The biochemistry of mineralizing extracellular vesicles. Part I: The role of phosphatases

CHAPTER 7

The biochemistry of mineralizing extracellular vesicles. Part I: the role of phosphatases

Flavia Amadeu de Oliveira[1], Cintia Tokuhara[1], Lucas Fabricio Bahia Nogueira[2,3], Juçara Gastaldi Cominal[3], Luiz Henrique da Silva Andrilli[1,3], Sonoko Narisawa[1], Colin Farquharson[4], Massimo Bottini[1,2], Ana Paula Ramos[3], Pietro Ciancaglini[3] and José Luis Millán[1]

[1]Human Genetics Program, Sanford Children's Health Research Center, Sanford Burnham Prebys Medical Discover Institute, La Jolla, CA, USA.
[2]Department of Experimental Medicine, University of Rome Tor Vergata, Rome, Italy
[3]Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto da Universidade de São Paulo (FFCLRP-USP), Ribeirão Preto, São Paulo, Brazil.
[4]Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, UK

Abstract
In this chapter, we will review some of the information regarding the functional significance of the inorganic phosphate (P_i)/pyrophosphate (PP_i) ratio for physiological mineralization of hard tissues. We will recount the structure and function of the phosphatases involved in the regulation of this ratio: Tissue-nonspecific alkaline phosphatase (TNAP); Nucleotide Pyrophosphatases/Phosphodiesterase 1 (ENPP1); Na,K-ATPase; Nucleoside triphosphate diphosphohydrolase 1 (CD39); ecto-5′-nucleotidase (CD73) and orphan phosphatase 1 (PHOSPHO1); and how this knowledge has guided the development of protein therapeutics and of small molecule inhibitors to affect the P_i/PP_i ratio in pathological conditions ranging from soft bones to ectopic calcification disorders.

Keywords
Tissue-nonspecific alkaline phosphatase; Nucleotide Pyrophosphatases/ Phosphodiesterases; Na,K-ATPase; Nucleoside triphosphate diphosphohydrolase 1; ecto-5′-nucleotidase; PHOSPHO1.

Content

1. Inorganic phosphate (P_i) and pyrophosphate (PP_i): a physicochemical balance

Ions are present throughout our body, circulating in the extracellular fluid. The concentration of Ca^{2+} is maintained at the narrow range of 2.2 to 2.7 mmol/L in the extracellular environment by the endocrine system [1]. On the other hand, the concentration of P_i varies considerably with age, being higher during childhood, but with values in the range of 1.12 to 1.45 mmol/L in adults [2]. In purely chemical terms, a salt will precipitate if its solubility is exceeded (supersaturation conditions), thus the concentration of circulating Ca^{2+} and P_i in the body fluid would be sufficient to explain apatitic mineral deposition in skeletal and dental tissues. However, despite the solubility product constant, log(Ksp), for hydroxyapatite being around 30 orders of magnitude smaller than the logarithm of the ionic product of the concentration of Ca^{2+} and P_i in human plasma under physiological conditions [3], direct precipitation of apatite or any metastable precursor does not occur in healthy soft tissues. Through very elaborate cellular mechanisms, our body can store ions and promote apatite mineralization only in bones and teeth, preventing calcification in soft tissues (except in pathological conditions). Furthermore, in certain regions of the body, such as the adhesion of tendons to bone, the interface between mineralized and non-mineralized tissue must be precisely controlled in order to maintain anatomical functionality. This fine control of biomineralization occurs through a cascade of spatially and temporally orchestrated events in which precursor ions are systemically prevented from precipitating as a solid mineral phase or are stabilized as precursor phases of amorphous minerals. Thus, in physiological conditions, mineralization occurs locally only in the extracellular matrix of skeletal and dental tissues, where osteogenic cells control the concentrations of mineralization inhibitors and create the ideal microenvironment for the nucleation and growth of the mineral phase [4].

As discussed in Chapter 2: Physiological biomineralization. Part I: the role of matrix vesicles in skeletal and dental calcifications, the characteristics of the extracellular matrix (collagen and non-collagenous proteins) influence mineral deposition. Non-collagenous macromolecules such as osteopontin (OPN) and vitronectin play a role in this process, either through their charged groups that act as sponges to attract precursor ions or through their ability to stabilize the amorphous precursor and allow its infiltration into fibrils of collagen and even act in the nucleation of the mineral phase. Thus, characteristics of the extracellular matrix could explain the existence of mineralized tissues, where charged groups create the favorable environment for overcoming the nucleation energy to establish a mineral phase [5–7]. However, in addition to the ability to secrete the extracellular matrix where the crystals will be deposited, osteogenic cells must be able to create a favorable environment to remove inhibitors and locally enhance the concentration of precursor ions (especially P_i). Mineralization is restricted to sites where cells can express a fibrillar matrix, such as type I collagen or elastin (the matrix) and enzymes capable of locally removing calcification inhibitors [8,9]. If the nucleation of the mineral phase is inhibited in a systemic way, the existence of mineralized tissues indicates cell specialization to a phenotype capable of creating the microenvironment necessary for the mineralization of the extracellular matrix. This concept recently enunciated by Marc McKee as the *Stenciling Principle* [10] describes that in the extracellular matrix, enzymes precisely control the selective removal (stenciling) of inhibitors – small molecules such as PP_i and inhibitory proteins such as OPN - to regulate mineral crystal growth. The *Stenciling Principle* implies a double-negative "inhibiting an inhibitor" paradigm that enables and regulates the growth of small mineral foci in the extracellular matrix [10].

PP_i, was the first extracellular calcification inhibitor identified, described in the 1960s as a potent inhibitor of mineralization [11]. PP_i is a phosphate ester formed by two P_i monomers joined by a P-O-P bond and is present in extracellular medium in concentrations of 1.5-3.0 µmol/L [12]. It acts as a mineralization inhibitor through complexation with Ca^{2+}, reducing the nucleation and growth rates of calcium phosphates [13]. Thus, maintaining an ideal P_i/PP_i ratio is widely accepted as a necessary condition for mineral formation in the extracellular matrix [14]. It has already been observed *in vitro* that different P_i/PP_i ratios promote the formation of different mineral phases. In fact, a ratio of around 140 results in the exclusive formation of apatites, while the reduction of this ratio to around 24 leads to the formation of calcium pyrophosphates [15]. The P_i/PP_i ratio has not been fully investigated under physiological conditions but considering that 1-2 mmol/L of P_i and 1-2 µmol/L of PP_i can be found in the extracellular medium, the resulting physiological P_i/PP_i ratio is around 1000. As described by Garimella *et al.* [16], a small variation in PP_i concentrations is enough to induce inhibition of apatite formation. In addition to PP_i being responsible for fine-tuning mineralization, phosphate is also stored in the form of polyphosphates, which is another way of controlling the concentration of free P_i necessary for the nucleation of the mineral phase [17].

