Indeed, while FGF23 binds with a modest affinity to multiple receptors belonging to the family of FGF receptors (FGF-R, mainly type 1,3 and 4), Klotho seems required *in vivo* for FGF23-mediated receptor activation (and mainly the FGF-R1c), thus stimulating the phosphorylation pathways downstream the receptor [23]. The highest expression of Klotho/FGF-R complex is in the distal tubule whereas the major biologic effects of FGF23 are located in the proximal tubule [24]; this discrepancy is not explained to date, but recent reports have also described an expression of Klotho in the proximal tubule, with a direct phosphaturic effect only by itself [25]. Of note, the association between Klotho and FGF23 is stabilized *in vitro* by heparin, but its potential impact in human physiology has not been evaluated.

Second, Klotho has also its own role in the regulation of phosphate/calcium metabolism: it can indeed function as an enzyme modifying the sugar chains of transient receptor potential vanilloid type 5 (TRPV5) in the distal tubule, preventing the calcium channel from internalization and inactivation, thus leading to an increased calcium reabsorption [26]. In such a case, Klotho has a β -glucuronidase activity. Moreover, Klotho can also directly regulate PTH synthesis: when intracellular calcium decreases in the parathyroid, the local expression of Klotho increases, therefore inducing an increased activity of the Na-K-ATPase channel, an increased PTH synthesis and a further correction of the hypocalcemic state [27].

Last, apart from its key role in phosphate/calcium metabolism regulation, Klotho has also many other roles in general metabolism. Klotho can regulate other ionic channels, such as the potassium channel ROMK1 (Renal Outer Medullary Potassium channel). In this later setting, its action is similar to the one observed with TRPV5: through a β -glucuronidase activity, it can stimulate urinary excretion of potassium [15]. Moreover, Fischer et al. have recently shown that mice with Klotho deficiency have a decreased extra-cellular volume with hyperaldosteronism ; however of interest, the underlying molecular pathways remain to be proven [28]. In terms of general metabolism, Klotho could also have a role in the regulation of the IGF1 / insulin axis, as well as in the Wnt pathway [15]. It could also have anti-apoptotic properties [29].

Although its expression is mainly located in the parathyroid and the kidney, an expression of Klotho has also been detected in other tissues (e.g., choroid plexus, placenta, endocrine organs such as testis, pancreas and ovary): it is therefore likely that other new roles for Klotho will be highlighted in the future [15].

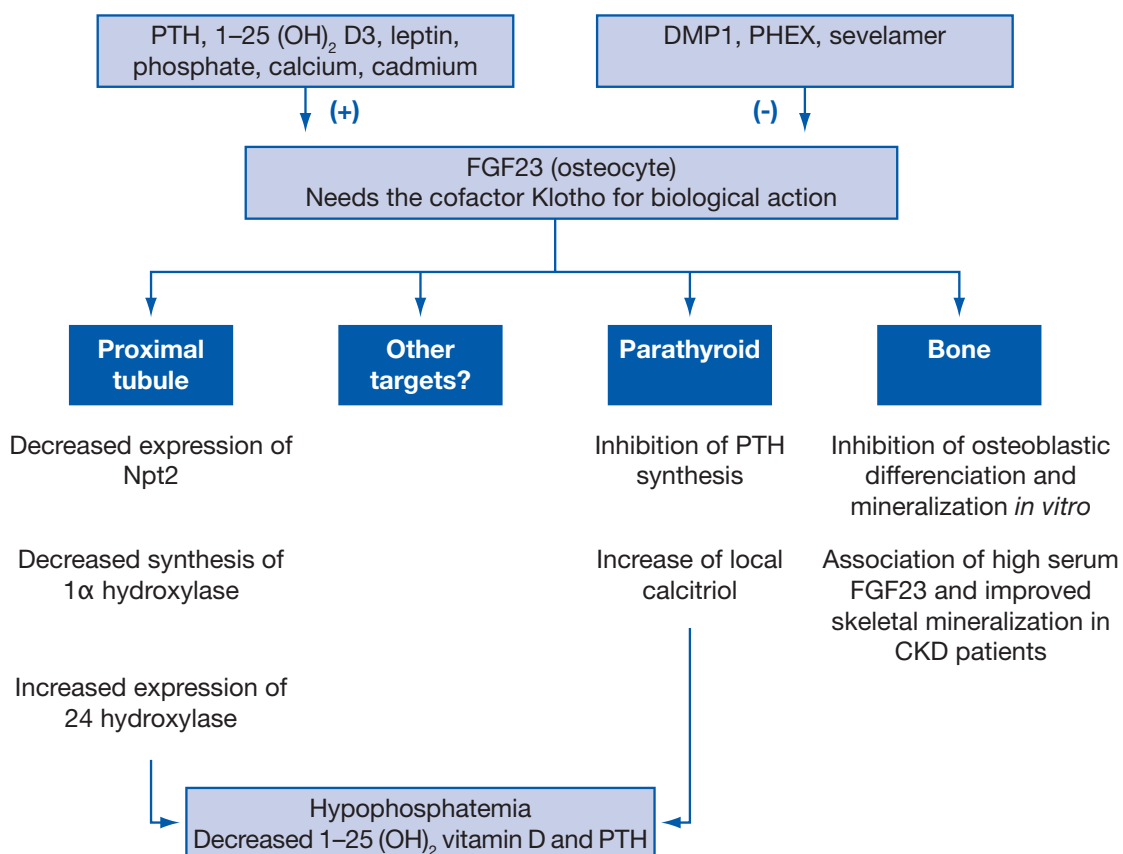
5. Overview of tubular reabsorption of phosphate

