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Extracellular pyrophosphate: the body's "water softener"

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Abstract

Extracellular pyrophosphate (ePP_i) was first identified as a key endogenous inhibitor of mineralisation in the 1960"s by Fleisch and colleagues. The main source of ePP_i seems to be extracellular ATP which is continually released from cells in a controlled way. ATP is rapidly broken down by enzymes including ecto-nucleotide pyrophosphastase /phosphodiesterases to produce ePP_i. The major function of ePP_i is to directly inhibit hydroxyapatite formation and growth meaning that this simple molecule acts as the body"s own “water softener”. However, studies have also shown that ePP_i can influence gene expression and regulate its own production and breakdown. This review will summarise our current knowledge of ePP_i metabolism and how it acts to prevent pathological soft tissue calcification and regulate physiological bone mineralisation.

Introduction

Inorganic pyrophosphate (PP_i) is a simple molecule comprised of two inorganic phosphates (PO_4^{3-} or P_i) joined by a hydrolysable ester bond. Intracellular PP_i is a by-product of over 200 different enzyme reactions and its hydrolysis plays a major role in driving fundamental biochemical reactions [1]. Every day large amounts of PP_i are produced within cells, particularly during the generation of nucleic acids, proteins, lipids and carbohydrates from their smaller precursors. Most of this PP_i remains within cells where it is hydrolysed by intracellular pyrophosphatases. In contrast, extracellular PP_i (ePP_i) is separately regulated and its levels are determined by the coordinated actions of several proteins. For further information about the biochemistry and biology of PP_i in nature the reader is referred to the 2001 monograph by Heinonen [2].

Body fluids are supersaturated with calcium and P_i which means that, in the presence of nucleating agents such as collagen, mineralisation can occur. It is important to note here that mineralisation is a complex process and the events that regulate its initiation and propagation are not fully understood. However, since most tissues do not readily mineralise under physiological conditions there must be inhibitors present. It was the pioneering studies by Fleisch and colleagues in the 1960"s that first discovered ePP_i acts as a key inhibitor of biomineralisation [3-7]. They found that ePP_i potently antagonises the ability of calcium to crystallise with P_i to form hydroxyapatite ($Ca_{10}(PO_4)(OH_2)$) [4, 6, 8]. ePP_i also binds strongly to the surface of hydroxyapatite and prevents further crystal growth [9]. This initial work helped to establish the concept that ePP_i is the body"s own "water softener" that acts to control mineralisation processes [6, 9].

These discoveries by Fleisch, Neuman, Bisaz, Russell and colleagues have laid the foundations for understanding the fundamental role of ePP_i in mineralisation. This article will summarise our current knowledge of how this simple molecule acts to regulate physiological bone mineralisation and prevent harmful soft tissue calcification. It will also discuss how the discovery of ePP_i and its role in bone contributed to the development of the widely used bisphosphonate drugs.

The generation and regulation of ePP_i

In vivo a balance between the rate of generation and hydrolysis ensures levels of ePP_i are carefully controlled (see **Figure 1**). Extracellular nucleotides such as adenosine triphosphate (ATP) represent an important source of ePP_i outside of cells. In particular, ATP and UTP are hydrolysed by ecto-nucleotide pyrophosphatase/phosphodiesterases (NPPs) to produce the corresponding monophosphate and ePP_i. The NPP enzymes are widely expressed and highly conserved between species. In humans, there are 7 members of the NPP family, each with different substrate specificity and expression patterns [10]. Of these, NPP1 (or PC-1), NPP2 (autotaxin) and NPP3 (B10) have been particularly well characterised with regard to their roles in ePP_i generation.

The intracellular concentration of ATP is between 2-5mM. Following membrane damage all cells can release ATP into the extracellular environment; however, controlled release has been reported from numerous cell types including neurons [11], bone cells [12, 13], vascular smooth muscle cells [14] and endothelial/epithelial cells [15, 16]. Once released, ATP can act via cell surface P2 receptors to regulate cell proliferation, differentiation and function [17]. The rapid hydrolysis of extracellular nucleotides by ecto-nucleotidases (including NPPs) means that autocrine/paracrine actions of ATP are limited to cells with close proximity of the release site. To date, a number of processes have been implicated in mediating ATP release (e.g. the P2X7 receptor, connexin/pannexin hemichannels), however, vesicular exocytosis appears to be the predominant mechanism (see review [18]). The level of cellular ATP release can be influenced by external factors such as mechanical stress [19, 20], hypoxia [21, 22] and vitamin D [23]. Extracellular nucleotides can also act via a positive feedback to further promote ATP release [15, 24, 25]. Since ATP hydrolysis is a key source of ePP_i, any factors which regulate ATP release may also indirectly ePP_i levels. Currently, the relationship between controlled ATP release and ePP_i levels is poorly investigated and represents an exciting area for future research.

Alkaline phosphatases (ALP) are broad spectrum ecto-nucleotidases that hydrolyse numerous phosphate containing molecules [10]. In particular, ALP displays pyrophosphatase activity and so will breakdown ePP_i to two P_i molecules. Whilst normally assayed under alkaline conditions, at physiological pH the Km for substrates like PP_i is very low and therefore these enzymes can „completely" hydrolyse ePP_i [10]. There are four different ALP enzymes some of which are tissue specific; the most widely expressed is the tissue non-specific form (TNSALP) which is found in the kidney, liver and bone.

ANK (progressive ankyloses or ANKH) is a membrane protein though to facilitate the transport of PP_i from the intra-to-extracellular environment [26]. However, since the intracellular PP_i concentration is in the micromolar range the relative contribution of ANK to ePP_i levels is likely to be less than the breakdown of ATP by NPPs [27]. Many tissues including bone, kidney, cartilage, brain, muscle and the vasculature have been reported to express Ank [26].

Biological mineralisation

In the literature, the terms “mineralisation” and “calcification” are often used interchangeably. Nevertheless, in journal articles mineralisation is most commonly used in the context of bone and refers to the process of laying down minerals within the organic matrix. Calcification, by definition, describes the accumulation of calcium salts in a tissue or material and is frequently used in papers to describe the pathological processes that can occur in soft tissues. The extracellular concentrations of calcium and P_i are major determinants of biomineralisation within both the skeleton (cartilage and bone) and soft tissues. In clinical conditions such as vitamin D deficiency, bone mineralisation is impaired by low calcium and P_i levels [28]. Conversely, ectopic calcification can occur when calcium or P_i levels are high, as seen in renal failure. Plasma P_i levels vary over a wider physiological range than calcium and are effected by dietary intake and renal excretion [29]. Numerous factors such as FGF23, Parathyroid hormone (PTH) and growth hormone regulate kidney function and therefore indirectly modulate P_i levels [29].

ePPi as an inhibitor of bone mineralisation

The seminal work by Fleisch and colleagues in the 1960"s has resulted in extensive research into the inhibitory actions of ePP_i on bone mineralisation. It is now accepted that the ePP_i-to-P_i ratio within the bone microenvironment is a fundamental regulator of skeletal mineralisation (see reviews [30, 31]). Osteoblasts express at least 3 members of the NPP family (NPP1, NPP2, NPP3), whilst NPP1 expression has been reported in osteocytes and osteoclasts [32-35]. Abundant evidence suggests that NPP1 is crucial for ePP_i generation, whilst TNSALP, which plays a central role in driving mineralisation processes, is the key enzyme involved in ePP_i breakdown [30, 32-35]. Thus the opposing actions of NPP1 and TNSALP are critical in determining local ePP_i and P_i levels [27, 33].