OPN, a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of acidic, phosphorylated, calcium-binding, matricellular proteins, is another potent inhibitor of mineralization known to regulate dental, skeletal, and pathologic mineralization [18–20]. OPN is physiologically degraded by the zinc-metallopeptidase encoding gene *PHEX* (phosphate-regulating gene with homologies to endopeptidases on the

X chromosome) and mutations in *PHEX* lead to accumulation of OPN and its proteolytic fragments that cause X-linked hypophosphatemic rickets [21]. Yet another potent systemic inhibitor of mineralization is fetuin-A, a circulating protein with a high affinity for Ca^{2+}, which prevents the growth of mineral nuclei in the blood by mobilizing Ca^{2+} and P_i in a complex with colloidal dimensions that can reach 150 nm [22,23]. A single molecule of fetuin-A sequesters up to 90-120 Ca^{2+} and 54-72 P_i ions [23]. In addition, fetuin-A is abundant in bone tissue, corresponding to about 25 wt.% of non-collagenous proteins [23]. The ossification process is accompanied by an intense vascularization of the nascent tissue [24], and the observation of mineral particles from the bloodstream in the bone matrix indicates that calciproteins can act as a source of precursor ions for calcification [25–27]. As an example, the human blood protein vitronectin is a major component of the abnormal deposits associated with age-related macular degeneration, Alzheimer's disease, and many other age-related disorders [28]. Shin *et al.* [6] recently demonstrated that vitronectin is capable of binding both soluble Ca^{2+} and crystalline hydroxyapatite with high affinity and chemical specificity. The authors proposed that vitronectin may act as a buffer for ionic Ca^{2+} in blood, a coordinator of calcium-phosphate deposition and mineralization on the surface of lipid droplets, and a regulator of hydroxyapatite crystal growth.

Osteogenic cells control mineral phase deposition through different mechanisms, ranging from the intracellular formation of mineral precursors [29–31] to the release of mineralizing extracellular vesicles [32]. Matrix vesicles are a special type of extracellular vesicles identified as responsible for triggering the formation of the mineral phase during bone mineralization and possessing high affinity for collagen fibrils. Extracellular vesicles are structures released by almost all cell types and ubiquitously present in biological fluids and classified according to their cellular origin into two classes: exosomes and microvesicles [33]. Exosomes have intracellular origin in the endosomal system, secreted into the extracellular environment after the fusion of multivesicular bodies with the cell membrane cell. Microvesicles are formed from directly budding of the cell membrane. Extracellular vesicles have aroused great interest due to their role in transporting and directing different cargoes (e.g., nucleic acids, lipids, and proteins) for delivery to cells, generating important responses for both physiological processes and pathological conditions [33]. The origin of the name "matrix vesicles" is related to their presence in the extracellular matrix. They are a special class of extracellular microvesicles found interacting specifically with the collagenous matrix and uniquely endowed with the specialized function of inducing mineralization [32]. Matrix vesicles were discovered in the 1960s by H. Clarke Anderson [34] and Ermanno Bonucci [35] who visualized vesicular structures containing mineral in the extracellular matrix of mineralizing cells using transmission electron microscopy. Matrix vesicles provide an appropriate microenvironment for the initiation of mineralization. All their lipidic and proteic components play important roles in this process, either by catalyzing reactions or creating specific interactions that result in the optimization of mineral phase formation [32,36]. In addition, they are identified as the only type of extracellular vesicle capable of binding to collagen [37]. Despite more than 50 years of study on matrix vesicles, the mechanism by which they are secreted, and how exactly they control mineralization remains elusive. In addition, there are still great challenges to fully understand the process: identifying matrix vesicles *in vivo*, differentiating them from other extracellular vesicles, and accurately characterizing the mineral formation mediated by these structures *in vivo* and *ex vivo*. Tracking mineral formation has been especially challenging, mainly due to artifacts in sample preparation for electron microscopy attributed to dissolution/recrystallization mechanisms or removal of metastable precursors.

Matrix vesicles can be isolated from biological tissues by ultracentrifugation (see Chapter 1X: Working with Mineralizing Extracellular Vesicles: Purification Techniques) after collagenase digestion, which allows obtaining a fraction rich in these vesicles, at the expense of other extracellular vesicles [38]. Collagenase digestion is the key point in distinguishing matrix vesicles from other extracellular vesicle populations. Using this procedure, a population of matrix vesicles is isolated and differentiated from other cellular components by their biochemical markers and properties: high tissue-nonspecific alkaline phosphatase (TNAP) activity; a characteristic proteic and lipidic profile and their ability to accumulate Ca^{2+} and P_i *in vitro* [32,36].

As can be seen in Figure 1, matrix vesicles have the ability to rapidly mineralize (5 hours), when dispersed in synthetic cartilage lymph in the presence of P_i and Ca^{2+}. Although phosphatases are not functional under these experimental conditions, Ca^{2+} and P_i transporters can act adjusting the ionic concentrations inside the matrix vesicles and generating the nucleational core to subsequently propagate mineral formation. In the presence of ATP, phosphatases are engaged in producing P_i (**Figure 1**). Since it is an enzymatic P_i-generation progress, mineralization initiation takes longer and begins after 10 hours. After 20 hours of incubation with ATP the turbidimetry reaches the same values found by incubation with free P_i **(Figure 1)**. This simple experiment evidences the role of the biochemical machinery in initiating mineralization [39]. Therefore, matrix vesicles create a microenvironment, inside the vesicles, that permit the initiation of mineralization (nucleation process), and in a second step the propagation of the mineral onto the collagenous matrix [32,40]. Evidence described in the

literature indicate that matrix vesicles act as nanoreactors during bone mineralization due to two main characteristics: [i] their elaborate enzymatic machinery that works in coordination to control the P_i/PP_i ratio and [ii] mineral phase formation within matrix vesicles that is specifically controlled by their lipidic and proteic composition.