Phosphate is a key element for several physiological pathways, such as skeletal development, bone mineralization, membrane composition, nucleotide structure, maintenance of plasma pH, and cellular signaling [30]. The kidney plays a key role in its regulation, mainly through two major hormonal regulators: PTH and FGF23. Indeed, these two hormones have hypophosphatemic effects through a decreased phosphate tubular reabsorption. The third main regulator of phosphate metabolism is the 1-25 dihydroxy-vitamin D that exerts hyperphosphatemic effects via a direct increased phosphate intestinal absorption and an inhibition of PTH synthesis.

Briefly, in terms of molecular regulation, three different families of sodium/phosphate cotransporters have been identified: the SLC17, the SLC20 and the SLC34 families. In humans, the SLC34 family has a key role in phosphate metabolism, with SLC34A1 (also Npt2a, or NaPi-IIa) and SLC34A3 (also Npt2c) expressed in the brush border of the tubular proximal renal cells, while SLC34A2 (also Npt2b) is expressed in intestinal cells. Npt2a, which allows phosphate tubular reabsorption, is probably the most important transporter; its regulation is complex, including oral phosphate intake, PTH, phosphatonins (such as FGF23, frizzled related protein 4 sFRP4, and matrix extracellular phosphoglycoprotein MEPE), estrogens and dopamine [31]. The stabilization on the membrane of Npt2a is done by the NHERF 1 protein (Sodium Hydrogen Exchanger Regulatory Factor) [32, 33]. To date, the role of the SLC17 family has not been demonstrated in humans; in contrast, in the SLC20 family, the SLC20A2 (or Pit2) transporter is also expressed in the proximal renal tubule, and could account for 2 to 3 % of the proximal reabsorption of phosphate [31].

6. FGF23 and Klotho in human physiology

The figure below summarizes the known roles of FGF23 in human physiology in 2011.



(+) corresponding to a positive effect

(-) corresponding to an inhibitory effect

DMP1: dental matrix protein 1

PHEX: phosphate-regulating gene with homologies to endopeptidases on X chromosome

Npt 2: type II sodium-phosphate cotransporters (type a and c)

FGF23 regulation involves both transcriptional and post-translational mechanisms, together with systemic and local bone-derived factors. PTH, vitamin D, phosphate and calcium stimulate FGF23 synthesis whereas glycoproteins synthesized by osteocytes can activate or inhibit FGF23 secretion (e.g., Matrix Extracellular Phosphoglycoprotein MEPE and Dentin Matrix Protein 1 DMP1, respectively) [34]. These two proteins are strongly involved in bone mineralization, but MEPE has also been recently described as a phosphaturic factor in the renal tubules [35].

The role of FGF23 on bone needs further evaluation but recent studies have demonstrated that FGF23 overexpression *in vitro* can suppress not only osteoblast differentiation but also matrix mineralization, independently of its systemic effect on phosphate metabolism [36], whereas Klotho is not expressed in bone. In contrast, other authors have reported a positive effect on FGF23 on bone: for example, Wesseling-Perry and al. have reported an association between high levels of circulating FGF23 and improved indices of skeletal mineralization (i.e., decreased osteoid thickness and shorter osteoid maturation time) in pediatric patients undergoing peritoneal dialysis [37]. In medical conditions such as hypophosphatemic rickets or TIO, the exact role of FGF23 on bone has not been yet established. A recent Japanese report has highlighted that patients with *osteogenesis imperfecta* receiving pamidronate infusions present an acute decrease of FGF23 circulating levels, but the underlying mechanisms remain to be demonstrated [38].