NPP1

Studies using three different mouse models have highlighted the importance of NPP1 in ePP_i generation and bone mineralisation. These are (1) the naturally occurring NPP1 “knockout” referred to as the tip-toe walking (*ttw/ttw*) mouse; (2) the genetically altered NPP1 knockout (*Enpp1*$^{-/-}$) and (3) the alternative *Enpp1*^{asj} knockout. Of these, the *Enpp1*$^{-/-}$ model has been most widely studied; these mice display aberrant calcification of the spine, joints, tendons and other collagen rich soft tissues which progressively worsens with age and is associated with altered gait and reduced movement [32, 34, 36, 37]. In keeping with reduced ePP_i levels, cultured osteoblasts isolated from *Enpp1*$^{-/-}$ mice display an increased ability to mineralise matrix *in vitro* [38]. However, somewhat surprisingly, given these *in vitro* observations, *Enpp1*$^{-/-}$ animals exhibit decreased levels of trabecular and cortical bone and reduced bone strength in the femur/tibia [34, 37, 39]. High resolution analysis of the cortical bone showed that these mice also display a reduction in the number and size of their blood vessel channels and osteocyte lacunae [34]. This failure to maintain lacunar size was attributed to the inability of *Enpp1*$^{-/-}$ osteocytes to hydrolyse constitutively released ATP to generate ePP_i [34]. Increased levels of sclerostin (an inhibitor of bone formation) and FGF-23 (a regulator of phosphate metabolism) have also been reported in *Enpp1*$^{-/-}$ mice [34, 37]. Thus the unexpected *in vivo* skeletal phenotype of these animals is most likely a consequence of changes and/or defects in multiple tissues.

Enpp1^{asj} (ages with stiffened joints) mice are on a different genetic background to the *Enpp1*$^{-/-}$ animals, however, they display many of the same phenotypic characteristics including widespread soft tissue calcification [40, 41]. They also exhibit inflammation and ectopic calcification of the middle ear which leads to hearing impairment [42]. *Ttw/ttw* mice, which have a phenotype similar to the disease OPLL (ossification of the posterior ligament of the spine), display ossification of the spinal ligaments, articular cartilage calcification and peripheral joint hyperstosis [43].

TNSALP

TNSALP has long been used as a marker of osteoblast differentiation and measurements of enzyme activity are widely used in skeletal research. The phenotype of TNSALP knockout mice is evident within a few days of birth and includes smaller body size, defective bone mineralisation, skeletal deformations and

spontaneous fractures. The animals also develop epileptic seizures usually dying before weaning [44-46]. Osteoblasts isolated from knockout animals differentiate normally but fail to mineralise the deposited matrix because of the increased ePP_i levels; this further illustrates the importance of TNSALP in regulating ePP_i levels [47].

The central role of NPP1 and TNSALP in skeletal mineralisation is also highlighted by the human diseases which arise due to deletion or inactivation of one of these enzymes (see reviews [30, 48]). Patients with hypophosphatasia have missense mutations in the TNSALP gene that results in impaired or, in severe cases, no enzyme activity. This leads to increased ePP_i levels and impaired bone mineralisation [49-51]. In contrast, the disease OPLL, which is characterised by ectopic calcification of spinal ligaments, is caused by a mutation in NPP1 and reduced enzyme activity [43]. Recent advances in enzyme replacement therapy have led to the development of Asfotase Alfa, a life changing drug for patients with hypophosphatasia [52]. Whilst treatment of OPLL remains challenging, a recent preclinical study using *ttw/ttw* mice found that coadministration of the TNSALP inhibitor, levamisole, and ePP_i slowed the progression of spinal ligament calcification without exerting negative effects on bone [53].

ANK

The role of ANK in bone mineralisation has been studied using the *ank/ank* model; these mice have a mutation in the C-terminal cytosolic domain of the protein which reduces PP_i transport to the extracellular environment [26]. Consistent with reduced ePP_i levels, *ank/ank* animals display joint calcification and destruction, vertebral fusion characteristic of ankylosing spondylitis and altered gait [26]. A comparative study of *Enpp1*$^{-/-}$ and *ank/ank* mice reported that the ectopic calcification was worse in the *Enpp1*$^{-/-}$ animals suggesting that NPP1 is more important in ePP_i generation than ANK [27].

ePP_i and bone cell function

In vitro ePP_i inhibits bone mineralisation in the low micromolar range (≥1μM) with a complete abolition at 100μM [35] (**Figure 2**). Whilst restricted to only a few studies, there is some evidence to suggest that ePP_i can also act directly on bone cells to regulate function. Work using osteoblast-like cells has shown that ePP_i can promote differentiation [54] and act via the MAP-kinase pathway to increase the expression of the mineralisation inhibitor, osteopontin [55]. Furthermore, both *Enpp1*$^{-/-}$ and *ank/ank* mice display reduced osteoblast expression of osteopontin and lower serum levels of the protein [27, 36].

Information regarding any direct functional effects on osteoclasts is even more limited. However, earlier work has shown that ePP_i can promote apoptosis [56] and *Enpp1*$^{-/-}$ mice display increased osteoclast activity *in vivo* [37]. Whilst there are no studies describing direct effects of ePP_i on osteocytes, earlier work has shown that osteocytes-derived from *Enpp1*$^{-/-}$ mice display increased sclerostin expression [34]. This raises the possibility that ePP_i could also modulate osteocyte function and represents an interesting area for future study.

Controlling the levels of ePP_i in bone

In order to prevent hyper- or hypomineralisation it is essential that expression and activity of NPP1, TNSALP and ANK is regulated. Numerous factors have been shown to influence ePP_i levels via actions on these proteins including P_i [57], extracellular nucleotides [35, 58], neurofibromin [59], acidosis [38, 60], hypoxia-inducible factor proteins [61], vitamin D [62] and FGF2 [63, 64]. Additionally and perhaps most interestingly, is the apparent ability of ePP_i to regulate its own production and hydrolysis. Previous work has shown that ePP_i can downregulate *Enpp1* and *Ank* expression in osteoblasts [27, 35]. Extracellular nucleotides (ATP/UTP) also inhibit *Enpp1* expression, however, it is unclear whether this is due to an NPP1-mediated increase in ePP_i or because of purinergic signalling [35]. It has also been reported that ePP_i can increase TNSALP activity in osteoblast-like cells [54]. Taken together these findings suggest the presence of a negative feedback pathway by which ePP_i can regulate the expression/activity of the enzymes involved in its metabolism. It is unknown how ePP_i exerts these actions or the effects on bone cell function described above. However, its size and charge means that ePP_i cannot passively cross the cell membrane. This therefore raises the exciting possibility of a cell surface receptor or sensor for ePP_i.

The regulation of soft tissue calcification by ePP_i

Under normal conditions there are robust regulatory mechanisms in place to prevent pathological soft tissue calcification. Numerous studies have shown that NPP1 is particularly important in generating the ePP_i needed to prevent unwanted calcification [34, 37, 65]. High resolution *in vivo* micro-computed x-ray tomography scanning of *Enpp1*$^{-/-}$ mice illustrates how widespread the effects of reduced ePP_i levels are

(**Figure 3**). These images show the calcification of tissues including ear pinna, trachea, whisker vibrissae, eye, cartilage and tendons. They also show abnormal bone formation (**Figure 3)**.

ePP$_i$ and cartilage mineralisation

Joints contain both unmineralised articular cartilage and calcified cartilage, which forms the interface between the articular cartilage and the underlying subchondral bone. To maintain joint integrity and health, cartilage calcification must be tightly regulated and restricted to specific regions. The cells found in cartilage, chondrocytes, have been shown to constitutively release ATP [66], express NPP1 and are able to generate large amounts of ePP$_i$ [67, 68]. Osteoarthritis (OA) is a degenerative joint disease associated with articular cartilage calcification [69]. Patients with severe OA are reported to have lower NPP1 levels [70] and *Enpp1* polymorphisms have been associated with hand OA [71]. In addition, OA-like changes and pathological calcification have been described in the articular cartilage of *ttw/ttw* mice [70, 72]. More recently, decreased Ank expression has been associated with cartilage endplate calcification in intervertebral disc degeneration [73]. Taken together these findings suggest that ePP$_i$ plays an important yet not fully defined role in preventing cartilage calcification.