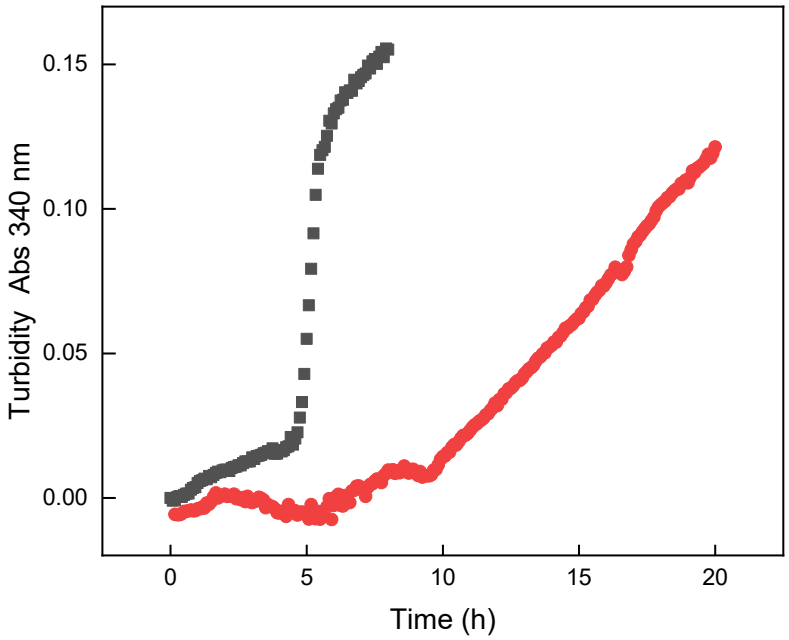

Figure 1. Mineralization curves obtained for matrix vesicles dispersed in synthetic cartilage lymph. Changes in the turbidimetry at 340 nm as a function of time for matrix vesicles dispersed in synthetic cartilage lymph in the presence of 2.0 mM $CaCl_2$ plus (■) 3.41 mM NaH_2PO_4 or (■) 2.0 mM ATP, as source of phosphate. As described by Buchet *et al*. [40], matrix vesicles were extracted from femurs epiphyses/growth plates embryo chicken in synthetic cartilage lymph constituted by: 1.83 mM $NaHCO_3$, 12.7 mM KCl, 0.57 mM $MgCl_2$, 5.55 mM D-glucose, 63.5 mM sucrose, 16.5 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol)/HCl, 100 mM NaCl, 0.7 mM Na_2SO_4, in deionized water at pH 7.6 [41]. The amount of vesicles added to the samples was normalized to the amount of protein.

2. The molecules regulating the P_i/PP_i ratio

Proteomic analysis of matrix vesicles (see Chapter 10: Working with mineralizing extracellular vesicles. Part II: proteomic profiling) revealed an important number of enzymes and transporters that work in synergy to enable the nucleation and propagation of the mineral, tightly controlling the concentration of P_i and PP_i outside and inside of the vesicles.

As schematically represented in the Figure 2, orphan phosphatase 1 (PHOSPHO1) produces P_i inside matrix vesicles through the hydrolysis of phosphocholine and phosphoethanolamine [42], derived from various sources including sphingomyelin by the action of sphingomyelin phosphodiesterase 3 (SMPD3) that acts in the inner leaflet of the vesicle´s membrane [43]. In addition, phosphate transporter 1 (P_iT-1), and possibly other not yet identified transporter(s), may also be responsible for increasing the concentration of P_i ions that is necessary for nucleation inside the matrix vesicles, forming hydroxyapatite [44]. In the membrane of matrix vesicles, TNAP and nucleotide pyrophosphatases/phosphodiesterase 1 (ENPP1) control the P_i/PP_i ratio by hydrolysis of phosphomonoesters, ATP and also PP_i [45]. ATP-binding cassette sub-family C member 6 (ABCC6) is one of the transporters that may export ATP, but possibly also AMP and PP_i, to the extracellular milieu [46]. In addition, Na,K-ATPase may contribute to intravesicular P_i formation acting to generate a Na^+ gradient that could be used to co-transport P_i by P_iT-1 and also to form P_i by ATP hydrolysis [39]. OPN binds to hydroxyapatite mineral as soon as it is exposed on the surface of matrix vesicles [47]. The exact mechanism by which matrix vesicles are generated and how they help to propagate hydroxyapatite mineral onto the collagenous matrix remains unclear and these are areas of active investigation in our laboratories. In the next sections, we will discuss the matrix vesicles biochemical machinery with emphasis on these phosphatases.

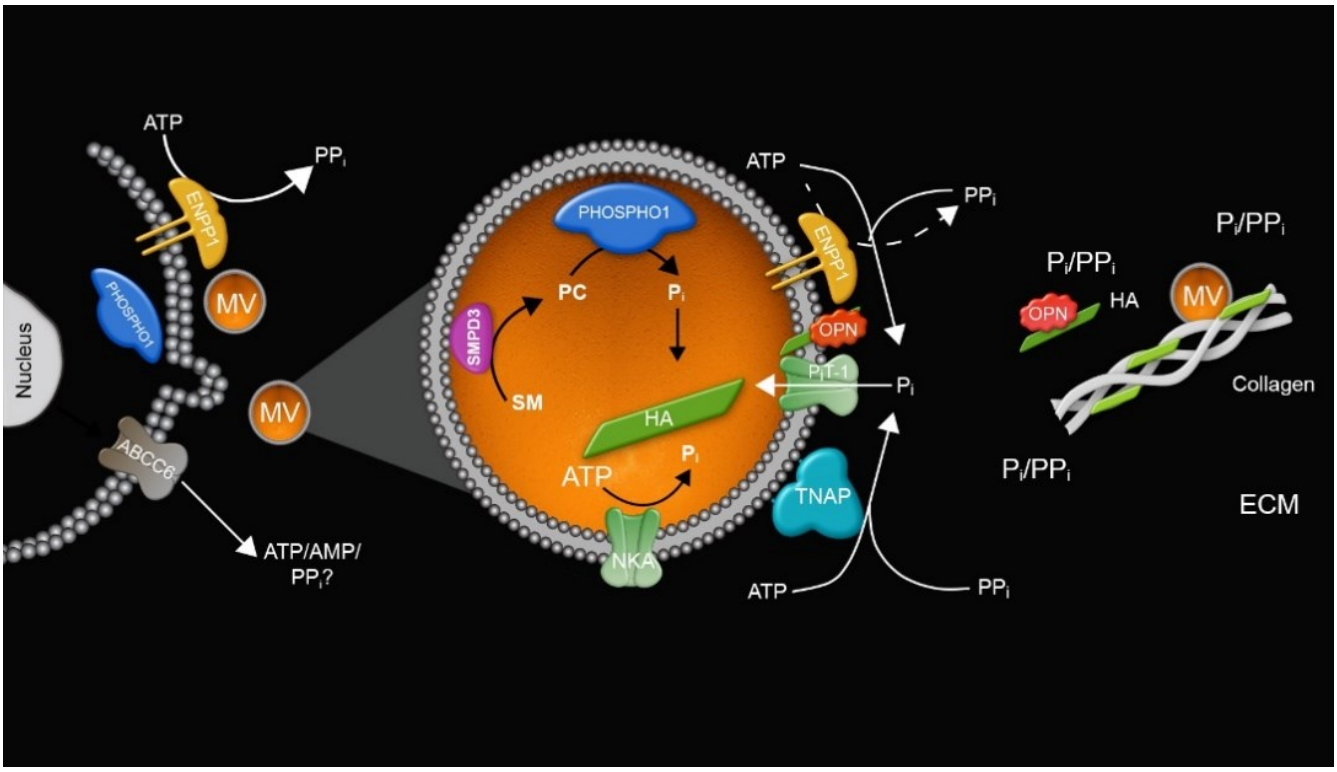

Figure 2. **Schematic representation of P_i/PP_i homeostasis during matrix vesicle-mediated mineralization**. Matrix vesicles provide an environment that allows the initial nucleation of apatite inside the vesicles. P_i is generated and the concentration of PP_i is controlled by the orchestrated action of phosphatases and phosphate transporters. Abbreviations in the figure: orphan phosphatase 1 (PHOSPHO1); phosphocholine (PC); sphingomyelin (SM); sphingomyelin phosphodiesterase 3 (SMPD3); phosphate transporter 1 (P_iT-1); apatitic mineral/hydroxyapatite (HA); Tissue-nonspecific alkaline phosphatase (TNAP); Nucleotide Pyrophosphatases/Phosphodiesterase 1 (ENPP1); Nucleoside triphosphate diphosphohydrolase 1 (CD39); ATP-binding cassette sub-family C member 6 (ABCC6), ecto-5′-nucleotidase (CD73); osteopontin (OPN); adenosine triphosphate (ATP); adenosine diphosphate (ADP) and Na,K-ATPase (NKA); matrix vesicles (MV).