7. FGF23 and Klotho in genetic human diseases

The initial description of FGF23 was done in the early 2000's, with studies focusing on hypophosphatemic rickets [39]. However, it has rapidly been understood that the spectrum of FGF23 deregulation was wider, with the description of its role in tumor induced osteomalacia, and then in patients with chronic kidney disease (CKD).

Mutations of FGF23 and most of its regulators have been reported to account for either hypophosphatemic either hyperphosphatemic diseases. The table below summarizes our current knowledge of genetic diseases associated directly or indirectly (through their regulators) to FGF23 and Klotho [5, 39 – 43].

| | Disease | Involved genes |
|-------------------|--------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hypophosphatemia | Hypophosphatemic rickets | Activating mutation of FGF23 *** Inactivating mutation of PHEX *** Inactivating mutation of DMP 1 *** Inactivating mutation of ENPP1 *** Inactivating mutation of Npt2c ** Activating translocation of Klotho * / ** |
| | With renal lithiasis and/or osteopenia and/or hypercalciuria | Inactivating mutation of Npt2a ** Inactivating mutation of Npt2c ** Inactivating mutation of NHERF1 |
| | Mac Cune Albright / fibrous dysplasia of bone | Overexpression of FGF23, GNAS *** |
| | Tumor induced osteomalacia | Overexpression of FGF23, MEPE, FGF7 and/or FRP4 *** |
| | Epidermal naevus syndrome | FGF-R3 *** |
| Hyperphosphatemia | Familial tumoral calcinosis | Inactivating mutation of FGF23 ** Inactivating mutation of Klotho *** Inactivating mutation of GALNT3 ** |

FGF: fibroblast growth factor

PHEX: phosphate-regulating gene with homologies to endopeptidases on the X chromosome

DMP1: Dentin matrix protein 1

ENPP1: ecto-nucleotide pyrophosphatase / phosphodiesterase 1

Npt2a: type IIa sodium-phosphate cotransporter (SLC34A1)

Npt2c: type IIc sodium-phosphate cotransporter (SLC34A3)

MEPE: matrix extracellular phosphoglycoprotein

FRP4: frizzled related protein 4

FGF-R3: fibroblast growth factor receptor 3

GALNT3: UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3

* with associated hyperparathyroidism

** disease associated with a low FGF23 serum level

*** disease associated with a high FGF23 serum level

Briefly, hypophosphatemic rickets correspond to a heterogeneous genetic pathology, affecting 1/20 000 children, resulting from mutations in FGF23, Klotho or their regulators to induce hypophosphatemia, rickets, dental abnormalities and bone deformations. Patients present with hypophosphatemia, decreased tubular phosphate reabsorption, normal 25OH vitamin D and PTH, increased alkaline phosphatase. The clinical management combines high oral phosphate intake and active vitamin D therapy, allowing a better growth and correcting the bone deformations, but explaining the two main complications often observed in this disease (i.e., secondary hyperparathyroidism secondary to phosphate intake, and nephrocalcinosis secondary to active vitamin D therapy) [44].

While hypophosphatemic rickets are usually diagnosed during early childhood, an acquired disease with the same phenotype can also affect adults (more exceptionally children or teenagers), i.e., tumor-induced osteomalacia (TIO) [45]. These TIO correspond to mesenchymal tumors, usually benign and located in the appendicular skeleton ; they result from an acquired hypersecretion of phosphatonins (mostly FGF23, sometimes sFRP4, MEPE or FGF7) [46]. Patients present with osteomalacia, bone pains, fractures and muscular weakness; a normal phosphate level years or months before the onset of the clinical picture is a strong rationale for an acquired cause of FGF23 hypersecretion. In such a case, the search for the tumor should be performed with accurate tools (tomography, MRI, Tc^{99m} bone scintigraphy, octreotide scintigraphy, F18-fluorodeoxyglucose scan), since these tumors are small and difficult to diagnose in one hand, and since hypophosphatemia will be corrected within days after surgery on the other hand [46].

