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REVIEW

Role of Phosphate in Biomineralization

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Abstract

Inorganic phosphate is a vital constituent of cells and cell membranes, body fluids, and hard tissues. It is a major intracellular divalent anion, participates in many genetic, energy and intermediary metabolic pathways, and is important for bone health. Although we usually think of phosphate mostly in terms of its level in the serum, it is needed for many biological and structural functions of the body. Availability of adequate calcium and inorganic phosphate in the right proportions at the right place is essential for proper acquisition, biomineralization, and maintenance of mass and strength of the skeleton. The three specialized mineralized tissues, bones, teeth, and ossicles, differ from all other tissues in the human body because of their unique ability to mineralize, and the degree and process of mineralization in these tissues also differ to suit the specific functions: locomotion, chewing, and hearing, respectively. Biomineralization is a dynamic, complex, and lifelong process by which precipitations of inorganic calcium and inorganic phosphate divalent ions form biological hard tissues. Understanding the biomineralization process is important for the management of diseases caused by both defective and abnormal mineralization. *Hypophosphatemia* results in mineralization defects and osteomalacia, and *hyperphosphatemia* is implicated in abnormal excess calcification and/or ossification, but the exact mechanisms underlying these processes are not fully understood. In this review, we summarize available evidence on the role of phosphate in biomineralization. Other manuscripts in this issue of the journal deal with other relevant aspects of phosphate homeostasis, phosphate signaling and sensing, and disorders resulting from hypo- and hyperphosphatemic states.

Keywords Biomineralization · Hypophosphatemia · Hyperphosphatemia · Vascular calcification · Soft tissue calcification · Calciphylaxis · Enthesopathy · Heterotopic ossification

Introduction

Phosphate (Pi) is a vital constituent of cells and cell membranes as well as of body fluids and tissues, it is a major intracellular divalent anion, participates in many genetic, energy and intermediary metabolic pathways, and is important for bone health [1–3]. Availability of calcium (Ca) and inorganic phosphate (Pi) in the right proportions at the right place is crucial for proper acquisition, biomineralization of collagen fibrils, and maintenance of mass and strength of bones and teeth [4], the two hardest tissues in human body. Among the various micronutrients in bone, Ca and Pi are the two major components of hydroxyapatite, the crystalline mineral component of the extracellular organic matrix of bone. Phosphate metabolism, distribution, intracellular signaling, physiological and pathological perturbations causing hypo- and hyperphosphatemia, and clinical aspects of phosphate excess and depletion are dealt with in other sections in this issue of the journal. Accordingly, in this section, we will focus exclusively on the role of phosphate in biomineralization, and more specifically, in bone and other tissue mineralization. A knowledge and understanding the role of phosphate in biomineralization processes are essential to manage patients with disorders of phosphate metabolism and the associated abnormal biomineralization of bone and other tissues.

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Distribution and Role of Phosphate in the Body

Although we think of phosphate mostly in terms of its level in the serum, which is maintained within a narrow range for many biological and structural functions of the body, phosphate is also an integral part of bones, tissues, cells and cell membranes. In a typical Western diet contains 1000–1600 mg of Pi/day, of which 3 mg/kg bodyweight/day enters the extracellular fluid with consequent exchange with bone as required [5, 6]. Bone contains about 99 and 80% of the total body content of Ca and Pi with a mass ratio of 2:2. Both these divalent ions exist in soluble and semi-soluble form in body fluids, cells and cell membranes, and phosphate circulates in the blood in free form, bound to protein, and as complex with Ca. A 70 kg individual has approximately 500–800 g of total body phosphate, 80% of which is in the form of hydroxyapatite crystal in bone and 20% as intracellular component. Intracellularly, phosphate is present predominantly in the organic form in nucleic acids and nucleoproteins. In contrast, both organic and inorganic forms of phosphate are present in serum, the latter is measured routinely by standard methods in clinical laboratories. At physiological pH of 7.40, phosphate exists as a mixture of ions (orthophosphates): HPO^{2-} and H_2PO^{4-} in a ratio of 4:1. Normal physiological functions of phosphate are manifold: it is the major source of high energy phosphate bonds (ATP) required ubiquitously for cellular homeostasis-muscle contraction, electrolyte transport, etc.; it is an integral part of intracellular messenger system including cyclic adenosine and guanosine monophosphates (c-AMP; c-GMP); it is required, for the synthesis phospholipid bilayer of all cell membranes; it is involved in the formation of 2,3-diphosphoglycerol that regulates oxygen delivery to tissues, and acts as a buffer to maintain normal blood pH and plays a significant role in immune functions and coagulation cascade (also see other chapters in this issue).

