# Phosphatonins: physiological role and pathological changes

**Loredana Cavalli**
**Celestina Mazzotta**
**Maria Luisa Brandi**

Division of Mineral and Bone Metabolism Diseases,
Department of Internal Medicine, University of Florence,
Orthopedic Trauma Centre, Florence, Italy

Address for correspondence:
Loredana Cavalli, MD
Division of Mineral and Bone Metabolism Diseases
Orthopedic Trauma Centre, Largo Palagi 1
50139 Firenze, Italy
Phone: +39 055 7948087
Fax: +39 055 7948320
E-mail: loredana.doc@gmail.com

**Summary**

**Maintenance of proper serum phosphate concentrations is required for healthy life, and critical for normal skeletal development and integrity. Several hormones and regulatory factors such as vitamin D, parathyroid hormone (PTH), and the phosphatonins (FGF-23, sFRP-4, MEPE) among others, may play a role only in the long-term regulation of phosphorus homeostasis.**
**FGF23 is part of a previously unrecognized hormonal bone-parathyroid-kidney axis. Its synthesis and secretion by osteocytes are positively regulated by 1,25(OH)2D and serum phosphorus and negatively by the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), and the enzyme N-acetyl galactosamine trasferase 3 (PPGGalNacT3), encoded by GALnT3 gene, prevents its degradation.**
**FGF23 requires Klotho protein as a coreceptor for high affinity binding to cognate FGF receptors (FGFRs). Mutations of any of FGF23, Klotho or GALnT3 genes can lead to a syndrome characterized by hyperphosphatemia, ectopic calcifications and recurrent long bone lesions with hyperostosis. Phosphatonin have been shown to be implicated in several common diseases involving kidney and mineral metabolism. FGF23 might also represent a promising putative marker for bone healing.**

*KEY WORDS: phosphatonins; FGF-23; sFRP-4; MEPE; GALnT3; Klotho; phosphate metabolism; PHEX; ADHR; XLH.*

## Introduction and context

The regulation of phosphorus balance is fundamental for healthy life. Intracellular phosphate is involved in essential cellular functions, such as energy provision in the form of ATP, and it is an integral molecule in DNA and RNA, and a substrate for kinase and phosphatase regulation of intracellular signalling; extracellular phosphate is necessary for bone matrix mineralization.
Adaptative mechanisms have evolved to protect organisms from hypophosphatemia and hyperphosphatemia and to coordinate the changing phosphate needs for bone mineralization and phosphate homeostasis (1). Reductions in serum phosphorus concentrations can acutely result in myopathy, cardiac dysfunction, neutrophil dysfunction, platelet dysfunction and red cell membrane fragility. Chronic P insufficiency results in impaired bone mineralization, rickets and osteomalacia. Elevated Pi concentrations contribute significantly to the pathogenesis of secondary hyperparathyroidism seen in patients with chronic renal failure (2).
The regulation of phosphorous homeostasis is therefore of great importance for the well-being of the organism (3). Processes of absorption of dietary phosphate from the intestine, mobilization from bone and excretion from the kidney into urine are coordinately regulated by several endocrine factors.
Calcitriol, the active form of vitamin D (1,25-dihydroxyvitamin D3) synthesized in the kidney, increases gut absorption of dietary calcium and phosphate, and on bone to stimulate osteoclastogenesis, thereby increasing blood levels of both calcium and phosphate. PTH acts on the kidney to promote both vitamin D synthesis throughout the activation of 1$\alpha$-hidroxilase, and phosphaturia. As a result, unlike vitamin D, PTH can selectively increase blood calcium levels without concomitant increase in blood phosphate levels (4).
The cells sense changes in extra- or intracellular Pi concentrations via specific “phosphate sensors” (5) with subsequent changes of the phosphorilation state of intracellular proteins and nuclear transcription events.
Non-epithelial cells such as osteoblasts and marrow stromal cells are capable of responding to changes in medium Pi concentrations by altering BPM-4 expression, Runx2/Cbfa1 localization and alkaline phosphatase secretion, demonstrating the presence of sensor in mammalian cells. However, the exact biochemical nature of this sensor is not known (6, 7).
Several hormones and regulatory factors such as vitamin D, parathyroid hormone, and the phosphatonins (FGF-23, sFRP-4, MEPE) among others, may play a role only in the long-term regulation of phosphorus homeostasis. Recently, new bone-renal phosphate regulating factors have been identified, which are phosphaturic hormones called phosphatonins.