8. FGF23 and Klotho dysregulation during chronic kidney disease

FGF23 metabolism is strongly modified by CKD, and most studies have focused on this dysregulation: FGF23 increases as GFR decreases, from early stages of CKD, even before serum phosphate and PTH have become abnormal [47, 48].

In healthy volunteers, oral phosphate loading stimulates FGF23 synthesis while it is the contrary for dietary phosphate restriction [49]. FGF23 levels increase progressively as GFR decreases, some papers reporting significant increases already by stage 2 and 3, i.e., well before the onset of a critical reduction in the nephron number [50, 51]. Although many studies have clearly shown the deregulation of FGF23 metabolism in the advanced stages of CKD, the mechanisms by which FGF23 serum levels increase during the early stages of CKD, even before serum phosphate or PTH increase or 1-25 vitamin D decreases, remain mysterious [52]; current hypotheses propose increased FGF23 as the earliest alteration in mineral metabolism in CKD, resulting from an increased bone production rather than a decreased renal clearance [48, 50, 51]. This increase could be explained by different factors, such as:

- a decreased clearance of FGF23
- a compensatory mechanism in an attempt to excrete the excess serum phosphate and keep serum phosphate within the normal range,
- a response to the treatment with active vitamin D analogs,
- a compensatory mechanism to the loss of the kidney-secreted Klotho protein, but it is not clear whether the biological effects of FGF23 are increased or decreased in this situation, or
- an increased production of FGF23 in bone cells [5, 40]. Pereira et al. have well demonstrated in 32 children and young adults with CKD stage 2 – 5 that the expression of FGF23 in bone was increased at all stages of CKD, with a positive association between bone expression and circulating levels of FGF23 [53], therefore giving strength to the increased bone production hypothesis. However, the trigger of this over-production remains to be determined...

During CKD, serum FGF23 levels are positively correlated with serum phosphate and negatively with serum calcitriol and PTH. The role of FGF23 to explain the onset of hyperparathyroidism can be explained by different and various direct and indirect effects:

- First, since FGF23 has a counter-regulatory effect on vitamin D, the increased FGF23 during CKD has the potential to reduce vitamin D activity, and thus to facilitate the development of secondary hyperparathyroidism [5].
- Second, FGF23 can also stimulate the local expression of 1 α hydroxylase in the parathyroid, suggesting that it could also indirectly down regulate PTH synthesis through an increased local production of calcitriol [7].
- Last, it has been well demonstrated both in CKD rats, in dialysis patients, and in CKD patients (among them some patients with a past of renal transplant) that there was a down-regulation of the FGF23 signaling pathway in the parathyroid glands, with :

- 1) a decreased expression of FGFR1 and Klotho in parathyroid cells, and
- 2) a resistance to FGF23 administration in CKD rats, i.e., the absence of decreased PTH synthesis [54 – 57]. All these observations can therefore explain, at least partly, the refractory secondary hyperparathyroidism observed in CKD patients.

Interestingly, iron infusions (often used in CKD and dialysis patients to treat anemia in addition to erythropoietin-stimulating agents) have been showed to induce hypophosphatemia, and to increase FGF23 in patients with iron deficiency and normal renal function [58, 59]. In CKD patients, one report was recently published, with a life-threatening hypophosphatemia following iron infusion in a woman with a past of renal transplantation [60]. The clinical implications of such observations and the underlying molecular mechanisms remain to be determined, but the overall role of systemic inflammation on FGF23 levels could probably be discussed. Of note, patients with polycystic kidney disease have also been shown to have increased FGF23 levels in comparison to patients with CKD of other etiology, even at early stages of CKD [61].

As detailed above, there is an accumulation of FGF23 in CKD. However, it remains questionable whether this accumulation corresponds to active or inactive fragments. In a small series of 14 end-stage renal disease (ESRD) adult patients, Weber et al. initially demonstrated an accumulation of C-term FGF23 fragments, suggesting that less than one quarter of the circulating FGF23 was bioactive in patients with end-stage renal disease, but recent data strongly support the conclusion that all circulating FGF23 in children and adults undergoing peritoneal dialysis is intact and biologically active [62]. While several authors have discussed a modification of the intact/C-terminal FGF23 ratio in CKD adults [63], this ratio does not seem useful in all CKD populations [64].