3. Tissue-nonspecific alkaline phosphatase (TNAP)

TNAP is an enzyme that has phosphomonohydrolytic activity. It produces P_i from the hydrolysis of many phosphorylated substrates: ATP, adenosine diphosphate (ADP), glucose-1-phosphate, glucose-6-phosphate, glyceraldehyde-phosphate, among others [48]. More efficiently and selectively, it converts PP_i into two molecules of P_i, or one molecule of ATP into up to three molecules of P_i and adenosine as the final reaction product. The existence of an enzyme with phosphomonohydrolytic activity capable of producing P_i locally during ossification was proposed in the 1920s [49]. TNAP belongs to a family of isozymes, widely expressed in the body. In humans, alkaline phosphatases are encoded by four genes traditionally named referring to the tissues where they are predominantly expressed [50]. TNAP is encoded by the *ALPL* gene and expressed at highest levels in the liver, bones, and kidney, the reason why it is called tissue-nonspecific. The other three isozymes are tissue-specific: placental (PLAP, *ALPP* gene), germ cell (GCAP, *ALPPL2* gene) and intestinal (IAP, *ALPI* gene) alkaline phosphatase. Regardless of their origin, alkaline phosphatases are homodimeric enzymes consisting of two identical subunits of 484 amino acids with a molecular weight of approximately 60 kDa, containing three active site metal ions (two Zn^{2+} and one Mg^{2+}), necessary for catalytic activity [50]. A non-catalytic site occupied by Ca^{2+} was discovered after resolving the three-dimensional structure of PLAP [51,52] providing structural confirmation of previous studies that indicated that, in cartilage, alkaline phosphatase is a Ca^{2+}-binding glycoprotein [53]. Since its identification, TNAP has been confirmed as one of the main enzymes responsible for calcification of the bone matrix [14]. Despite the presence of alkaline phosphatases in several tissues in the body, mineralization is physiologically restricted to the skeleton and teeth since they require extracellular matrices of specific composition (collagens) to serve as a scaffold for mineral deposition. Moreover, the co-expression of TNAP and collagen fibers has been described as necessary and sufficient to calcify any

extracellular matrix [8]. Thus, not surprisingly, many pathological conditions that result in soft tissue calcification involve the upregulation of TNAP [54].

TNAP is found at the outer leaflet of both the plasma membrane of osteogenic cells and the membrane of matrix vesicles. It is a marker of a mineralizing phenotype, given the role of this enzyme in PP_i removal and local P_i generation during mineralization. TNAP-deficient mice produce matrix vesicles that lack TNAP [45] and *in vivo* analyzes of bone matrix in these animals demonstrate deficient propagation of bone mineral onto the extracellular matrix due to accumulation of extracellular PP_i [37]. In addition, TNAP is also involved in the removal of phosphate groups from some proteins such as OPN, being able to modify its phosphorylation level and, consequently, affecting its inhibitory properties during calcification [55-57]. Furthermore, the extracellular PP_i concentrations regulate the expression of OPN [58] and in turn OPN regulates the expression of genes that control the production and degradation of PP_i in a counter-regulatory loop [59]. The ability of TNAP to locally control the PP_i concentration and the phosphorylation of non-collagenous proteins points to the fundamental role of this enzyme in the complex mechanisms of mineralization [4].

The importance of TNAP in controlling skeletal and dental mineralization is further evidenced by the pathophysiology of hypophosphatasia, a hereditary bone disease caused by hypomorphic mutation in the *ALPL* gene [60], that will be discussed in more detail later on in this chapter. TNAP deficiency in hypophosphatasia leads to soft bones, in the form of rickets in children and osteomalacia in adults, due to the accumulation of extracellular PP_i that suppresses the propagation of apatitic mineral onto the collagenous matrix [60]. Skeletal as well as dental hypomineralization characterizes hypophosphatasia, with disease severity varying from life-threatening perinatal or infantile forms to milder forms that manifest in adulthood or only affect the dentition [61].

In contrast, upregulation of TNAP in soft tissues leads to sinking PP_i levels and wide-spread ectopic calcification. Overexpressing TNAP in the medial layer of arteries in mice leads to medial artery calcification [62] (see Chapter 3: Pathological biomineralization. Part I: Mineralizing extracellular vesicles in cardiovascular diseases). Elevated expression of TNAP causes media calcification in patients with diabetes, obesity, chronic kidney disease-mineral bone disorder and simply during aging. Overexpression of TNAP in the intimal layer of arteries is also a key event in the pathophysiology of atherosclerosis [63,64] where rupture of a plaque is considered the primary reason for cardiovascular death, accounting for most myocardial infarction cases and about 20% of ischemic strokes [65]. Recent data suggest that microcalcifications, characterized by size smaller than 10 μm, negatively impact plaque stability [66,67]. The potential beneficial effects of TNAP inhibition have been investigated in two models of atherosclerosis, one with the *Ldlr*$^{-/-}$ atherogenic mutation superimposed onto the Tie2-Cre TNAP overexpressor mice [64] and in the *ApoE*$^{-/-}$ atherogenic mice, both fed a high-fat diet. In both models, calcification was the initiating event in the pathophysiology of atheroma formation and administration of a TNAP inhibitor by food admixture prevented vascular calcification and reduced the development of microcalcifications [68].

These data clearly illustrate the physiological role of TNAP in controlling the extracellular P_i/PP_i ratio conducive for proper skeletal and dental mineralization and the consequences of a pathophysiological upregulation of TNAP that results in sinking PP_i concentrations and calcification of soft tissues.

4. Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) and other ENPPs

ENPP1 belongs to a class of enzymes (ENPPs) expressed in different anatomical sites such as bone, cartilage, and adipose tissue, having specific functions in each tissue/cell type. Among the main enzymes of this large family, ENPP1 (also known as NPP1 or PC-1) is an integral membrane protein [69,70], with a molecular weight of approximately 125 kDa and a total of 906 amino acid residues. This enzyme stands out for its ability to hydrolyze different types of polyphosphorylated nucleotides and phosphodiesters, ATP in particular, to generate PP_i [69,71,72]. Thus, ENPP1 (the main generator of PP_i) together with TNAP (that controls the P_i/PP_i ratio in the extracellular matrix), have crucial functions in the production and regulation of the P_i /PP_i ratio in the extracellular matrix, influencing the generation of the mineral phase [73]. Failures in the expression of ENPP1 result in abnormalities related to bone hypermineralization and pathological soft tissue mineralization, including arterial calcification [44]. However, elevated expression of ENPP1 results in calcification of cartilaginous tissues, due to the formation of calcium pyrophosphate dihydrate, which occurs mainly during the aging process of the knee meniscus cartilage [74,75]. Abnormal precipitation of calcium pyrophosphate dihydrate has also been reported associated with TNAP deficiency [44].