The Process of Biomineralization

The three most mineralized tissues (bones, teeth, and ossicles) are specialized organs that differ from all other tissues in the human body because of their unique ability to mineralize [7, 8]. Interestingly, the degree and process of mineralization in bone, teeth, and ossicles also differ to suit the specific functions of these hard tissues: locomotion, chewing, and hearing, respectively. Biomineralization is a dynamic, complex, and lifelong process by which precipitations of inorganic Ca and Pi to form biological hard tissues such as bone, cementum, dentin, and enamel [8, 9]. Understanding the biomineralization process is important for the management of diseases caused by both defective and abnormal mineralization [10–15]. Throughout life bone and teeth, but not ossicles, are subject to processes of mineralization and demineralization on a constant and continual basis required for renovation of these hard tissues [9]. Notwithstanding the significant progress made in our understanding of bone biology several questions remain: What are the initial steps in biomineralization? How is the temporal and spatial regulation of matrix production and biomineralization integrated and accomplished? Why does biologic mineralization normally occur only in certain types of tissues but not in others? Why, and under what circumstances, does abnormal biomineralization occur? Is non-skeletal biomineralization such as that occurs in muscles, tendons, cartilage, and blood vessels dependent on ambient Pi or due to underlying tissue characteristics? Are any other cells involved in biomineralization besides the osteogenic cells? Nevertheless, considering that mineralized hard tissue formation in vivo is governed by a combination of cellularly driven processes and thermodynamics, biomineralization should be considered both biological and chemical in nature [16].

Phosphate, Ca and type-1 collagen fibrils are the major building blocks of bone tissue aided by key enzymes [17–20]. In humans, free phosphates also have control over the formation of new mineral by influencing a wide variety of cells (chondrocytes, osteoblasts, and osteocytes), signaling molecules, and enzymes [16] (see other chapters in this issue). Not surprisingly serum phosphate concentrations vary considerably with age, higher in infants and children (1.5–2.65 mM) and decline during adulthood (0.8–1.5 mM) [21]. This is most likely because of higher requirements for phosphate needed for bone growth, optimal bone mineralization, and to achieve peak bone mass in infants and growing children.

The first step in biomineralization process, at least in bone, appears to be the nucleation of Ca–Pi crystals within the matrix vesicles (Fig. 1) [4, 22, 23], followed by formation of amorphous Ca–Pi (ACP) phase with gradual transition to Ca–Pi crystal nucleation resulting in hydroxyapatite crystal [4, 24]. The Ca–Pi crystal nucleation takes place within the matrix vesicles that bud from the plasma membrane of osteogenic cells [25]. The matrix vesicles are endowed with two key enzymes, tissues non-specific alkaline phosphatase (TNSALP) and PHOSPHO-1, and Na/Pi cotransporter to generate and accumulate Pi from organic phosphate compounds (Fig. 1). TNSALP hydrolyzes inorganic pyrophosphate (PPi), adenosine triphosphate (ATP) and protein-P, whereas PHOSPHO-1 generates Pi from phosphatidyl choline (PC) and phospho-ethanol amine (PEA). In addition, Pi is actively transported into the matrix vesicles from the