## FGF23

The key phosphatonin appears to be fibroblast growth factor-23 (FGF23) (8-10).
The FGF23 is a member of the fibroblast growth factor FGFs family that possess broad mitogenic and cell survival activities and are involved in a variety of biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion.
The FGF23 gene encodes a protein of 251 amino acids and includes a 24-aminoacid peptide (11, 12). The secreted form of FGF23 is 227 amino acids in length. The protein FGF23 is proteolytically cleaved between $^{179}$Arg and $^{180}$Ser by subtilisin-like enzymes that recognize $^{176}$Arg-X-X$^{179}$ Arg motif (13). This processing

occurs intracellularly, either before or during the process of secretion of FGF23 (14).
FGF23 is produced by osteoblasts and osteocytes (15) and acts on kidney indicating that FGF23 is a systemic factor in contrast to many other members of FGF family. In fact when phosphate is in excess FGF23 is secreted and appears in the systemic circulation, and act in renal proximal tubule to reduce the expression of the type II a and c sodium-dependent phosphate co-transporters (NPT2a and c), leading to decreased phosphate reabsorption (16, 17). FGF23 also functions as a counterregulatory hormone for vitamin D: it suppresses the expression of the Cyp27b1 gene, which encodes $\alpha$1-hydroxylase, an essential enzyme for synthesis of the active vitamin D metabolite, $1,25(OH)_2D$ which enhances intestinal phosphate absorption; furthermore, FGF23 upregulates expression of the Cyp24 gene that encodes 24- hydroxylase, the enzyme that hydrolyzes and inactivates $1,25(OH)_2D$. Together, these two pathways account for the hypophosphatemia caused by an excess of serum FGF23 (18).
FGF23 binds to and activates FGF receptors (FGFR) 1c, 3c, and 4 in cell lines that co-expresses Klotho, a co-factor that determines the tissue specificity of FGF23 (19, 20). Klotho, a single-pass transmembrane protein that belongs to a family 1 glycosidase (21), expressed primarily in renal distal tubule, parathyroid, pituitary, and sinoatrial node of the heart (22) and choroid plexus, forms a binary complex with several FGF receptor isoforms (FGFR1c, 3c, and 4) and significantly increases their affinity to FGF23 (20). Klotho therefore determines the tissue specificity of its ligand FGF23. Studies *in vitro* with parathyroid cell cultures of rat demonstrated that Klotho mRNA is high in parathyroid and kidney, low in thyroid, duodenum and liver, not expressed at all in the spleen and also showed the presence of FGFR1 and FGFR3 in the parathyroid tissue. These results show that the parathyroid expresses the FGFR-Klotho receptor complex and suggest that the parathyroid is a target organ for FGF23 (23).
Moreover, FGF23 inhibits PTH expression and secretion through activation of the MAPK pathway (24, 25).
The *Klotho* gene, named after a Greek goddess who spins the thread of life, was originally identified as a gene mutated in a mouse strain that inherits a premature-aging syndrome in an autosomal recessive manner (26). Mice defective in *klotho* gene expression develop multiple aging-like phenotypes around 3-4 weeks after birth, including growth retardation, hypogonadotropic hypogonadism, rapid thymus atrophy, skin atrophy, sarcopenia, vascular calcification, osteopenia, pulmonary emphysema, cognition impairment, hearing disturbance, and motor neuron degeneration, and die around 2 months of age. In contrast, transgenic mice that overexpress *Klotho* live longer than wild-type mice. Thus, the *klotho* gene may be an aging suppressor gene that extends life span when overexpressed and accelerates aging when disrupted. Furthermore, polymorphisms in the human *Klotho* gene are associated with life span as well as bone mineral density, high-density lipoprotein (HDL) cholesterol level, blood pressure, stroke, coronary artery disease, and cognitive function, suggesting that Klotho may be involved in the regulation of aging processes in humans.
FGF23-deficient mice not only exhibit phosphate retention but also develop multiple aging-like phenotypes (27), which is reminiscent of Klotho-deficient mice. Conversely, Klotho-deficient mice not only develop a premature-aging syndrome but also exhibit hyperphosphatemia (28, 29), which is reminiscent of FGF23-deficient mice. These observations suggested that Klotho and FGF23 might function in a common signal transduction pathway (30).

## Implications for clinical practice

Clinically relevant pathologies are related to altered expression of FGF23, either for mutation of FGF23 gene or other genes which regulate its signalling pathway.