Although ePP$_i$ can act to prevent unwanted cartilage mineralisation, in excess it may be detrimental because it can promote calcium pyrophosphate dehydrate (CPPD) crystal formation and the development of chondrocalcinosis. This condition is very common in ageing populations where it can lead to significant morbidity [74]. Previous work has suggested that the excessive ePP$_i$ levels could involve ANK since activating mutations in *Ankh* have been associated with familial forms of chondrocalcinosis and protein expression is increased in patients with CPPD deposits [75, 76].

ePP$_i$ and vascular calcification

Vascular calcification is a common consequence of chronic kidney disease, diabetes, atherosclerosis and ageing. It is characterised by the pathological deposition of calcium phosphate mineral, most often as hydroxyapatite, in the intimal and/or medial layer of the arteries and heart valves. Arterial medial calcification (AMC) is the calcification which develops within the tunica media of blood vessels and its development a complex, cell-mediated process which is thought to share some similarities with physiological bone mineralisation. Vascular smooth muscle cells (VSMC) are the predominant cell type driving AMC and in calcifying conditions these cells undergo phenotypic changes to take on some limited osteoblast-like characteristics [77].

ePP$_i$ is well established as an inhibitor of AMC and valve calcification [65, 78, 79]. *In vitro,* ePP$_i$ inhibits VSMC calcification in the low micromolar range [80, 81] (**Figure 2**). It also acts to reduce apoptosis in calcifying VSMCs, an effect which is most likely a direct consequence of the physiochemical inhibition of calcification [80]. Similar to osteoblasts, ePP$_i$ can increase VSMC TNSALP activity suggesting the presence of feedback mechanisms to regulate extracellular levels [80].

The importance of NPP1 in preventing AMC has been shown by numerous studies. *In vitro* work has shown that by hydrolysing released ATP, NPP1 is a key source of ePP$_i$ in VSMC cultures [14, 81]. Furthermore, the inhibitory effects of ATP and UTP on AMC are mediated via both purinergic signalling and the non-receptor mediated breakdown to produce ePP$_i$ [80]. Both *Enpp1*$^{-/-}$ and *Enpp1*^{asj} mice display AMC *in vivo* and *Enpp1*$^{-/-}$ VSMCs have a reduced ability to generate ePP$_i$ from ATP leading to increased calcification *in vitro* [37, 40, 82]. *Enpp1*$^{-/-}$ VSMCs also exhibit higher expression of osteoblast and osteocyte marker genes [83]. ANK may also contribute to the ePP$_i$ levels needed to prevent AMC however evidence suggests it is less important than NPP1 [14, 82]. Interestingly, a recent study reported that Wnt1 can inhibit AMC by upregulating Ank and increasing ePP$_i$ levels [84].

Mutations in the *Enpp1* gene are associated with a very rare autosomal recessive condition called generalised arterial calcification of infancy (GACI) [85, 86]. Children with GACI usually die in infancy because of substantial vascular calcification. However, recent work has shown that treatment with bisphosphonates can increase the life expectancy of infants with GACI [87, 88]. The *Enpp1*^{asj} mouse is widely used as a preclinical model for GACI [40] and has been used in several studies investigating treatments for this disorder. Subcutaneous administration of an NPP1 fusion protein (ENPP1-FC) can prevent the mortality and AMC in *Enpp1*^{asj} animals [89]. Whilst more recently, NPP1 enzyme replacement therapy was shown to have beneficial effects on AMC in this experimental model [90].

In patients with advanced chronic kidney disease, AMC is inversely correlated with circulating ePP$_i$ levels [65, 78, 91]. Evidence from pre-clinical studies has shown that AMC can be inhibited by daily injections of ePP$_i$ without adversely affecting the bone [92, 93]. Together this work suggests a potential therapeutic use for ePP in treating AMC.

Pseudoxanthoma elasticum (PXE) and ePP$_i$

PXE is an autosomal recessive condition characterised by decreased plasma ePP$_i$ levels and progressively worsening calcification of the skin, arteries and eyes [94, 95]. The primary cause of the condition is inactivating mutations in the gene encoding the ATP-binding cassette subfamily C member 6 (*ABCC6*) [96]. However, polymorphism in the NPP1, TNAP and ANK genes have also been identified as risk factors for developing PXE [97].

ABCC6 is primarily expressed in the liver where it has be implicated in the controlled release of ATP from hepatocytes. The *Abcc6*$^{-/-}$ mouse model displays the symptoms of PXE including a 40% decrease in plasma ePP$_i$ levels [95]. Mechanistic studies using these animals suggested that cells lacking ABCC6 release less ATP, which results in a lack of substrate for NPP1 and consequently lower ePP$_i$ levels and the development of PXE [98]. In agreement, a recent investigation found that a deficiency in plasma ePP$_i$ is the major but not the sole cause of the ectopic calcification that occurs in the ABCC6 model of PXE [99]. Furthermore, treatment of *Abcc6*$^{-/-}$ mice with daily injections of ePP$_i$ had no effects on established calcification but prevented the development of further calcific lesions [100]. Inhibition of TNSALP also attenuates the formation of ectopic calcification in this mouse model [101, 102]. Taken together, this work serves to further illustrate the importance of circulating ePP$_i$ in preventing unwanted calcification.

Hutchinson-Gilford progeria syndrome and ePP$_i$

The rare, premature ageing disease Hutchinson-Gilford progeria syndrome effects multiple organs and is characterised by high levels of atherosclerosis and AMC [103, 104]. It is caused by a mutation in the prolamin A gene that results in the production of progerin, a mutant form of the lamin A protein. Overexpression of progerin in a mouse model resulted in decreased circulating ePP$_i$ levels and AMC, effects attributed to increased TNSALP activity, reduced extracellular ATP levels [105] and lower ePP$_i$ generation from ATP [106]. Treatment with ePP$_i$ or ATP and ecto-nucleotidase inhibitors decreased the AMC that developed in these animals [105] and increased longevity [106].

ePP$_i$ in the circulation

The concentration of ePP$_i$ in the plasma is reported to be in the range 1-6μM/litre [107]. Early studies using ^{32}P labelled PP$_i$ in dogs suggested that the daily turnover of ePP$_i$ was in the range of 100mg/day, a small amount compared to the many grams generated during intracellular biosynthetic reactions [108]. Furthermore, work by Russell *et al* [109] revealed that the ePP$_i$ in bodily fluids, including urine, is endogenous and does not come from dietary sources. They showed that feeding large amounts of PP$_i$ did not increase levels any more than giving the same level of P$_i$. However, a recent study challenged this idea by reporting that ePP$_i$ has bioavailability when administered orally [110]. Using *ttw/ttw* and *Abcc6*$^{-/-}$ mice, Dedinszki *et al* showed that oral ePP$_i$ reduced the ectopic calcification that develops in these animals [110]. A low level of oral bioavailability (versus none) increases the potential for use of ePP$_i$ therapeutically and therefore this warrants further work to fully understand the pharmacology and kinetics involved.

The tissue source of circulating ePP$_i$ is also an area of some debate. Whilst there is evidence to suggest that the skeleton may be an important source [32], more recent work has implicated the liver in this process [98]. Nonetheless, it has become evident that systemic ePP$_i$ plays a key role in preventing pathological soft tissue calcification. This is illustrated by the observation that plasma ePP$_i$ is decreased in patients with AMC [78, 91]. Furthermore, it has been reported that transplanting *Enpp1*$^{-/-}$ aortas into *Enpp1*$^{+/+}$ littermates stopped the development of AMC; conversely, if normal aortas were transplanted into *Enpp1*$^{-/-}$ mice they began to calcify [65]. As a result it has been suggested that systemic ePP$_i$ levels could represent a measureable risk factor for AMC [65].