ENPP1 deficiency in mice leads to ossification of the posterior longitudinal ligament of the spine, peripheral joint hyperostosis and calcification of articular cartilage as well as generalized arterial calcification of infancy in humans but can also manifest phenotypic changes characteristic of pseudoxanthoma elasticum, that is caused by a deficiency in the ABCC6 transporter. *Enpp1*$^{-/-}$ mice have almost undetectable levels of plasma PP_i leading

to inappropriate deposition of apatitic mineral in soft tissues [73,74]. Surprisingly, *Enpp1*$^{-/-}$ mice also display reduced trabecular and cortical bone in the long bones and decreased bone strength [76] corresponding to autosomal recessive hypophosphatemic rickets type 2 (ARHR2) in humans [77] and in fact, those children that survive generalized arterial calcification of infancy (experiencing the characteristic calcification of their arteries, heart, kidneys, and joints) will develop ARHR2 (displaying rickets, bone and muscle pain, bowing of the legs, short stature, and an increased risk of fractures). Pseudoxanthoma elasticum is a genetic disorder caused by mutations in the human *ABCC6* gene that is characterized by Ca^{2+} deposition outside of the skeletal system, specifically in the blood vessels, skin, and eyes, but can also manifest skeletal mineralization abnormalities [78].

The ENPP family members share a homologous catalytic core, phosphodiesterase domain, and previously they were named and grouped based on the nature of their substrates, extracellular location, and chronological discovery. The family members are divided into two subgroups, ENPP1-3 and ENPP4-7, reflecting their structure and domain constitution [79]. ENPP1-3 have multi-domain proteins, with two tandem N-terminal somatomedin B-like domains, a phosphodiesterase domain, a linker region (“lasso loop”), and a C-terminal nuclease-like domain [79]. The ENPP1 and 3 are considered single pass type II membrane proteins. However, Autotaxin (also known as ENPP2) is a secreted protein, processed by removal of its signal peptide [80,81]. The other four ENPP members (4,5,6,7), just have the signature phosphodiesterase domain. On the other hand, ENPP4, ENPP5, and ENPP7 are single-pass type I membrane proteins, while ENPP6 is glycosylphosphatidylinositol-anchored to the plasma membrane [82]. Therefore, all the ENPP members are implicated in purinergic signaling and can hydrolyze extracellular nucleotides. They have biological importance due to their broad substrate repertoire and structural diversity, as revealed by animal and human data [79].

ENPP1 is a member of the ENPP family of proteins with two somatomedin B-like domains, a catalytic domain, and nuclease-like domain. The catalytic and nuclease-like domains are related to bone mineralization while, the somatomedin B-like domains are important for insulin signaling. ENPP1 cleaves a variety of substrates, including phosphodiester and pyrophosphate bonds of nucleotides and nucleotide sugars [83].

It has been established that ENPP1 is a regulator of soft tissue and skeletal mineralization and is involved in bone remodeling [76,84]. Studies have reported that *Enpp1*$^{-/-}$ mice are protected against obesity and diabetes through alterations to their osteocalcin carboxylation status [85]. In turn, bone is a key endocrine regulator of metabolism and energy balance mediated by an osteocalcin pathway [86–88]. On the cell surface, ENPP1 generates PP_i by hydrolyzing ATP, which inhibits apatitic crystal formation and deposition, inhibiting the mineralization by binding to newly formed crystals, therefore, preventing the growth of these crystals [83,89]. Also, ENPP1 can act as a potent ATPase in the matrix vesicles microenvironment in absence of TNAP [45,60,72,90].

5. Nucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5′-nucleotidase (CD73)

Ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1 or CD39) belongs to one of the largest family of ectonucleotidases present in the human body. The fundamental characteristic that distinguishes this family of transmembrane enzymes is the use of Ca^{2+} and Mg^{2+} ions as cofactors of their catalytic sites. Within this family there are eight different types of E-NTPDase, among which E-NTPDase1-3 and 8 stand out as the main enzymes capable of hydrolyzing ATP to generate PP_i and AMP [91]. Therefore, they share very similar protein structures, as well as similar tertiary structure with actin/HSP70/sugar kinase families. The E-NTPDase1 encoded by *ENTPD1* gene on the 10q24.1 chromosome, are constituted by 510 amino acids residues, and contains a C-terminal and an N-terminal α-helical transmembrane domain [92]. The formation of its catalytic site comes from two oligomeric complexations, resulting in a strong interaction with substrates between these complexed domains [91].

Moreover, the presence of E-NTPDase1 stands out in polymorphonuclear neutrophils, and in HL-60 cells, and hydrolyzing ATP for the production of antibodies [91,93]. E-NTPDase1 corresponds to antigen CD39 in lymphoid cells, especially when associated with the quiescent vascular endothelium [91]. Due to its presence in cells of the immune system, CD39 is also found in the microenvironment of tumors, due to its possible correlation with the production of adenosine during the process of tumor proliferation by various immunosuppression mechanisms [92]. The formation of adenosine, under non-pathological conditions, is intertwined with the function of another family of nucleotides, the Ecto-5'-Nucleotidase (CD73 or ecto-5'-NT) and TNAP.

Ecto-5'-NT is a glycoprotein bound directly to the cell membrane through a glycosylphosphatidylinositol anchor [94]. Purified Ecto-5'-NT is a 70 kDa protein, with 574 amino acid residues, forming three structural domains: i) an N-terminal domain with binding site for metal ions (metal binding site); ii) a C-terminal domain, basically consisting of the catalytic activity of the enzyme, and a hydrophobic interaction region that results in the stabilization of its homodimerization; and iii) the α-helix domain bridge [92]. Its catalytic site has an

extracellular orientation and is activated with the coordination of two Zn^{2+} ions, hydrolyzing AMP molecules to produce adenosine and P_i [92,95]. Therefore, its adenosine-forming property makes Ecto-5'-NT a rate-limiting second step in the metabolism of purine-based nucleotides.

Recently, both ENPD1 and Ecto-5'-NT were found expressed in hypertrophic chondrocytes and differentiated osteoblasts of 7-days-old wild-type mice, indicating that they likely act together with TNAP in maintaining the ATP/adenosine and the P_i/PP_i ratios during bone biomineralization [95]. However, while TNAP is transferred from the cell membrane to the matrix vesicles' membranes, neither CD39 nor CD73 were found on the membranes of matrix vesicles, but remained at the cell membrane [95], so while both enzymes affect ATP metabolism and generate P_i, they are not matrix vesicles' enzymes, per se.

6. PHOSPHO1

PHOSPHO1, discovered in the Farquharson laboratory, is a phosphomonohydrolase found in the cytosol of mineralizing cells and in the lumen of their blebbed matrix vesicles [42,96]. The PHOSPHO1 gene of humans and mice has three exons of which exon 3 contains the conserved motif of the haloacid dehalogenase superfamily [96,97]. A PHOSPHO1-3a transcript has been identified in human osteoblasts. The 127 bp sequence in intron 2 of PHOSPHO1-3a forms the starting point of an 879 bp open reading frame with a predicted protein, which encodes 292 amino acids resulting in a protein of ~ 29 kDa [42,98].