Autosomal-dominant hypophosphatemic rickets (ADHR) is caused by a missense mutation of FGF23, which becomes resistant to proteolysis (31), resulting in high serum FGF23 levels and inappropriately normal 1,25-dihydroxyvitamin D3, with phosphate-wasting phenotypes including hypophosphatemia and defects in bone mineralization (rickets) (32).
FGF23 production in bone as well as the mineralization of extracellular matrix (30) is regulated by PHEX, (phosphate regulating gene with homologies to endopeptidases on the X chromosome), a cell surface endopeptidase providing signaling pathways for coordinating bone phosphate accretion with renal phosphate conservation. The PHEX gene is localized at chromosome Xp22.1 and comprises 22 exons, spanning 220 kb. PHEX is expressed mainly in cartilage, osteoblasts, and odontoblasts. Deletion of either *Phex* or the gene coding for dentin matrix acidic phosphoprotein 1 (*Dmp1*) in mice results in nearly identical increments in FGF23 production by osteocytes, leading to overlapping phenotypes characterized by hypophosphatemia, aberrant vitamin D metabolism, and rickets/osteomalacia (33, 34).
Inactivating mutations in the PHEX gene lead to X-linked hypophosphatemia (XLH) characterized by defective renal phosphate handling, aberrant vitamin D metabolism, and defective calcification of bone.
Inactivating mutations in DMP1 and PHEX increase, through yet unknown mechanisms, FGF23 synthesis and thus enhance renal phosphate excretion (35). Loss of PHEX increases the uncleaved full-length FGF23 and/or abnormal processing of MEPE (Matrix Extracellular Phosphoglycoprotein). MEPE is a member of the SIBLING (small integrin binding ligand *N*-glycosylated) family of extracellular matrix proteins and plays a role in cell signaling, mineral homeostasis, and mineralization. MEPE expression is bone cell-specific and induced by the bone morphogenetic protein-2 (BMP-2) signaling pathway. Its C-terminal proteolytic cleavage product is an acidic-serine-asparate-rich-MEPE-associated motif (ASARM), a strong regulator of body phosphate metabolism and mineralization. PHEX enzyme prevents the release of the ASARM motif, a mineralization inhibitor, from the MEPE molecule. Thus, the MEPE/PHEX ratio may be a good indicator of mineralization progression because we found that the mRNA ratio and protein levels were low when osteoblasts were actively differentiating to the mineralization stage and the ratio was high when the cells reached the mineralization stage when it is assumed that osteocytes may protect themselves and make a space to survive from the mineralized matrix by releasing the ASARM motif. In addition, the MEPE/PHEX ratio of the cell could be a very important barometer indicating the progression of tissue mineralization (36, 37).
Oncogenic hypophosphatemic osteomalacia is caused by tumor-expressed proteins, MEPE and/or FGF23, whose overexpression results in abnormal renal phosphate metabolism and bone mineralization. Based on these data, FGF23, PHEX, and MEPE are generally accepted as the main regulators of systemic phosphate levels and tissue mineralization.
Mutations of different genes can lead to the same disease. A typical example is Familial Tumoral Calcinosis (FTC), a group of disorders inherited in an autosomal recessive fashion for mutation of FGF23, *Klotho* or *GALnT3* genes. Defective function of any one of these proteins results in hyperphosphatemia and ectopic calcification around major joints.
The GALnT3 gene encodes N-acetyl galactosamine trasferase 3 (PPGGalNacT3), a Golgi-associated biosynthetic enzyme that mediates mucin type O-linked glycan biosynthesis by instating an initial N-acetyl-a-galactosamine (GalNAc) residue on the protein scaffold (38). This enzyme prevents degradation of the phosphaturic hormone FGF23. Biallelic mutations in either GALNT3 or FGF23 result in hyperphosphatemic familial tumoral calcinosis (FTC) or its variant, hyperostosis-hyperphosphatemia syndrome, characterized by recurrent long bone lesions with hyperostosis. Both these syndromes represent a continuous spectrum of the same di-

sease caused by increased phosphate levels, rather than two distinct disorders (39).

Recent observations revealed FGF23 implications in more common pathologic conditions, such as atherosclerosis and chronic kidney disease (CKD). In patients with end-stage renal disease on maintenance hemodialysis, FGF23 resulted positively correlated with phosphate, calcium x phosphate product and intact PTH, and inversely correlated with total and non-HDL cholesterol. The higher FGF23 tertile was independently associated with decreases in cholesterol levels and with a less-pronounced increase in carotid intima-medial thickness. Thus FGF23 appears to be negatively associated with atherosclerosis on hemodialysis (40).

In particular, the plasma FGF-23 level was shown to be a significant predictor of vascular calcification of the hand artery, but not of the aorta, in both non-DM and DM hemodialysis patients, independently of the Ca×Pi product. Inaba et al. hypothized that locally generated FGF-23 acts inside the vessel wall. Coincident with the correlation of hyperphosphatemia and vascular calcification in hemodialysis patients (41), the treatment of human smooth muscle cells with elevated Pi concentrations has been demonstrated to induce human smooth muscle cells to differentiate into osteoblast-like cells, as shown by the expression of the osteoblast differentiation markers osteocalcin and Osf2/ Cbfa-1 (42).

In patients with CKD, FGF23 levels increase progressively to compensate for phosphate retention, but these elevated levels of FGF23 fail to suppress the secretion of PTH. Parathyroid resistance to FGF23 may be caused by decreased expression of Klotho-FGFR1 complex in hyperplastic parathyroid glands (43, 44).

Moreover, serum FGF23 levels might be an indicator for fracture healing processes prone to reunion versus non-union. FGF23 (C-term) resulted elevated on day 3 postoperatively in patients undergoing an exchange of total hip implants. A cohort of patients sustaining primary hip arthroplasty also showed elevated FGF23 (C-term) but no change in FGF23 (intact), with normal values of phosphate clearance. FGF23 mRNA expression in ovine callus was compared between a standard and delayed course of osteotomy healing. It resulted markedly more increased in the standard model. Therefore FGF23 is a promising putative marker for bone regeneration (45).

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