Bisphosphonates and ePP$_i$

Bisphosphonates (BPs) are potent inhibitors of osteoclast activity that are widely used clinically to prevent the bone loss associated with conditions such as osteoporosis, metastatic bone disease and Paget"s disease. They are chemically stable analogues of PP$_i$, in which a carbon atom replaces the central oxygen to form a P-C-P moiety. The individual BPs are produced by differences in the R1 and R2 side chains off the central carbon atom [111]. Like PP$_i$, BPs bind strongly to bone mineral and inhibit the formation and propagation of hydroxyapatite crystals [112]. However, the binding affinity of individual BPs and consequently their uptake and persistence is affected by the R1 and R2 groups [113].

The ability of BPs to inhibit skeletal and soft tissue mineralisation was problematic with some of the early BPs such as etidronate. The newer BPs display a larger therapeutic window between the inhibition of bone resorption and mineralisation, meaning this is no longer a clinical problem. Several studies have shown that BPs inhibit bone mineralisation *in vitro* [114, 115]. However, these actions may prove beneficial if BPs are to be used as potential treatments for treating conditions associated with unwanted calcification including AMC, PXE and GACI. Indeed there are now a several pre-clinical studies which report beneficial effects of BPs in mouse models of these conditions. In the *Enpp1*^{asj} model of GACI, treatment with etidronate and alendronate reduced ectopic soft tissue calcification and restored bone architecture [116]. Etidronate also decreased the development of ectopic calcification in the *Abcc6*$^{-/-}$ model of PXE, however it could not reverse existing mineralisation [117]. BP treatment has also been shown to extend the lifespan of children with GACI [87, 88]. A recently published study also reported beneficial effects of a novel BP compound (FYB-931) on VSMC calcification *in vitro* and in a rat model of AMC [118].

The ability of nitrogen containing BPs to inhibit protein prenylation means that they can have additional clinical effects. For example, in a mouse model of Hutchinson-Gilford progeria syndrome treatment with zoledronate and a statin lead to increased lifespan and an attenuation of many of the tissue ageing effects [119]. These data led to the use of this drug combination in patients with Hutchinson-Gilford progeria syndrome with encouraging results [120, 121].

Concluding remarks

Understanding of how ePP_i prevents pathological mineralisation has increased significantly since the initial seminal work describing its physicochemical effects on hydroxyapatite formation. It is now evident that several proteins are involved in the formation, transport and metabolism of ePP_i and defects in any of these can have a major impact on the level of mineralisation. ATP hydrolysis appears to be the key source of ePP_i and further work is required to determine whether alterations in controlled ATP release indirectly influence ePP_i levels. Additional studies are also warranted to establish the mechanisms by which ePP_i can induce intracellular signalling pathways and the potential therapeutic uses of this simple molecule.

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Figure Legends

Figure 1. ***The regulation of ePP_i levels***

ATP is continually released from cells via controlled mechanisms such as vesicular exocytosis. Once outside the cell, ATP is rapidly hydrolysed by NPP1 to produce ePP_i and AMP. The membrane protein ANK also contributes to ePP_i levels by transporting it from the inside to the outside of the cell. TNSALP hydrolyses ePP_i to generate 2 x P_i molecules. The main action of ePP_i is to prevent mineralisation /calcification by inhibiting hydroxyapatite crystal formation and growth. ePP_i can also regulate gene expression suggesting the presence of an unidentified receptor/sensor.

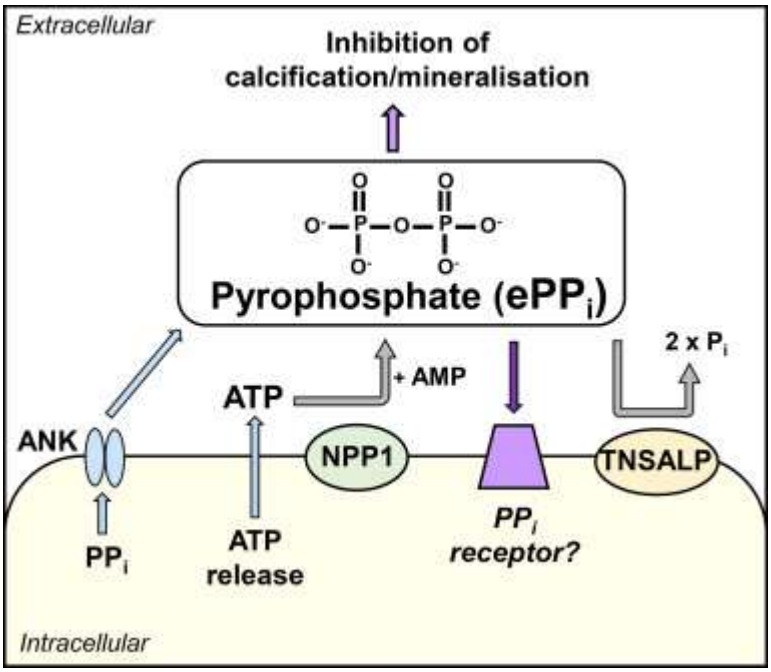


Figure 2. ***The effect of ePP$_i$ on bone mineralisation and VSMC calcification***

(A) Treatment with 1μM ePP$_i$ inhibits bone mineralisation by 45% with complete inhibition of mineralisation at 100μM ePP$_i$. **(B)** ePP$_i$ (≥10μM) decreases VSMC calcification by up to 90%. Data shown as box and whisker plots (min-to-max values) and is from 5 independent experiments; * = p<0.05, ** = p<0.01, *** = p<0.001. **(C)** Representative phase contrast, alizarin red stained images showing the inhibition of bone mineralisation and VSMC calcification by ePP$_i$. The arrow indicates regions of unmineralised matrix in the osteoblast cultures. Scale bars: osteoblast = 200μm, calcifying VSMC = 50μm.