Last, Klotho circulating levels appear to be decreased during CKD, and Klotho polymorphisms have been associated to overall prognosis in this population: one specific Klotho polymorphism could be a protective factor against CKD progression of non-diabetic ESRD in African Americans [65], while another Klotho specific variant could increase global mortality at one year [66], and another one has been found to be associated with increased uric acid levels and decreased low density lipoprotein cholesterol in hemodialysis patients [67]. Interestingly, this effect was even more marked in patients not receiving active vitamin D supplementation.

9. FGF23 and cardio-vascular morbidity and mortality

CKD patients often present with a phenotype close to the one observed in Klotho or FGF23 deficient mice, i.e., hyperphosphatemia, ectopic calcifications, vascular calcifications, hypogonadism and premature death [68]. In that setting, circulating FGF23 levels can also provide prognostic information in CKD patients, mainly in terms of CKD progression, therapeutic response and cardio-vascular mortality. In a prospective cohort of 177 non diabetic CKD patients, FGF23 was an independent predictor of CKD progression, with a cut-off serum level of 104 RU/mL at inclusion for the C-term assay [69]. In a prospective study of 62 dialysis patients, baseline intact FGF23 levels in association with baseline PTH serum levels were found to be good predictors of refractoriness to intravenous calcitriol therapy at 24 weeks [70]. Moreover, baseline intact FGF23 serum level (cut-off 7500 ng/L) was described as a potential predictor at two years of refractory hyperparathyroidism in 103 non diabetic dialysis patients with mild hyperparathyroidism at baseline [71]. More recently, Gutierrez et al. demonstrated that higher quartiles of serum FGF23 were associated with an increased risk of mortality in hemodialysis adult patients [72]; Jean et al. obtained similar results in a cohort of 219 hemodialysis patients, with an increased risk of mortality and vascular calcifications in patients with higher quartiles of FGF23 serum levels two years after inclusion [73]. One may thus hypothesize that a therapeutic reduction of FGF23 could have a clinical importance to delay the onset of secondary hyperparathyroidism, the onset of mineral and bone disorders associated to CKD (CKD-MBD), and maybe the global morbi-mortality in CKD patients [74].

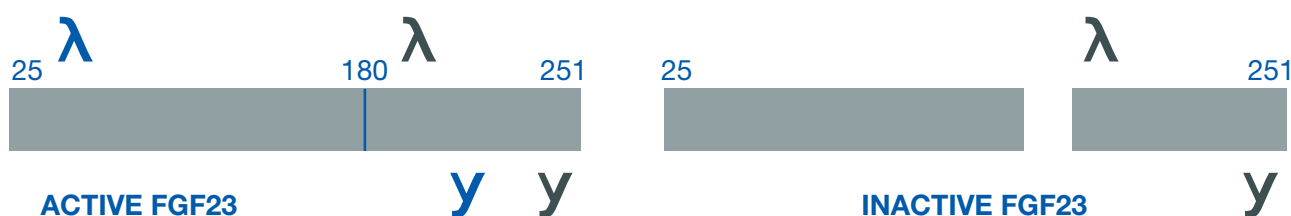
In general populations, the same trends have also been demonstrated: Parker et al. have described an increased risk of mortality and cardiovascular events in patients with stable coronary disease in patients with higher FGF23 levels [75], while elevated serum FGF23 levels, even within the normal range, are associated with increased left ventricular mass index and increased risk for the presence of left ventricular hypertrophy in a cohort of 795 Swedish elderly subjects [76]. In terms of bone status, the same team has also showed in a prospective cohort of 2868 men (75±3 years, median follow-up 3.4 years) that baseline FGF23 levels were directly correlated to the overall fracture risk, with the strongest relation when FGF23 was above 56 pg/mL; this relationship remained after adjustment on all other fracture risk factors [77]. Secondary analyses from these two previous cohorts have also showed that FGF23 circulating levels were also negatively associated with high-density lipoprotein and apolipoprotein A1 and positively with triglycerides. FGF23 levels were higher in subjects with metabolic syndrome compared with those without [78].

Similar data are accumulating in the literature, in different sub-groups of patients [79]. However, all these data result from epidemiological studies, and demonstrate the presence of an association between FGF23, left cardiac hypertrophy and vascular calcifications; however, to date, there are very few published reports showing a direct effect of FGF23 on the vessel and the cardiac cells, and the question of whether FGF23 could exert both direct and systemic toxic effects will need further longitudinal studies [5, 80]. Anyway, Klotho, FGF-R1 and FGF-R3 appear to be expressed in human normal arteries; moreover, human smooth muscle cells (normally expressing Klotho) cultured with FGF23 up-regulated the downstream kinases p-ERK and p-AKT [81]. Another step in the future will be to confirm that strategies lowering FGF23 levels can also decrease cardiovascular and overall mortality in such sub-groups of patients.