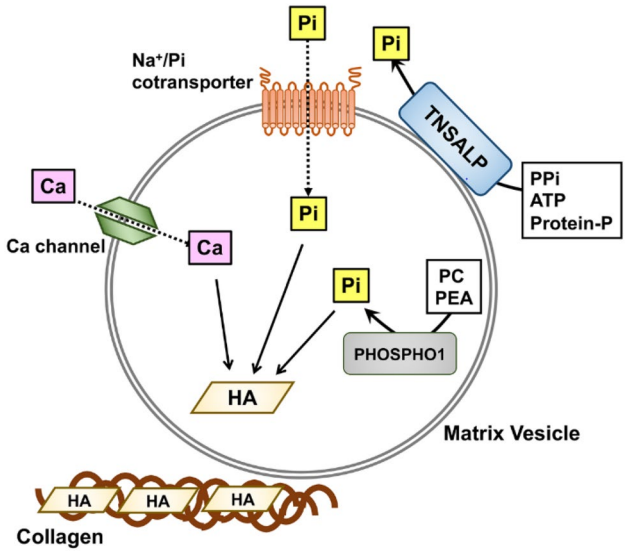

Fig. 1 Schematic depiction of initial step in bone mineralization. *ATP* adenosine triphosphate; *Ca* calcium; *HA* hydroxyapatite; *Na* sodium; *Pi* inorganic phosphate; *PPi* inorganic pyrophosphate; *PEA* phosphoethanolamine; *TNSLAP* tissue non-specific alkaline phosphatase

plasma by Na/Pi cotransporters (Fig. 1). In humans, biologic mineralization occurs by precipitation Ca and Pi in the presence of collagenous and non-collagenous proteins, polysaccharides at a pH of 7.4, and an ambient temperature of 37 °C [16]. Both $H_2PO_4^-$ and HPO_4^{2-}, the two most important orthophosphate ions, react avidly with aqueous Ca ions resulting in the nucleation and subsequent growth of biologically relevant mineral complexes, providing a chemical basis for biomineralization [16]. Whether such a sequence occurs in tissues other than bone is less clear.

Additional supporting evidence for biomineralization process comes from the classical in vivo electron microprobe studies that demonstrated a rapid initial deposition of mineral when the Ca–Pi ratio of 1.35 with a further slow increase in the deposition of minerals up to a ratio of 1.6 over a few days [26]. One possible sequence in the formation of bone mineral (or hydroxyapatite crystals) is that brushite, tricalcium phosphate, octocalcium phosphate, and hydroxyapatite are formed in succession (Fig. 2a, b). An alternate pathway is that a trimer of amorphous tricalcium brushite or three dimers of amorphous brushite are intermediate products before transitioning to hydroxyapatite crystal in bone. In either case, there is sequential addition of calcium and phosphate from the extracellular fluid to bone, but these steps are phase transformations [27], not exactly true chemical reactions. In bone, the mineral ultrastructure organization, morphology, and composition are crucial for its mechanical and biological functions. Osteocalcin and osteopontin play specific roles in the biomolecular regulation of mineral content in bone, the quality of bone mineral, and regulate bone mineral crystal size, shape, and organization. While osteocalcin predominantly regulates the physical properties of bone mineral, osteopontin plays a major role in the regulation of mineral composition [28].

Bone Mineralization

As discussed broadly with respect to the process of biomineralization, the basic template for bone formation is osteoid deposited by osteoblasts [29]. This unmineralized matrix, or osteoid, forms a scaffold for subsequent mineral deposition and bone formation. Osteoid is composed of organic materials, the major component of which is type-1 collagen [30]. The exact role of this phase in the infiltration of mineral precursors and the subsequent evolution of highly oriented hydroxyapatite crystals remains unknown. Several non-collagenous proteins, pH, and enzymes influence hydroxyapatite crystal formation in addition to the availability of appropriate ratio of Ca–Pi ions. There is growing evidence that orthophosphate mineral precursors are formed separately before integrating with collagen [16, 18]. The orthophosphates required for biomineralization is provided in the form of inorganic phosphate (Pi) and the optimal Ca × Pi product for proper mineralization is ~ 40. At a Ca × P product of 60 represents the saturation product above which spontaneous precipitation of ca-phosphate salt may occur in non-skeletal tissues [31].