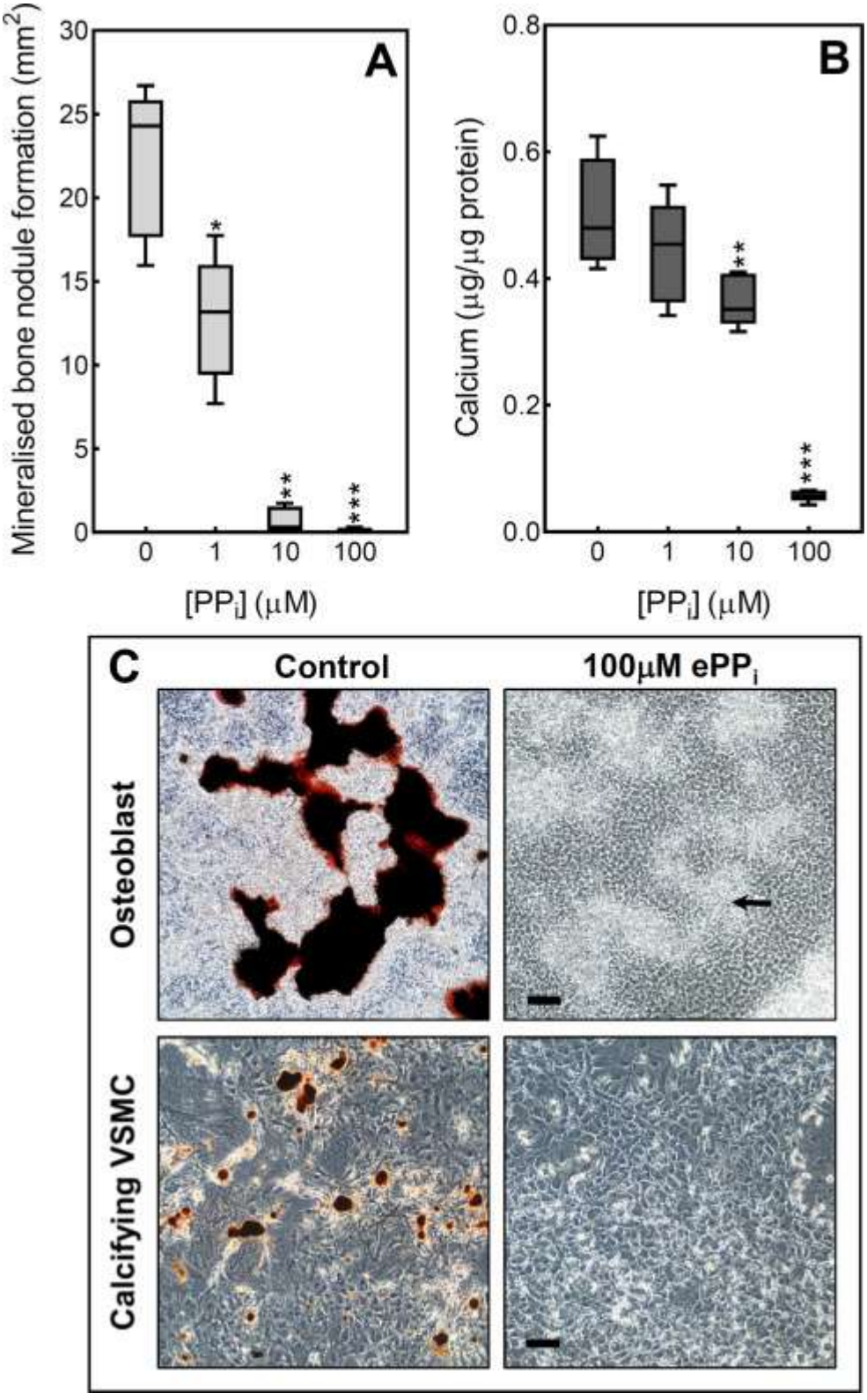
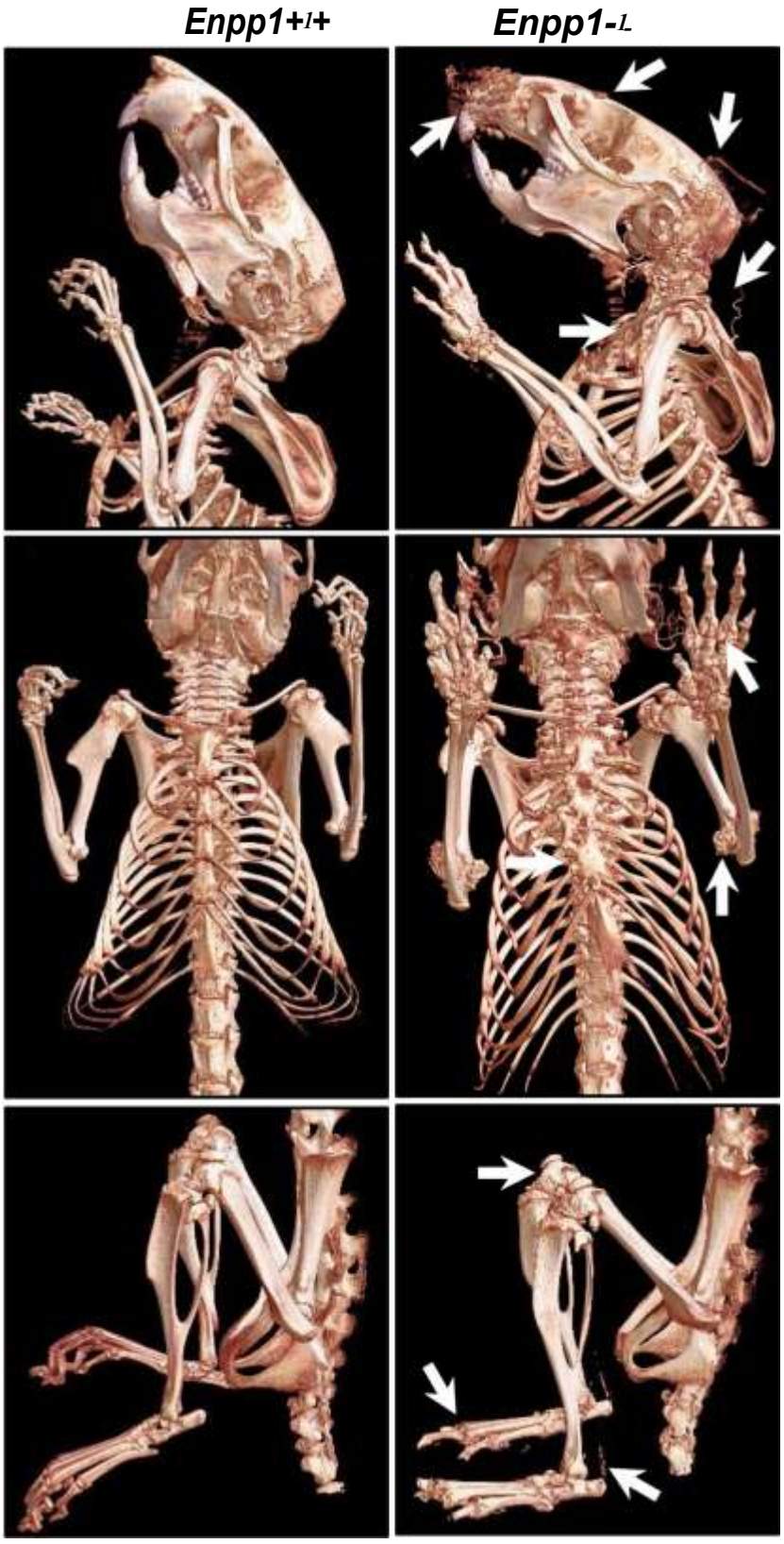


Figure 3. ***Ectopic calcification in Enpp1$^{-/-}$ mice***

In vivo microcomputed x-ray tomography scans showing the widespread ectopic calcification that develops in *Enpp1$^{-/-}$* knockout mice. The arrows highlight the calcification of the whisker vibrissae, eye, ear pinna, trachea, ligaments and joints. The images are of 20 week-old animals at 9μm resolution and were obtained with a SkyScan 1176 high resolution *in vivo* scanner (Bruker MicroCT, Kontich, Belgium).