The crystal structure of the PHOSPHO1 protein has not been reported to-date but a three-dimensional model of human PHOSPHO1 protein shows two domains, a catalysis-related α/β structure and a Rossmann-like fold with a four-spiral bundle domain [99]. The Rossmann-like fold had six parallel β-sheets and six surrounding α-helices. The predicted human PHOSPHO1 protein contained three conserved peptide motifs. The motif I comprised threonine and valine residues and two aspartic acids (Asp43 and Asp123) residues. Mutation of Asp123 reduced the catalytic activity of PHOSPHO1 with phosphatidylcholine by 20 and 60 times, respectively. Mutation of Asp43 reduced the catalytic activity of PHOSPHO1 with phosphatidylethanolamine and abolished the reactivity of PHOSPHO1 with phosphatidylcholine. These results indicated that Asp123 and Asp43 of motif I might be the active enzymatic sites of PHOSPHO1 protein in catalyzing different substrates [99].

Regarding its role in bone mineralization, it has been reported that PHOSPHO1 acts in concert with TNAP to accumulate P_i for initiation of mineralization. In fact, animals doubly deficient in TNAP and PHOSPHO1 exhibit complete ablation of skeletal mineralization and embryonic lethality [100]. Despite concrete evidence for the role of PHOSPHO1 in ossification, the biochemical pathway by which this enzyme aids in the accumulation of P_i within matrix vesicles remains unclear. PHOSPHO1 generates P_i through the hydrolysis of two substrates: phosphocholine and phosphoethanolamine. Different pathways have been proposed for the local production of these substrates, either through the action of phospholipase A2 (PLA_2), ectonucleotide pyrophosphatase/phosphodiesterase 6 (ENPP6) or SMPD3 as recently reviewed [101]. Stewart *et al.*[99] proposed a mechanism through which phosphatidylethanolamine and phosphatidylcholine may be generated intravesicularly by the enzymatic action of PHOSPHO1 on the vesicle's phospholipid membrane, as mediated by a PLA_2 and ENPP6 [101,102]. The PLA_2 family of enzymes catalyze cleavage of the acyl group at the *sn*-2 acyl position of glycerophospholipids resulting in a free fatty acid and lysophospholipid [41,48,103–105]. These enzymes may therefore act to breakdown phosphatidylcholine and phosphatidylethanolamine present in the matrix vesicles' membrane, forming lysophosphatidylcholine and lysophosphatidylethanolamine, respectively, along with arachidonic acid [102]. Indeed, the matrix vesicle membrane has been shown to be enriched in phospholipids containing phosphatidylcholine and phosphatidylethanolamine which progressively decline during mineralization [106–108], while phosphatidylcholine was also identified as an abundant metabolite in developing mouse long bones by matrix-assisted laser desorption/ionization-imaging mass spectrometry [109]. Interestingly, a recent spatial lipidomic study reported that lysophosphatidylcholine and lysophosphatidylethanolamine were upregulated in the growth plate of PHOSPHO1 null mice supporting a role for these lipids in the formation of PHOSPHO1 substrates [110]. There are upwards of 30 identified mammalian PLA_2 enzymes which exhibit a wide range of localizations (including secreted, cytosolic and lysosomal groups) and have been shown to be involved with many physiological and pathological processes [111–113]. Mebarek et al. [114] comprehensively reviewed the evidence for the role of phospholipases in mineralization, noting several experimental studies confirming expression of both secreted and cytosolic PLA_2s in chondrocytes and osteoblasts where they play several roles [114]. While some specific PLA_2s have been shown to influence bone formation [115] it is currently unclear whether they act directly in the mineralization process. A second enzymatic processing phase is hypothesized to convert generated lysophospholipid to phosphatidylcholine for direct hydrolysis by PHOSPHO1, mediated by ENPP6 [116]. ENPP6 has been shown to possess lysophospholipase C activity, catalyzing the conversion of lysophosphocholine with a monoacylglycerol by-product [69,117,118]. Expression of ENPP6 has

been demonstrated in bone tissue lysate and was immunolocalized to hypertrophic chondrocytes and forming bone surfaces[116]. Specific localization of ENPP6 to matrix vesicles has yet to be established, however. PHOSPHO1-deficient mice presented, in addition to compromised ossification, a reduced number of matrix vesicles, indicating that this enzyme may also be involved in matrix vesicle biogenesis[44].

7. Can Na,K-ATPase act as a phosphatase?

Finally, Na,K-ATPase is also found in the matrix vesicles' membranes, and can promote mineralization by increasing the local concentration of P_i and consequently changing the P_i/PP_i ratios. Na,K-ATPase is an active cationic transporter found in the cell membrane of all mammals, acting to transport three Na^+ out and two K^+ into cells for each hydrolyzed ATP molecule [119]. The functional structure of the enzyme is a heterodimer formed by two major α and β subunits. The α subunit (110 kDa) has ten transmembrane segments and three cytoplasmic domains, while the β subunit (55 kDa) has only one transmembrane segment and a highly glycosylated extracellular portion [120,121]. There are 2 isoforms for each of the α and β subunits that can associate with each other in dimers of distinct enzymatic and inhibition properties. The presence of α1, α2, β1 and β2 isoforms were identified in chondrocytes isolated from bovine cartilaginous joint [122] and in matrix vesicles isolated from hypertrophic chondrocytes from femurs of chicken embryos [123,124]. The fact that in the membranes of matrix vesicles there may be a dimeric form of the Na,K-ATPase combined with the fact that osteoblasts are able to mineralize from ATP hydrolysis, may show an important role of this enzyme in biomineralization [125]. Ongoing *in vitro* experiments in the Ciancaglini´s laboratory suggest that all ATP inside matrix vesicles could be hydrolyzed by Na,K-ATPase contributing to the P_i concentration necessary for apatite formation [36]. In this way, the Na,K-ATPase adds P_i to the pool of ions produced by PHOSPHO1.

8. Phosphate transporters

Phosphate transporters P_iT-1 and P_iT-2 also called Na^+/P_i type III cotransporters have been proposed as proteins capable of mediating the loading of extracellular P_i into the matrix vesicles [44]. P_iT-1 was found in hypertrophic chondrocytes during endochondral ossification in mice [126], evidencing its important role in regulating P_i concentrations in bones and cartilage. In addition, P_i has been shown to modulate the differentiation of chondrocytes and osteoblasts [127]. Sugita *et al*. [128] suggested that ATP synthesis mediated by the transport of P_i into cells via P_iT-1 is critical for the regulation of apoptosis and mineralization of chondrocytes. Lau *et al*. [129] demonstrated that Na^+/P_i co-transport activity through P_iT-1 is associated with osteoblast differentiation and that increased extracellular P_i concentration affects chondrocyte differentiation. Suzuki *et al.* [130] also investigated the effects of transgenic P_iT-1 overexpression on the Ca^{2+}/P_i ratio and bone metabolism. P_iT-1 transgenic mice exhibited abnormal mineral metabolism and reduced TNAP activity in osteoblasts, although bone matrix mineralization and skeletal development were normal [130]. Adult P_iT-1 transgenic mice exhibited hyperphosphatemia, associated with reduced bone mass [130]. In this *in vitro* study, overexpression of P_iT-1 in osteoblasts led to a marked increase in P_i transport and downregulation of TNAP expression [130].