As best as we currently understand, for proper and optimal mineralization of bone, at a minimum, requires two principal processes: synthesis of mature lamellar bone matrix by osteoblasts and exposure of the newly synthesized lamellar bone matrix to optimal calcium × phosphate product insured by the mineral homeostatic system regulated by parathyroid hormone (PTH), vitamin D, and fibroblast growth factor-23 (FGF-23; and see other chapters) [32, 33]. Any abnormality in either component will result in defective mineralization (Tables 1, 2).

Role of Phosphate in Chondrocytes and Bone Cells

Attaining the full potential of adult height and achieving maximal peak bone mass require longitudinal bone growth and maximal consolidation of mineral into bones during growth period. Therefore, it is not surprising that these two important biological processes require participation of cells, hormones and minerals, each of which are interconnected and interdependent [2, 7, 34–39]. Both Ca and Pi influence bone cells, and their differentiation and function as well as mineralization process. Since hypophosphatemia is common in all types of rickets, much of the research is focussed on

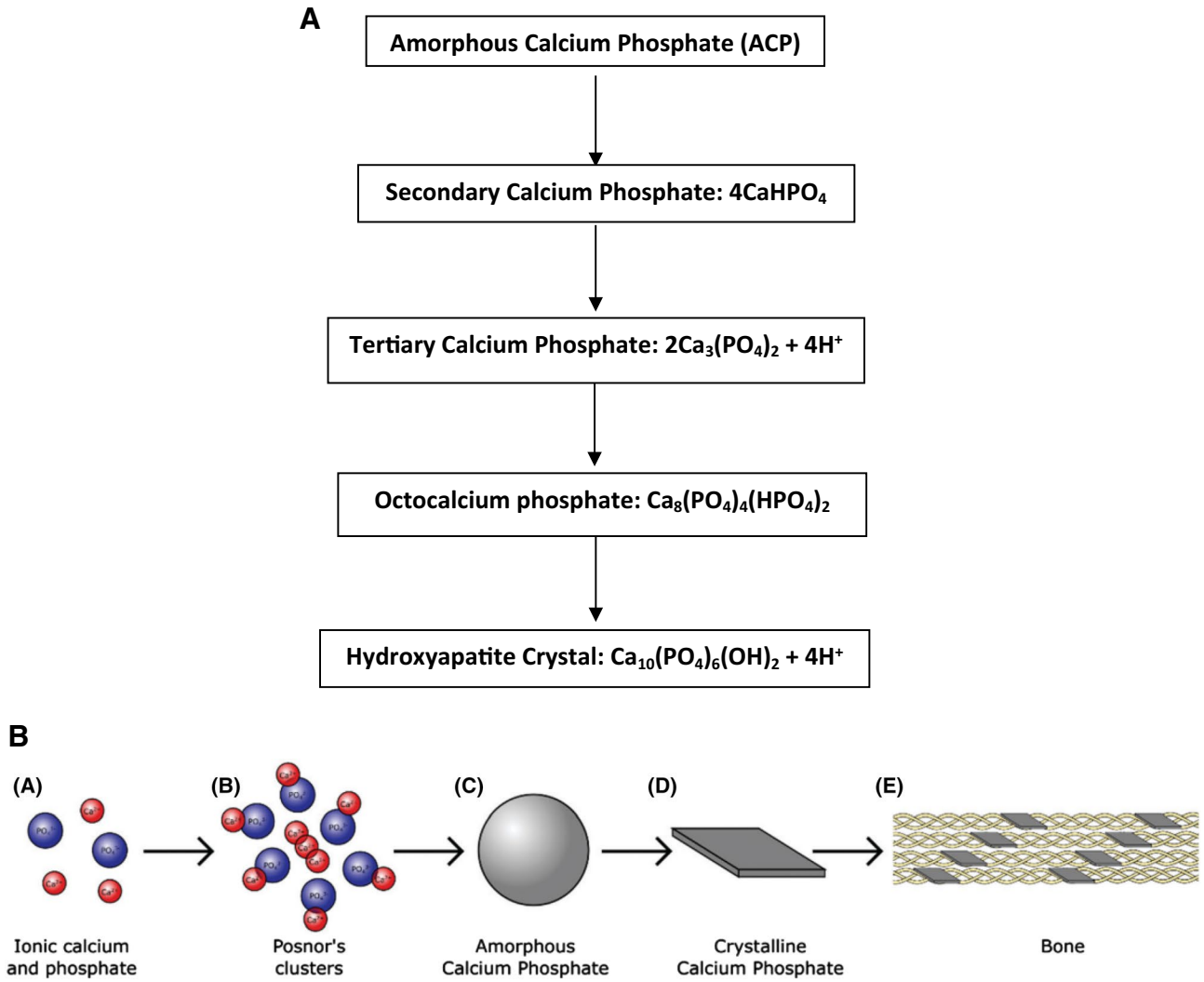

Fig. 2 **a** A simplified schematic depiction of sequential phase transformation of amorphous calcium phosphate to hydroxyapatite crystal formation. **b** Schematic depiction of different phases in bone mineralization. *A* Ca (red color filled circles) and Pi (lavender color filled circles) ions are co-localized in the physiological milieu. *B* The two divalent ions associate in a ratio of 1.5 to form Posner's clusters. *C* Posner's clusters agglomerate to form amorphous calcium phosphate (ACP) particulates. *D* ACP transforms into crystalline hydroxyapatite (HA) phase with a Ca:Pi ratio of 1.67. *E* The HA nanocrystals are then incorporated into collagen fibrils, mineralizing the organic scaffold (modified from [16]