References

1. Kornberg A. Pyrophosphorylases and phosphorylases in biosynthetic reactions. Adv Enzymol Relat Subj Biochem. 1957;18:191-240.
2. Heinonen JK. Biological role of inorganic pyrophosphate: Kluwer Academic Publishers; 2001. 250 p.
3. Fleisch H, Bisaz S. Isolation from the plasma of pyrophosphate, an inhibitor of calcification. Helv Physiol Pharmacol Acta. 1962;20:C52-C3.
4. Fleisch H, Bisaz S. Mechanism of calcification: inhibitory role of pyrophosphate. Nature. 1962;195:911.
5. Fleisch H, Bisaz S. The inhibitory role of pyrophosphate in calcification. J Physiol (Paris). 1962;54:340-1.
6. Fleisch H, Maerki J, Russell RG. Effect of pyrophosphate on dissolution of hydroxyapatite and its possible importance in calcium homeostasis. Proc Soc Exp Biol Med. 1966;122:317-20..
7. Fleisch H, Neuman W. The role of phosphatase and polyphosphates in calcification of collagen. Helv Physiol Pharmacol Acta. 1961;19:C17-C8.
8. Fleisch H, Bisaz S. Isolation from urine of pyrophosphate, a calcification inhibitor. Am J Physiol. 1962;203:671-5.
9. Fleisch H, Russell RG, Straumann F. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. Nature. 1966;212:901-3.
10. Zimmermann H, Zebisch M, Strater N. Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal. 2012;8:437-502.
11. Burnstock G, Cocks T, Kasakov L, Wong HK. Direct evidence for ATP release from non-adrenergic, non-cholinergic ("purinergic") nerves in the guinea-pig taenia coli and bladder. Eur J Pharmacol. 1978;49:145-9.
12. Brandao-Burch A, Key ML, Patel JJ, Arnett TR, Orriss IR. The P2X7 receptor is an important regulator of extracellular ATP levels. Front Endocrinol. 2012;3:41.
13. Orriss IR. The role of purinergic signalling in the musculoskeletal system. Auton Neurosci. 2015;124-134.
14. Prosdocimo DA, Douglas DC, Romani AM, O'Neill WC, Dubyak GR. Autocrine ATP release coupled to extracellular pyrophosphate accumulation in vascular smooth muscle cells. Am J Physiol Cell Physiol. 2009;296:C828-C839.
15. Bodin P, Burnstock G. ATP-stimulated release of ATP by human endothelial cells. J Cardiovasc Pharmacol. 1996;27:872-5.
16. Knight GE, Bodin P, De Groat WC, Burnstock G. ATP is released from guinea pig ureter epithelium on distension. Am J Physiol Renal Physiol. 2002;282:F281-F288.
17. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev. 2007;87:659-797.
18. Lazarowski ER. Vesicular and conductive mechanisms of nucleotide release. Purinergic Signal. 2012;8:359-373.
19. Hecht E, Liedert A, Ignatius A, Mizaikoff B, Kranz C. Local detection of mechanically induced ATP release from bone cells with ATP microbiosensors. Biosens Bioelectron. 2013;44:27-33.
20. R Rumney RM, Sunters A, Reilly GC, Gartland A. Application of multiple forms of mechanical loading to human osteoblasts reveals increased ATP release in response to fluid flow in 3D cultures and differential regulation of immediate early genes. J Biomech. 2012;45:549-554.
21. Bodin P, Burnstock G. Synergistic effect of acute hypoxia on flow-induced release of ATP from cultured endothelial cells. Experientia. 1995;51:256-259.
22. Orriss IR, Knight GE, Utting JC, Taylor SE, Burnstock G, Arnett TR. Hypoxia stimulates vesicular ATP release from rat osteoblasts. J Cell Physiol. 2009;220:155-162.
23. Biswas P, Zanello LP. 1alpha,25(OH)(2) vitamin D(3) induction of ATP secretion in osteoblasts. J Bone Miner Res. 2009;24:1450-1460.
24. Kringelbach TM, Aslan D, Novak I, Schwarz P, Jorgensen NR. UTP-induced ATP release is a fine-tuned signalling pathway in osteocytes. Purinergic Signal. 2014;10:337-347.
25. Orriss IR, Gueneri D, Hajjawi MO, Shaw K, Patel JJ, Arnett TR. Activation of the $P2Y_2$ receptor regulates bone cell function by enhancing ATP release. J Endocrinol. 2017; 233:341-356

26. Ho AM, Johnson MD, Kingsley DM. Role of the mouse ank gene in control of tissue calcification and arthritis. Science. 2000;289:265-70.
27. Harmey D, Hessle L, Narisawa S, Johnson KA, Terkeltaub R, Millan JL. Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: an integrated model of the pathogenesis of mineralization disorders. Am J Pathol. 2004;164:1199-1209.
28. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357:266-281.
29. Favus MJ, Bushinsky DA, Lemann J. Chapter 13: Regulation of calcium, magnesium and phosphate metabolism. In Primer on the metabolic bone diseases and disorders of mineral metabolism. 6th ed: The American Society for Bone and Mineral Research; 2006. p. 76-83.
30. Millan JL. The role of phosphatases in the initiation of skeletal mineralization. Calcif Tissue Int. 2013;93:299-306.
31. O Orriss IR, Arnett TR, Russell RG. Pyrophosphate: a key inhibitor of mineralisation. Curr Opin Pharmacol. 2016;28:57-68.
32. H Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R, Millan JL. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proc Natl Acad Sci USA. 2002;99:9445-9449.
33. Johnson KA, Hessle L, Vaingankar S, Wennberg C, Mauro S, Narisawa S, Goding JW, Sano K, Millan JL, Terkeltaub R. Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PC-1. Am J Physiol Regul Integr Comp Physiol. 2000;279:R1365-R1377.
34. Hajjawi MO, MacRae VE, Huesa C, Boyde A, Millan JL, Arnett TR, Orriss IR. Mineralisation of collagen rich soft tissues and osteocyte lacunae in *Enpp1*$^{-/-}$ mice. Bone. 2014;69C:139-147.
35. O Orriss IR, Utting JC, Brandao-Burch A, Colston K, Grubb BR, Burnstock G, Arnett TR. Extracellular nucleotides block bone mineralization in vitro: evidence for dual inhibitory mechanisms involving both $P2Y_2$ receptors and pyrophosphate. Endocrinology. 2007;148:4208-4216.
36. Johnson K, Goding J, Van Etten D, Sali A, Hu SI, Farley D, Krug H, Hessle L, Millan JL, Terkeltaub R. Linked deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. J Bone Mine rRes. 2003;18:994-1004.
37. Mackenzie NC, Zhu D, Milne EM, van 't HR, Martin A, Darryl QL, Millan JL, Farquharson C, Macrae VE. Altered bone development and an increase in FGF-23 expression in Enpp1(-/-) mice. PLoS ONE. 2012;7:e32177.
38. Orriss IR, Key ML, Hajjawi MO, Millan JL, Arnett TR. Acidosis is a key regulatior of osteoblast ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) expression and activity. J Cell Physiol. 2015;230:3049-3056.
39. Anderson HC, Harmey D, Camacho NP, Garimella R, Sipe JB, Tague S, Bi X, Johnson K, Terkeltaub R, Millan JL. Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. Am J Pathol. 2005;166:1711-1720.
40. Li Q, Guo H, Chou DW, Berndt A, Sundberg JP, Uitto J. Mutant Enpp1asj mice as a model for generalized arterial calcification of infancy. Dis Mod Mech. 2013;6:1227-1235.
41. Zhang J, Dyment NA, Rowe DW, Siu SY, Sundberg JP, Uitto J, Li Q. Ectopic mineralization of cartilage and collagen-rich tendons and ligaments in Enpp1asj-2J mice. Oncotarget. 2016;7:12000-12009.
42. Tian C, Harris BS, Johnson KR. Ectopic Mineralization and Conductive Hearing Loss in Enpp1asj Mutant Mice, a New Model for Otitis Media and Tympanosclerosis. PloS one. 2016;11:e0168159.
43. Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y, Ikegawa S. Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. Nat Genet. 1998;19:271-273.
44. Fedde KN, Blair L, Silverstein J, Coburn SP, Ryan LM, Weinstein RS, Waymire RS, Narisawa S, Millan JL, MacGregor GR, Whyte MP. Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. J Bone Miner Res. 1999;14:2015-2026.
45. Narisawa S, Frohlander N, Millan JL. Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Dev Dyn. 1997;208:432-446.

46. Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, MacGregor GR. Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. Nat Genet. 1995;11:45-51.
47. Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, Millan JL. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res. 2000;15:1879-1888.
48. Mackenzie NC, Huesa C, Rutsch F, Macrae VE. New insights into NPP1 function: lessons from clinical and animal studies. Bone. 2012;51:961-968.
49. Caswell AM, Whyte MP, Russell RG. Hypophosphatasia and the extracellular metabolism of inorganic pyrophosphate: clinical and laboratory aspects. Crit Rev Clin Lab Sci. 1991;28:175-232.
50. Russell RG. Excretion of inorganic pyrophosphate in hypophosphatasia. Lancet. 1965;2:461-464.
51. Henthorn PS, Whyte MP. Missense mutations of the tissue-nonspecific alkaline phosphatase gene in hypophosphatasia. Clin Chem. 1992;38:2501-2505.
52. Whyte MP, Rockman-Greenberg C, Ozono K, Riese R, Moseley S, Melian A, Thompson DD, Bishop N, Hofmann C. Asfotase Alfa Treatment Improves Survival for Perinatal and Infantile Hypophosphatasia. J Clin Endocrinol Metab. 2016;101:334-342.
53. Hiratsuka S, Takahata M, Shimizu T, Hamano H, Ota M, Sato D, Iwasaki N. Drug therapy targeting pyrophosphate slows the ossification of spinal ligaments in twy mice. J Orthop Res. 2018;36:1256-1261.
54. Pujari-Palmer M, Pujari-Palmer S, Lu X, Lind T, Melhus H, Engstrand T, Karlsson-Ott, M, Engqvist, H. Pyrophosphate stimulates differentiation, matrix gene expression and alkaline phosphatase activity in osteoblasts. PloS one. 2016;11:e0163530.
55. Addison WN, Azari F, Sorensen ES, Kaartinen MT, McKee MD. Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. J Biol Chem. 2007;282:15872-15883.
56. Sun JS, Huang YC, Lin FH, Chen LT. The effect of sintered dicalcium pyrophosphate on osteoclast metabolism: an ultrastructural study. J Biomed Mater Res A. 2003;64:616-621.
57. Ito N, Findlay DM, Anderson PH, Bonewald LF, Atkins GJ. Extracellular phosphate modulates the effect of 1alpha,25-dihydroxy vitamin D3 (1,25D) on osteocyte like cells. J Steroid Biochem Mol Biol. 2013;136:183-186.
58. Orriss IR, Key ML, Brandao-Burch A, Patel JJ, Burnstock G, Arnett TR. The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of P2X receptors. Bone. 2012;51:389-400..
59. de la Croix Ndong J, Makowski AJ, Uppuganti S, Vignaux G, Ono K, Perrien DS, Joubert S, Baglio SR, Granchi D, Stevenson DA, Rios JJ, Nyman JS, Elefteriou F. Asfotase-alpha improves bone growth, mineralization and strength in mouse models of neurofibromatosis type-1. Nat Med. 2014;20:904-910.
60. Brandao-Burch A, Utting JC, Orriss IR, Arnett TR. Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcif Tissue Int. 2005;77:167-74..
61. Skubutyte R, Markova D, Freeman TA, Anderson DG, Dion AS, Williams CJ, Shapiro IM, Risbud MV. Hypoxia-inducible factor regulation of ANK expression in nucleus pulposus cells: possible implications in controlling dystrophic mineralization in the intervertebral disc. Arthritis Rheum. 2010;62:2707-2715.
62. Yang D, Turner AG, Wijenayaka AR, Anderson PH, Morris HA, Atkins GJ. 1,25-Dihydroxyvitamin D3 and extracellular calcium promote mineral deposition via NPP1 activity in a mature osteoblast cell line MLO-A5. Mol Cell Endocrinol. 2015;412:140-147.
63. Hatch NE, Li Y, Franceschi RT. FGF2 stimulation of the pyrophosphate-generating enzyme, PC-1, in pre-osteoblast cells is mediated by RUNX2. J Bone Miner Res. 2009;24:652-662.
64. Hatch NE, Nociti F, Swanson E, Bothwell M, Somerman M. FGF2 alters expression of the pyrophosphate/phosphate regulating proteins, PC-1, ANK and TNAP, in the calvarial osteoblastic cell line, MC3T3E1(C4). Connect Tissue Res. 2005;46:184-192.
65. Lomashvili KA, Narisawa S, Millan JL, O'Neill WC. Vascular calcification is dependent on plasma levels of pyrophosphate. Kidney Int. 2014;85:1351-1356..
66. Graff RD, Lazarowski ER, Banes AJ, Lee GM. ATP release by mechanically loaded porcine chondrons in pellet culture. Arthritis Rheum. 2000;43:1571-1579.

67. Johnson K, Terkeltaub R. Inorganic pyrophosphate (PPI) in pathologic calcification of articular cartilage. Front Biosci. 2005;10:988-997.
68. Johnson K, Vaingankar S, Chen Y, Moffa A, Goldring MB, Sano K, et al. Differential mechanisms of inorganic pyrophosphate production by plasma cell membrane glycoprotein-1 and B10 in chondrocytes. Arthritis Rheum. 1999;42:1986-1997.
69. Boyde A, Davis GR, Mills D, Zikmund T, Cox TM, Adams VL, Niker A, Wilson PJ, Dillon JP, Ranganath LR, Jeffery N, Jarvis JC, Gallagher JA. On fragmenting, densely mineralised acellular protrusions into articular cartilage and their possible role in osteoarthritis. J Anat. 2014;225:436-446.
70. Bertrand J, Nitschke Y, Fuerst M, Hermann S, Schafers M, Sherwood J,Nalesso G, Ruether W, Rutsch F, Dell'Accio F, Pap T. Decreased levels of nucleotide pyrophosphatase phosphodiesterase 1 are associated with cartilage calcification in osteoarthritis and trigger osteoarthritic changes in mice. Ann Rheum Dis. 2012;71:1249-1253.
71. Suk EK, Malkin I, Dahm S, Kalichman L, Ruf N, Kobyliansky E, Toliat M, Rutsch F, Nurnberg P, Livshits G. Association of ENPP1 gene polymorphisms with hand osteoarthritis in a Chuvasha population. Arthritis Res Ther. 2005;7:R1082-1090.
72. Sakamoto M, Hosoda Y, Kojimahara K, Yamazaki T, Yoshimura Y. Arthritis and ankylosis in twy mice with hereditary multiple osteochondral lesions: with special reference to calcium deposition. Pathol Int. 1994;44:420-427.
73. Liu MH, Sun C, Yao Y, Fan X, Liu H, Cui YH, Bian XW, Huang B, Zhou Y. Matrix stiffness promotes cartilage endplate chondrocyte calcification in disc degeneration via miR-20a targeting ANKH expression. Sci Rep. 2016;6:25401.
74. Abhishek A, Doherty M. Epidemiology of calcium pyrophosphate crystal arthritis and basic calcium phosphate crystal arthropathy. Rheum Dis Clin North Am. 2014;40:177-191.
75. Uzuki M, Sawai T, Ryan LM, Rosenthal AK, Masuda I. Upregulation of ANK protein expression in joint tissue in calcium pyrophosphate dihydrate crystal deposition disease. J Rheumatol. 2014;41:65-74.
76. Netter P, Bardin T, Bianchi A, Richette P, Loeuille D. The ANKH gene and familial calcium pyrophosphate dihydrate deposition disease. Joint Bone Spine. 2004;71:365-368.
77. Patel JJ, Bourne LE, Davies BK, Arnett TR, MacRae VE, Wheeler-Jones CP, Orriss IR. Differing calcification processes in cultured vascular smooth muscle cells and osteoblasts. Exp Cell Res 2019;380:100-113.
78. O'Neill WC, Sigrist MK, McIntyre CW. Plasma pyrophosphate and vascular calcification in chronic kidney disease. Nephrol Dial Transplant. 2010;25:187-191.
79. Rathan S, Yoganathan AP, O'Neill CW. The role of inorganic pyrophosphate in aortic valve calcification. J Heart Valve Dis. 2014;23:387-394.
80. Patel JJ, Zhu D, Opdebeeck B, D'Haese P, Millan JL, Bourne LE, Wheeler-Jones CPD, Arnett TR, Macrae VE, Orriss IR. Inhibition of arterial medial calcification and bone mineralization by extracellular nucleotides: The same functional effect mediated by different cellular mechanisms. J Cell Physiol. 2018;233:3230-3243.
81. Prosdocimo DA, Wyler SC, Romani AM, O'Neill WC, Dubyak GR. Regulation of vascular smooth muscle cell calcification by extracellular pyrophosphate homeostasis: synergistic modulation by cyclic AMP and hyperphosphatemia. Am J Physiol Cell Physiol. 2010;298:C702-713.
82. Villa-Bellosta R, Wang X, Millan JL, Dubyak GR, O'Neill WC. Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle. Am J Physiol Heart Circ Physiol. 2011;301H61-68.
83. Zhu D, Mackenzie NC, Millan JL, Farquharson C, Macrae VE. The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells. PLoS ONE. 2011;6:e19595.
84. Chen B, Zhao Y, Han D, Zhao B, Mao Y, Cui ZK, Chu YC, Feng L, Yin S, Wang CY, Wang X, Xu MJ, Zhao G. Wnt1 inhibits vascular smooth muscle cell calcification by promoting ANKH expression. J Mol Cell Cardiol. 2019;135:10-21.
85. Nitschke Y, Baujat G, Botschen U, Wittkampf T, du Moulin M, Stella J,Le Merrer M, Guest G, Lambot K, Tazaroute-Pinturier MF, Chassaing N, Roche O, Feenstra I, Loechner K, Deshpande C, Garber SJ, Chikarmane R, Steinmann B, Shahinyan T, Martorell L, Davies J, Smith WE, Kahler SG, McCulloch M, Wraige E, Loidi L, Hohne W, Martin L, Hadj-Rabia S, Terkeltaub R, Rutsch F. Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCC6. Am J Hum Genet. 2012;90:25-39.