10. Different assays for measuring circulating FGF23 and Klotho

A | FGF23

Following the description of an active and an inactive form of FGF23, **different assays** have been developed for serum FGF23 measurement: the ‘intact’ assay that measures only active FGF23 and the ‘C-terminal’ assay that measures both the active and inactive FGF23. The figure below summarizes the two different types of assays for measuring FGF23 concentrations.



in blue, antibodies used to measure only the active FGF23: intact assay

in gray, antibodies used to measure both the active and the inactive FGF23: C-terminal assay

At least three studies have well demonstrated the absence of circadian intra-individual variability for FGF23 [64, 82, 83]. Racial differences could account for differences in FGF23 circulating levels: for example, in a cohort of 1099 CKD patients undergoing conservative therapies, it has been well shown after adjustment that African American patients had decreased FGF23 and 25OH vitamin D levels and increased PTH levels [84]. In the same time, FGF23 levels are greater in women, in persons with tobacco exposure and diabetes [75]. It remains questionable whether factors such systemic inflammation, infection or therapies can influence FGF23 serum levels by themselves; however, body weight (positive association between BMI and serum FGF23 levels), a past of solid organ transplantation as well as a corticosteroids treatment appear to influence FGF23 circulating levels [64].

Even though the manufacturers do not provide reference values, highlighting the fact that these assays should be used only for research purposes, reference values have nevertheless been proposed in healthy population: in adults, the reference values for C-terminal FGF23 are 55±50 RU/ml [82], and 10 to 50 pg/mL for intact FGF23 [85]. There are very few pediatric data on FGF23. An initial study reported normal values of C-terminal FGF23 serum levels of 69±36 RU/mL in 26 children (10.9 ± 5.5 years), however without specific data confirming normal renal function [82]; since this initial report, other teams have proposed reference values according to age, gender and glomerular filtration rate with the first generation C-terminal assay and the intact one [64]: the table below summarizes the reference values (results expressed as mean with 95 % confidence interval) for C-terminal and intact FGF23 according to age in the 115 children with a normal renal function (i.e., glomerular filtration rate between 90 and 139 mL/min per 1.73 m²). Of note, gender did not influence the results in this pediatric population, in contrast to the Heart and Soul study [64, 75].

| Age (years) | 5 – 10 | 10 – 15 | 15 – 20 |
|--------------------------|--------------|--------------|--------------|
| N | 57 | 33 | 25 |
| C terminal FGF23 (RU/mL) | 65 (39 – 91) | 49 (25 – 73) | 76 (6 – 146) |
| Intact FGF23 (pg/mL) | 32 (28 – 37) | 36 (30 – 41) | 45 (37 – 54) |

In younger children, Brown et al. recently described an elevated FGF23 serum level in a child with Jansen's metaphyseal chondrodysplasia in comparison to 5 healthy children aged 10 – 18 months; in that study, reference FGF23 serum levels were 30 ± 17 pg/mL with an intact assay, and 104 ± 36 RU/mL with a C-terminal assay [86]. Recent papers have also studied FGF23 levels in newborns, showing a rapid decrease of the intact / C-term ratio during the first 5 days after birth [87].

In conclusion, FGF23 circulating levels on EDTA plasma can now be measured with immunometric assays that detect either the intact hormone alone or either the intact FGF23 as well as the C-terminal fragments; it seems preferable to use an intact FGF23 assay to establish the diagnosis of FGF23-dependent hypophosphatemic disorders [51].

B | Klotho

Recent data have reported the possibility to measure α -Klotho circulating levels in human blood [83, 88]. However, by definition, this kit measures the portion of α -Klotho that is circulating in the systemic circulation after its release from the cell membrane after shedding. The clinical relevance of such a circulating α -Klotho remains to be fully understood, since the main biological effects of Klotho are probably mediated by the trans-membrane form of the protein.