the study of the effect of hypophosphatemia on chondrocytes. An orderly process of proliferation of resting chondrocytes and their differentiation into pre-hypertrophic, hypertrophic and terminally differentiated mature chondrocytes is necessary for longitudinal bone growth [40]. Adequate amounts of Pi are critical for the induction of apoptosis of mature chondrocytes in the growth plate [36, 38], without which the normal physiological chain of events fail resulting in expansion of growth plate, which is manifested as rickets and delayed growth [41, 42]. Under normal conditions, hypertrophic chondrocytes secrete angiogenic factors that promote vascular invasion [37], undergo apoptosis [36–38], and are replaced by mineralized bone. The chondrocyte apoptotic pathway is facilitated by phosphate-regulated activation of the caspase-9-mediated mitochondrial pathway [38]. Since rickets caused either by vitamin D deficiency or ablation of vitamin D receptor (VDR) can be rescued by adequate dietary Ca and Pi suggest that rickets is not a direct consequence of impaired VDR action, but rather, is due to the resultant hypocalcemia, hypophosphatemia, or hyperparathyroidism [38, 39].

Other osteogenic cells are also involved in bone mineralization. Crucial to the activity of osteoblasts and osteocytes in the process of matrix mineralization is the maintenance of adequate ambient Pi levels [2, 3]. Matrix vesicles arise from the cell membranes of osteoblasts and osteoblast lineage [7,

Table 1 Contrasting biochemical and bone histomorphometric features of vitamin D and phosphate deficiency osteomalacia

Measurement	Vitamin D deficiency	Phosphate deficiency
Serum calcium	Normal or low	Almost always normal[¶]
Serum phosphate	Normal or low*	By definition < 2.5 mg/dl
Serum PTH	↑ or ↑↑	Normal[¶]
Serum alkaline phosphatase	Almost always elevated	Almost always elevated
Osteoclast surface	↑↑	Normal[¶]
Marrow fibrosis	Frequent	Almost never[¶]
Cortical thickness	↓↓	Normal or ↑ or ↓[§]
Cancellous bone volume	Normal or↓	Normal or ↑ or ↓[§]

* Occasionally high due to severe hypocalcemia causing renal resistance to PTH action [85]

[¶]Except in patients with tertiary hyperparathyroidism due to long term oral phosphate therapy [86]

[§]Decreased only in acquired forms of hypophosphatemia most likely due to associated deficiency of vitamin D or calcium or both. Modified from reference [87]

Table 2 Representative values for bone histomorphometry in vitamin D and phosphate deficiency osteomalacia