9. Genetic diseases caused by altered P_i/PP_i ratio

Hypophosphatasia is an inborn error-of-metabolism caused by loss-of-function mutations in the *ALPL* gene, which encodes TNAP [131–134]. To date, 416 mutations spread across the *ALPL* gene have been documented (https://alplmutationdatabase.jku.at/) but their genotype/phenotype correlations are not well understood [135,136]. Studies in mice have demonstrated that mineralizing skeletal and dental cells, including osteoblasts, chondrocytes, ameloblasts, odontoblasts, and cementoblasts, express TNAP and thus would be affected in hypophosphatasia [137,138]. Hypophosphatasia patients suffer from distinctive rickets and/or osteomalacia with a broad range of severity, as well as dental defects. There are seven major forms the disease: life-threatening perinatal and infantile (OMIM#241500), benign perinatal, mild and severe childhood (OMIM#241510), adult (OMIM#136300), and odonto-hypophosphatasia (OMIM#146300) [133,134]. Patients with perinatal hypophosphatasia, the gravest form of the disease, often die in utero or soon after birth because of severe skeletal hypomineralization, respiratory failure due to thoracic cage dysplasia and hypoplastic lungs, and elevated intracranial pressure due to craniosynostosis [139–143]. Dentoalveolar phenotypes, including premature exfoliation of primary teeth, periodontal disease, and enamel alternations, are commonly observed in patients with all forms of hypophosphatasia [144–146]. The inheritance pattern of perinatal and infantile hypophosphatasia is often autosomal recessive, with most patients being compound heterozygotes for pathogenic *ALPL* mutations that result in almost null TNAP activity, but some are homozygous for recessive alleles and most adult and odonto hypophosphatasia patients harbor a single dominant-negative *ALPL* allele [147,148].

Phospho1$^{-/-}$ mice exhibit growth plate and skeletal abnormalities as well as thoracic scoliosis that becomes apparent shortly after birth [100]. The long bones in the *Phospho1*$^{-/-}$ mice show a distinctive patchy osteomalacia with frequent bowing and displaying spontaneous fractures, they contain less mineral, with smaller mineral crystals [149]. Three-point bending studies showed that the *Phospho1*$^{-/-}$ fractured bones had a more elastic characteristic than the control bones [150,151]. Biochemically, the lack of PHOSPHO1 leads to downregulation of *Alpl* gene expression, resulting in reduced levels of TNAP in matrix vesicles, chondrocytes/osteoblasts and plasma and an increase in plasma PP_i and OPN concentrations [152]. In addition, deficiency in PHOSPHO1 leads to a marked decrease in the production of matrix vesicles by chondro-osteogenic cells, and those matrix vesicles that are still produced are smaller in size and volume and largely devoid of the nucleational core characteristically observed in mineralizing wild-type matrix vesicles [44,153]. Consequently, both matrix vesicles-mediated initiation and propagation of mineralization onto the extracellular matrix are compromised in the PHOSPHO1 deficiency, phenocopying a form of pseudo-hypophosphatasia [154].

Conversely, several genetic disorders that feature ectopic calcification are associated with dysregulated extracellular PP_i homeostasis [155]. They include pseudoxanthoma elasticum (PXE), generalized arterial calcification of infancy (GACI), ankylosis, and arterial calcification due to deficiency of CD73 (ACDC), all transmitted via autosomal recessive modes. PXE (OMIM 264800) is characterized by late-onset yet progressive accumulation of calcium mineral at ectopic sites, including the skin, eyes, and cardiovascular system [156]. The classical forms of PXE are caused by loss-of-function mutations in the *ABCC6* gene, encoding a transmembrane efflux transporter ABCC6 protein expressed primarily in the liver. GACI is an extremely severe, early-onset vascular calcification disease often diagnosed by prenatal ultrasound revealing calcium deposits in the fetal heart and arteries [78]. GACI is classified into two types, GACI type 1 (OMIM 208000) and GACI type 2 (OMIM 614473) which are associated with inactivating mutations in the *ENPP1* and *ABCC6* genes, respectively. Regardless of the two types, most patients with GACI die before six months of age. In contrast to GACI, ankylosis and ACDC (OMIM 211800) are adult-onset calcification disorders of elderly individuals. Patients with ankylosis develop calcification of tissues and poorly perfused bodily fluids, such as cartilage, intervertebral disc, and synovial fluid of joints [157]. Patients with ACDC (also known as CALJA develop arterial calcification in the lower extremities and calcification in joint capsules of the hands and feet causing severe pain [158]. Ankylosis and ACDC are caused by biallelic inactivating mutations in the *ANKH* and *NT5E* genes, encoding ANKH (ANK in mice) and CD73 proteins, respectively.

These rare diseases clearly demonstrate the fundamental role of maintaining a properly regulate P_i/PP_i ratio to ensure physiological mineralization in skeletal and dental tissues, while preventing unwanted ectopic calcification of soft tissues. Next, we will briefly summarize ongoing therapeutic strategies being explored to normalize the P_i/PP_i ratio for the treatment of these rare diseases.

10. Therapeutic approaches to normalizing the P_i/PP_i ratio

Asfotase alfa is a recombinant fusion protein comprising the human TNAP ectodomain, a human IgG1 Fc fragment to enable one-step purification and enhance its pharmacokinetic properties, and a terminal deca-aspartate (D_{10}) motif to confer mineral-targeting properties to this biologic. Treatment of the *Alpl*$^{-/-}$ mouse model of infantile hypophosphatasia with daily subcutaneous injections of asfotase alfa normalized plasma PP_i concentrations, preserved life, and prevented the skeletal defects, the epileptic seizures and the dental defects [159–161]. In humans, subcutaneous injections of asfotase alfa, up to seven times a week in children or adults with hypophosphatasia have demonstrated substantial and sustained efficacy with a good safety profile [139,142,143,162–165]. Asfotase alfa was approved world-wide for pediatric-onset hypophosphatasia in 2015 under the name Strensiq (Alexion/AstraZeneca). However, patient burden related to almost daily injections to maintain the efficacy of asfotase alfa and the associated medical cost have prompted preclinical studies of alternative strategies for treating HPP [166].