In contrast to the stability of FGF23 circulating levels among time, Klotho has a circadian rhythm, with a nadir around midnight and a maximum concentration in the early morning [83]. Yamazaki et al. have reported reference serum α -Klotho values in 142 healthy volunteers (66 men, 61 ± 19 years, serum creatinine 61 ± 12 μ mol/L): the values ranged from 239 to 1266 pg/mL (mean \pm SD: 562 ± 146 pg/mL). The levels of α -Klotho were not influenced by gender or indices of skeletal metabolism, but were inversely related to serum creatinine and age. Moreover, in that study, Klotho seems to decrease with age [83, 88] and the additional analysis of 39 Asiatic children (23 boys, age 7 ± 5 years; serum creatinine 32 ± 11 μ mol/L) showed that the concentration of the soluble form of α -Klotho was 952 ± 282 pg/mL (i.e., significantly greater than in adults), with an intact FGF23 of 24 ± 12 pg/mL. Analyses including both adults and children showed a positive relationship between α -Klotho and serum phosphate, whereas the association was inverse between α -Klotho and both FGF23, calcium, creatinine and age [88]. Recently, Ohata et al. have also shown that the levels of α -Klotho were markedly higher in cord blood than in neonates at four days of life, in mothers and adult volunteers, while the fetal levels of FGF23 were lower; the levels of soluble α -Klotho and FGF23 were inversely correlated in cord blood [89].

In conclusion, α -Klotho circulating levels on EDTA plasma or serum can now be measured with immunometric assays but their use should be restricted for research use in 2011.

11. Hot topics in research

Even though many questions remain unsolved [50], FGF23 and Klotho have obviously modified our understanding of phosphate / calcium metabolism, and a therapeutic targeting of this axis will probably be the next step, at least in CKD patients. Economic and public health consequences of such strategies can be important, since the prevalence of CKD is growing worldwide, as well as the number of patients reaching ESRD. FGF23 is a negative predictor of survival and cardio-vascular morbidity in different sub-groups of patients, but its direct toxicity has not been demonstrated to date. However, even though it remains debatable whether FGF23 is a direct culprit or an innocent bystander [80], strategies aiming at decreasing FGF23 levels or at restoring Klotho circulating levels could be of interest in CKD patients [5, 9]. In such a setting, FGF23 could also represent a biomarker to adapt all the therapies aiming at controlling CKD-MBD. Some trials have been recently reported, showing a decrease of FGF23 levels:

- in early stages of CKD in adults receiving the non-calcium phosphate binder sevelamer [90],
- in early stages of CKD in adults lowering dietary phosphate intake [91], and
- in hemodialysis patients receiving the calcimimetic cinacalcet [92].

Moreover, a post-hoc analysis of a one-year randomized trial of phosphate binders (sevelamer vs. calcium acetate, with or without calcitriol) in 72 hemodialysis patients has recently shown that a more significant FGF23 decrease was obtained in patients taking sevelamer, not receiving calcitriol, and on a 2.5 mEq/l calcium dialysate [93]. Other trials evaluating the effect of other non-calcium phosphate binders (i.e., lanthanum carbonate) or calcium carbonate in early CKD stages have not shown a decrease of FGF23 levels [90, 94], but a recent paper has highlighted decreased FGF23 levels in 18 patients with early CKD (stage 3) receiving lanthanum carbonate for 4 weeks [95]. Moreover, in rat models of early CKD, antibodies directed against FGF23 decrease PTH levels, increase calcium and phosphate levels, and normalize 1-25 vitamin D levels [96]. In a pediatric population, strategies aiming at decreasing FGF23 levels could also be of interest for patients with hypophosphatemic rickets, a rare genetic condition affecting 1/20 000 children, and which is currently managed with active vitamin D sterols and oral phosphate intakes, nephrocalcinosis and secondary hyperparathyroidism remaining unfortunately the two main complications of these therapies [44]. In a mouse model of this model (Hyp mice), the administration of specific antibodies directed against FGF23 led to an improvement of the phenotype [97, 98].

In addition to these human and animal therapeutic trials, the underlying molecular mechanisms and cellular targets of FGF23 remain to be more accurately identified, while Klotho appears in 2011 to be a systemic hormone with systemic and very various effects.

12. Conclusion

In less than one decade, the description of the key role of FGF23 and Klotho in the the „bone-kidney/parathyroid“ axis had led to a better understanding of genetic conditions associated with hypophosphatemia and phosphate calcium disorders as well as CKD-MBD. However, although a therapeutic targeting of this pathway seems very likely in a next future, many questions remain unresolved, and the availability of FGF23 and Klotho assays in daily practice for bench and clinical research will probably allow us in a next future to better understand this fascinating and global pathway.