Measurement	Vitamin D deficiency	Phosphate deficiency	Reference range
OS/BS (%)	61.3 ± 18.0	70.1 ± 15.9	21 ± 11
O.Th (μm)	29.7 ± 10.5	38.0 ± 12.2	< 12.5
OV/BV (%)	21.7 ± 11.5	33.6 ± 19.1	2.6 ± 1.4
ES/NOS (%)	5.27 ± 3.59	1.99 ± 1.53	4 ± 2
TBV/TV (% TV)	24.4 ± 9.97	26.4 ± 16.1	20 ± 6
CBV/TV (%)	84.1 ± 23.4	92.5 ± 45.7	94.5 ± 2.5
C.Th (cm)	0.51 ± 0.35	0.94 ± 0.71	1.27 ± 0.37

Note *higher mean values* for OS, O.Th, OV, TBV, and CBV in phosphate deficiency osteomalacia, and *lower mean value* for C.Th in vitamin D deficiency osteomalacia, a characteristic feature due to associated secondary hyperparathyroidism

Differences in bone volumes and C.Th are not significant since the phosphate deficiency osteomalacia group is a mixture of both hereditary and acquired (tumor induced and tenofovir treated) hypophosphatemic osteomalacia. Bone volumes C.Th. are high in hereditary forms, but are low in the acquired forms [10]

OS osteoid surface, *BS* bone surface, *O.Th* osteoid thickness, *OV* osteoid volume, *ES* eroded surface, *NOS* non-osteoid surface, *BV* trabecular bone volume, *TV* total tissue volume, *CBV* cortical bone volume, *C.Th* cortical thickness

23, 25], and osteocytes produce FGF-23 to regulate phosphate homeostasis to protect osteogenic cells from hyperphosphatemia, which negatively impacts osteoblasts and practically result in cell death [2, 32].

Abnormal Biomineralization

In contrast to some understanding of the physiological normal mineralization of bone, teeth and ossicles, the pathogenesis of abnormal biomineralization is poorly understood. Nevertheless, research in the last two decades has shed light on our understanding of the non-skeletal calcifications and mineralization processes. The most extensively studied is the vascular calcifications in the context of chronic kidney disease (CKD) in which hyperphosphatemia plays a dominant role [43–48]. Several complex pathological mechanisms are implicated in the development of vascular, valvular and soft tissue calcifications, including trans-differentiation of vascular smooth muscle cells to osteo/chondrogenic phenotype [49], apoptosis of vascular smooth muscle cells [50], instability and release of extracellular vesicles loaded calcium and phosphate [51], and elastin degradation [52]. Also, deficiency of inhibitors of mineralization such as pyrophosphate contributes to abnormal calcification process [47, 49]. However, it is unclear why abnormal biomineralization outside the skeleton necessarily does not become bone.

In classical osteomalacia, deficiency of key divalent ions (calcium and phosphate), however produced, results in the accumulation of unmineralized bone matrix (or osteoid) [10, 11, 41]. In contrast, in bone disorders that resemble osteomalacia or "osteomalacia like" or sometimes referred to interchangeably as "hyperosteoidosis", the osteoid accumulation is a consequence of disturbances outside of these two principal components. In hypophosphatasia [53], it is the

enzyme deficiency, whereas in Paget disease of bone [54], fibrous dysplasia [55], fibrogenesis imperfecta ossium [56], and osteogenesis imperfecta [57], it is the abnormal bone matrix due either to abnormal collagen fibrillar arrangement or to mutations in type-1 collagen genes. In drug-induced bone disorders that are associated with prolonged treatment with etidronate [58], fluoride [59, 60], aluminum [61, 62], or iron excess [63], the mineralization defect is the result of the toxic effects of these drugs inhibiting matrix mineralization [64]. Understanding the fundamental differences in the pathogenesis of defective mineralization of bone in different disease states and conditions is critical for clinical management as many disorders that mimic osteomalacia do not respond to vitamin D therapy as the word *osteomalacia* might imply [41]. With a very few exceptions (adefovir, adefovir, and tenofovir-induced osteomalacia [65–67], and that associated with renal failure [62], the serum phosphate levels are generally normal in these various bone disorders.