Gene therapy is a powerful approach for the treatment of genetic diseases. Recent studies have shown that a single administration of either a lentiviral vector or adeno-associated viral vectors expressing TNAP-D_{10} caused continued expression of TNAP and the improvement in bone and dentoalveolar phenotype in a severe infantile hypophosphatasia mouse model [60,61,73,74,76–78]. This AAV8-TNAP-D_{10} viral-mediated enzyme replacement was also efficacious in halting the progression of disease in the *Alpl*$^{Prx1/Prx1}$ model of late-onset hypophosphatasia and prevented the development of scoliosis and ameliorated skeletal disease in the *Phospho1*$^{-/-}$ model of osteomalacia [167]. Enzyme replacement with a non-mineral-targeted, soluble, chimeric form of alkaline phosphatase composed of moieties from the intestinal and placental isozymes [168], has also shown effectiveness in reducing plasma PP_i levels and extending life and ameliorating skeletal and dental deficits in the *Alpl*$^{-/-}$ model of infantile hypophosphatasia [169]. Finally, another potential strategy that could be developed

therapeutically is to reduce PP_i production by inhibiting ENPP1. Genetic experiments documented considerable improvement in the skeleton of [*Alpl*$^{-/-}$; *Enpp1*$^{-/-}$] double knockout mice [73]. The improvements were more pronounced in the axial than in the appendicular skeleton, due to the different ratios of expression of TNAP and ENPP1 at those anatomical sites [90,170].

Given that overexpression of TNAP in the arterial media or intima is sufficient to cause severe vascular calcification, inhibiting the inappropriately elevated levels of TNAP would represent a viable therapeutic strategy for ectopic calcification diseases. The Millan laboratory developed and characterized an arylsulphonamide uncompetitive inhibitor of TNAP, SBI-425 [171,172], capable of suppressing the inappropriately enhanced activity of TNAP in the vasculature [62,64] and restoring the local concentration of PP_i, thus preventing further ectopic mineral deposition. They showed that systemic administration of SBI-425 by food admixture effectively prevented ectopic calcification in the muzzle of the *Abcc6*$^{-/-}$ model of PXE [46,173] and prevented vascular calcification in a murine model of chronic kidney disease [174]. DS-1211 (Daiichi Sankyo, Tokyo, Japan), a derivative of SBI-425, also showed efficacy in increasing plasma PP_i concentrations and preventing ectopic calcification in the mouse model of PXE [175]. DS-1211 has now completed Phase I clinical trials [176] and has entered Phase II clinical trials as of November 2022 in patients with PXE (ClinicalTrials.gov Identifier: NCT05569252).

Another obvious therapeutic strategy to enhance the production of PP_i is to administer ENPP1, the enzyme responsible for the production of PP_i from ATP. ENPP1-Fc is a recombinant protein, resulting from the fusion of the entire human active ENPP1 molecule and the Fc region of immunoglobulin to enhance its plasma half-life and facilitate its purification [177]. The subcutaneous administration of the ENPP1-Fc fusion protein prevented mortality and vascular calcification in the *Enpp1*$^{asj/asj}$ animal model of GACI [177] and has recently been shown to affect muzzle calcification in the *Abcc6*$^{-/-}$ model of PXE [178]. That initial construct, now called ENZ-701 (Inozyme Pharma, Boston, MA) has progressed into ongoing Phase II clinical trials in patients with GACI and PXE (ClinicalTrials.gov Identifier: NCT05030831, ClinicalTrials.gov Identifier: NCT05030831). BL-1118 is a longer-lasting and more potent version of recombinant ENPP1-Fc characterized in 2021 [179,180], that has greatly improved pharmacodynamic properties compared to the original fusion enzyme used for proof-of-concept studies in the *Enpp1*$^{asj/asj}$ mouse model [177]. The use of the ENPP1-Fc biologic to generate systemic PP_i from available systemic ATP levels has been shown effective in preventing heart, coronary artery, and kidney calcification in the *Enpp1*$^{asj/asj}$ model of GACI after weekly intraperitoneal injections [177] and muzzle calcification in the *Abcc6*$^{-/-}$ [178].

Conclusions

Overwhelming evidence, both from animal models and clinical data, point to the fundamental role of the P_i/PP_i ratio as the single-most important determinant of physiological mineralization and pathological calcification. Phosphatases present in matrix vesicles are crucial in controlling this all-important ratio and we have gained a deeper understanding of their interplay in recent years. ENPP1 and TNAP clearly control extracellular PP_i formation and degradation, respectively. The double genetic experiment showing that the double ablation of PHOSPHO1 and TNAP leads to complete suppression of skeletal/dental mineralization also points to the requirement for enzymatic P_i-generation as a determinant of the physiologic P_i/PP_i ratio. The phosphatases controlling the P_i/PP_i ratio are druggable and of pharmaceutical interest. The realization of the crucial role of TNAP as the enzyme responsible for maintaining physiological concentrations of extracellular PP_i has turned this enzyme into an approved life-saving treatment for hypophosphatasia as well as a pharmacological target for ectopic calcification, with a small molecule inhibitor now in clinical trials for PXE. ENPP1 enzyme replacement is currently in clinical trials for GACI and PXE. What remains poorly understood is how matrix vesicles are generated, and how they can propagate nascent apatitic mineral formed in their lumen onto the collagenous extracellular matrix. These are areas of very active current investigation in our laboratories.

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List of abbreviations

ABCC6= ATP-binding cassette sub-family C member 6
ACDC= arterial calcification due to deficiency of CD73
ADP= adenosine diphosphate
ALPI= gene encoding for intestinal alkaline phosphatase
ALPL= gene encoding for tissue non-specific alkaline phosphatase
ALPP= gene encoding for placental alkaline phosphatase
ALPPL2= gene encoding for germ cell alkaline phosphatase intestinal
AMP= adenosine monophosphate
ANK= inorganic pyrophosphate transport regulator
ANKH=genes encoding for ANKH
Apo-E= apolipoprotein E
ARHR2= autosomal recessive hypophosphatemic rickets type 2
ATP= adenosine triphosphate
CD39= nucleoside triphosphate diphosphohydrolase 1
CD73= ecto-5′-nucleotidase
ecto-5'-NT or CD73= ecto-5'-Nucleotidase
ENPP= ectonucleotide Pyrophosphatase/Phosphodiesterase
ENPP1= nucleotide Pyrophosphatases/Phosphodiesterase 1
ENPP6= nucleotide Pyrophosphatases/Phosphodiesterase 6
E-NTPDase1 or CD39= Ecto-nucleoside triphosphate diphosphohydrolase 1
ENTPD1= gene encoding for E-NTPDase1
GACI= generalized arterial calcification of infancy
GCAP= germ cell alkaline phosphatase
HL-60= human leukemia cell line
HSP70= 70 kD heat shock proteins
IAP= intestinal alkaline phosphatase
LDLR= low density lipoprotein receptor
NKA= Na,K-ATPase
NT5E= gene endocing for CD73
OPN= osteopontin
PHOSPHO1= orphan phosphatase 1
PHEX= phosphate-regulating gene with homologies to endopeptidases on the X chromosome
P_i= inorganic phosphate
P_iT-1= phosphate transporter 1
P_iT-2= phosphate transporter 2
PLAP= placental alkaline phosphatase
PP_i= pyrophosphate
SIBLING= small integrin-binding ligand N-linked glycoprotein
SMPD3= sphingomyelin phosphodiesterase 3
Tie2= Tyrosine-protein kinase receptor
TNAP= tissue-nonspecific alkaline phosphatase

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