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Fibroblast Growth Factor 23

FGF-23 is produced in osteoblast precursor cells and is a potent regulator of phosphate and vitamin D metabolism.

Phosphate plays an essential role in the stability of skeletal bones and energy metabolism as well as in DNA synthesis and intracellular signal cascades.

FGF-23 inhibits in combination with cofactor Klotho phosphate reabsorption in renal proximal tubular cells via FGF-23 receptors (increased phosphate loss, reduced serum phosphate) and decreases calcitriol synthesis by suppressing alpha-1-hydroxylase.

FGF-23 (C-Term) 2nd Generation

Fibroblast Growth Factor 23

C-terminal

| | |
|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cat. No.: | 60 – 6100 |
| Tests: | 96 |
| Method: | ELISA |
| Range: | 1.5 - 1500 RU/ml |
| Sensitivity: | 1.5 RU/ml |
| Incubation time: | 3.5 hours |
| Sample volume: | 100 µl |
| Sample type: | Plasma, cell culture |
| Sample preparation: | It is recommended to collect the sample in the morning after a 12-hour fasting period. Store the samples at -20°C, or at -80°C for a longer storage. Avoid repeated freezing and thawing of specimens. |
| Reference values: | Premenopausal women (<40 years): 20.9 - 91.1 RU/ml Postmenopausal women (>60 years): 44 - 139.9 RU/ml Men (27-76 years): 33.7 - 96.5 RU/ml |
| Species: | Human, Cynomolgus Macaque |

FGF-23 Intact, Human (Kainos)

Fibroblast Growth Factor 23

Intact

| | |
|----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cat. No.: | CY-4000 |
| Tests: | 96 |
| Method: | ELISA |
| Range: | 8 – 800 pg/ml |
| Sensitivity: | 3.0 pg/ml |
| Incubation time: | 3.5 hours |
| Sample volume: | 50 µl |
| Sample type: | Serum |
| Sample preparation: | It is recommended to collect the sample in the morning after a 12-hour fasting period. Intact FGF-23 is very instable. Therefore, collection and testing or storage should take place promptly. Store samples at -20 °C or below. Avoid repeated freezing and thawing of specimens. |
| Reference values: | 10 – 50 pg/ml |
| Species: | Human |

FGF-23 Intact, Human

Fibroblast Growth Factor 23

Intact

| | |
|----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cat. No.: | 60 – 6500 |
| Tests: | 96 |
| Method: | ELISA |
| Range: | 6 – 200 pg/ml (can be extended to 650 pg/ml) |
| Sensitivity: | 1.0 pg/ml |
| Incubation time: | 3.5 hours |
| Sample volume: | 150 µl |
| Sample type: | EDTA plasma, cell culture |
| Sample preparation: | It is recommended to collect the sample in the morning after a 12-hour fasting period. Intact FGF-23 is very instable. Therefore, collection and testing or storage should take place promptly. Store samples at -20 °C or below. Avoid repeated freezing and thawing of specimens. |

Reference values: 7 – 29.3 pg/ml

Species: Human

Specificity: Antibodies recognize FGF-23 amino acids 186-206 and 51-69.

Klotho, Human

| | |
|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cat. No.: | 27998 |
| Tests: | 96 |
| Method: | ELISA |
| Range: | 93.75 – 6000 pg/ml |
| Sensitivity: | 6.15 pg/ml |
| Incubation time: | 2 hours |
| Sample volume: | 100 µl |
| Sample type: | Serum, EDTA plasma |
| Sample preparation: | Collection, testing and samples storage should take place promptly. Store samples at -20 °C or below. Avoid repeated freezing and thawing of specimens. |

Reference values: 239 – 1266 pg/ml

Species: Human

Specificity: No cross-reactions observed with: osteopontin, human VEGF or PDGF.

Intended Use:

Klotho is a single-pass trans-membrane anti-aging protein (1014 amino-acids, 130 kDa, chromosomal location in 13q12 in humans) that has been recently shown to have wide and important biological effects. Its expression occurs mainly in the kidney and in the parathyroid. Its extra-cellular domain is wide whereas its intracellular part is very short; it also exists as a soluble protein.

In humans, Klotho polymorphisms have been associated both in a positive and negative way to bone mineral density, life expectancy, cardiovascular events (e.g., ischemic stroke, carotid atherosclerosis), biomarkers of metabolic syndrome (e.g., uric acid levels, lipid and glucose metabolisms), and even to cognitive ability.

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