Role of Phosphate in Abnormal Mineralization

Several types of calcifications, enthesopathy, and ossification occur in a variety of conditions and disorders; some are associated with hyperphosphatemia, others with hypophosphatemia, and still others without any abnormalities in calcium and phosphate homeostasis (Table 3). Calcification of tendons (calcific tendinitis) [68], cartilage (chondrocalcinosis) [69], and soft tissues (metastatic and dystrophic calcifications [70, 71], can occur in various conditions and in aging, but their pathogenesis is poorly understood. In most such instances the serum phosphate levels are normal except in patients with associated CKD [48, 49]. Chondrocalcinosis and corneal calcifications (band keratopathy) have been described both in patients with primary hyperparathyroidism with *hypophosphatemia*, but with significant hypercalcemia [72, 73], and in patients with uremic secondary hyperparathyroidism with *hyperphosphatemia* with variable concentrations of serum Ca levels [74, 75]. Calciphylaxis, an uncommon serious complication seen in patients with CKD is often, but not always, associated with hyperphosphatemia, and responds sometimes to parathyroidectomy [76]. Enthesopathy, a common complication of X-linked hypophosphatemic disorders (XLH), is associated with *hypophosphatemia* rather than *hyperphosphatemia*, and its pathogenesis remains largely elusive, but FGF-23-Klotho axis has been implicated [77, 78]. In contrast, basal ganglion calcifications, a characteristic feature of patients with all varieties of hypoparathyroidism, is associated with hyperphosphatemia and hypocalcemia [79, 80], but such intracranial calcifications have also been described in patients without the abnormalities in divalent ion mineral homeostasis [81]. More recently, therapeutic use of FGFR inhibitors to treat certain cancers is associated with hyperphosphatemia and calcinosis cutis [82]. Tumoral calcinosis is another interesting entity, first described in 1943, and occurs with or without hyperphosphatemia [83]. Even the least understood abnormal biomineralization is heterotopic ossification, the pathogenesis of which remains unknown [84].

Summary

Biomineralization is a complex and dynamic lifelong process necessary to maintain both the structural and the functional integrity of the skeleton. Inorganic phosphate is an essential nutrient needed for many genetic, energy, and intermediary metabolic pathways as well as function of the osteogenic cells. Availability of adequate calcium and inorganic phosphate in the right proportions, at the right place, and at the right time is critical for proper acquisition, biomineralization, and maintenance of mass and strength of the skeleton. *Hypophosphatemia* results in mineralization defects and osteomalacia, and *hyperphosphatemia* is implicated in abnormal excess calcification and/or ossification, but the exact mechanisms underlying these processes are not fully understood. In this review we

Table 3 Types of abnormal calcification and mineralization associated with or without abnormalities in serum phosphate levels

Type of abnormality	Hypophosphatemia	Hyperphosphatemia
Basal ganglion calcification	No	Often, but not always
Calciphylaxis	No	Yes, exclusively in CKD patients
Calcinosis cutis	No	Yes, but not always
Chondrocalcinosis	Yes	Yes
Corneal calcifications	Yes (with hypercalcemia)	Yes (with or without hypercalcemia)
Enthesopathy	Yes	Uncertain
FGF-receptor inhibitor therapy	No	Yes
Heterotopic ossification	No	No/yes
Metastatic/dystrophic	No	Yes, but not always
Tumoral calcinosis	No	Almost always elevated
Vascular and valvular calcification	No	Yes

summarize available evidence on the role of phosphate in biomineralization. Other papers in this issue of the journal deal with other relevant aspects of phosphate homeostasis, phosphate signaling and sensing, and disorders resulting from hypo- and hyperphosphatemic states.

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Compliance with Ethical Standards

Conflict of interest Sudhaker D. Rao and Sanjay Kumar Bhadada declare that they have no conflict of interest.

Ethical Approval The study was conducted in accordance with the ethical standards of the institutional research committee of Henry Ford Hospital and the 1964 Helsinki Declaration and its later amendments.

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