86. Rutsch F, Vaingankar S, Johnson K, Goldfine I, Maddux B, Schauerte P,Kalhoff H, Sano K, Boisvert WA, Superti-Furga A, Terkeltaub R. PC-1 nucleoside triphosphate pyrophosphohydrolase deficiency in idiopathic infantile arterial calcification. Am J Pathol. 2001;158:543-554.
87. Akhtar Ali S, Ng C, Votava-Smith JK, Randolph LM, Pitukcheewanont P. Bisphosphonate therapy in an infant with generalized arterial calcification with an ABCC6 mutation. Osteoporos Int 2018;2911:2575-2579..
88. Edouard T, Chabot G, Miro J, Buhas DC, Nitschke Y, Lapierre C, Rutsch F, Alos N. Efficacy and safety of 2-year etidronate treatment in a child with generalized arterial calcification of infancy. Eur J Pediatr. 2011;170:1585-1590.
89. Albright RA, Stabach P, Cao W, Kavanagh D, Mullen I, Braddock AA,Covo MS, Tehan M, Yang G, Cheng Z, Bouchard K, Yu ZX, Thorn S, Wang X, Folta-Stogniew EJ, Negrete A, Sinusas AJ, Shiloach J, Zubal G, Madri JA, De La Cruz EM, Braddock DT. ENPP1-Fc prevents mortality and vascular calcifications in rodent model of generalized arterial calcification of infancy. Nat Commun. 2015;6:10006
90. Khan T, Sinkevicius KW, Vong S, Avakian A, Leavitt MC, Malanson H, Marozsan A, Askew KL. ENPP1 enzyme replacement therapy improves blood pressure and cardiovascular function in a mouse model of generalized arterial calcification of infancy. Dis Model Mech. 2018;11:10.
91. Lomashvili KA, Khawandi W, O'Neill WC. Reduced plasma pyrophosphate levels in hemodialysis patients. J Am Soc Nephrol 2005;16:2495-2500..
92. O'Neill WC, Lomashvili KA, Malluche HH, Faugere MC, Riser BL. Treatment with pyrophosphate inhibits uremic vascular calcification. Kidney Int 2011;79:512-517.
93. Schibler D, Russell RG, Fleisch H. Inhibition by pyrophosphate and polyphosphate of aortic calcification induced by vitamin D3 in rats. Clin Sci. 1968;35:363-372.
94. Neldner KH. Pseudoxanthoma elasticum. Int J Dermatol. 1988;27:98-100.
95. J Jansen RS, Kucukosmanoglu A, de Haas M, Sapthu S, Otero JA, Hegman IE, Bergen AA, Gorgels TG, Borst P, van de Wetering K. ABCC6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. Proc Natl Acad Sci USA. 2013;110:20206-20211..
96. Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards A, Terry S, Bercovitch L, De Paepe A, Boyd CD. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet. 2000;25:223-227.
97. Dabisch-Ruthe M, Brock A, Kuzaj P, Charbel Issa P, Szliska C, Knabbe C, Hendig D. Variants in genes encoding pyrophosphate metabolizing enzymes are associated with Pseudoxanthoma elasticum. Clin Biochem. 2014;47:60-67.
98. Jansen RS, Duijst S, Mahakena S, Sommer D, Szeri F, Varadi A, Plomp A, Bergen AA, Oude Elferink PP, Borst P, van de Wetering K. ABCC6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation-brief report. Arterioscler, Thromb Vasc Biol 2014;34:1985-1989.
99. Zhao J, Kingman J, Sundberg JP, Uitto J, Li Q. Plasma PPi Deficiency Is the Major, but Not the Exclusive, Cause of Ectopic Mineralization in an Abcc6(-/-) Mouse Model of PXE. J Invest Dermatol. 2017;137:2336-2343.
100. Pomozi V, Brampton C, van de Wetering K, Zoll J, Calio B, Pham K, Owens JB, Marh J, Moisyadi S, Varadi A, Martin L, Bauer C, Erdmann J, Aherrahrou Z, Le Saux O. Pyrophosphate Supplementation Prevents Chronic and Acute Calcification in ABCC6-Deficient Mice. Am J Pathol. 2017;187:1258-1272.
101. Ziegler SG, Ferreira CR, MacFarlane EG, Riddle RC, Tomlinson RE, Chew EY, Martin L, Ma CT, Sergienko E, Pinkerton AB, Millan JL, Gahl, WA, Dietz HC. Ectopic calcification in pseudoxanthoma elasticum responds to inhibition of tissue-nonspecific alkaline phosphatase. Sci Transl Med. 2017;9 eaal1669.
102. Li Q, Huang J, Pinkerton AB, Millan JL, van Zelst BD, Levine MA, Sundberg JP, Uitto J. Inhibition of Tissue-Nonspecific Alkaline Phosphatase attenuates ectopic mineralization in the Abcc6(-/-) mouse model of PXE but not in the Enpp1 mutant mouse models of GACI. J Invest Dermatol. 2019;139:360-368.
103. Nair K, Ramachandran P, Krishnamoorthy KM, Dora S, Achuthan TJ. Hutchinson-Gilford progeria syndrome with severe calcific aortic valve stenosis and calcific mitral valve. J Heart Valve Dis.. 2004;13:866-869.
104. Salamat M, Dhar PK, Neagu DL, Lyon JB. Aortic calcification in a patient with hutchinson-gilford progeria syndrome. Pediatr Cardiol. 2010;31:925-926.

105. Villa-Bellosta R, Rivera-Torres J, Osorio FG, Acin-Perez R, Enriquez JA, Lopez-Otin C, Andres V. Defective extracellular pyrophosphate metabolism promotes vascular calcification in a mouse model of Hutchinson-Gilford progeria syndrome that is ameliorated on pyrophosphate treatment. Circulation. 2013;127:2442-2451.
106. Villa-Bellosta R. ATP-based therapy prevents vascular calcification and extends longevity in a mouse model of Hutchinson-Gilford progeria syndrome. Proc Nat Acad Sci USA. 2019;116:23698-23704.
107. Russell RG, Bisaz S, Donath A, Morgan DB, Fleisch H. Inorganic pyrophosphate in plasma in normal persons and in patients with hypophosphatasia, osteogenesis imperfecta, and other disorders of bone. J Clin Invest. 1971;50:961-969.
108. Jung A, Russel RG, Bisaz S, Morgan DB, Fleisch H. Fate of intravenously injected pyrophosphate-32P in dogs. Am J Physiol. 1970;218:1757-1764.
109. Russell RG, Wadstrom LB, Lindstedt S, Care AD, Bisaz S, Fleisch H. The origin of inorganic pyrophosphate in urine. Clin Sci. 1969;37:419-429.
110. Dedinszki D, Szeri F, Kozak E, Pomozi V, Tokesi N, Mezei TR,Mercel K, Letavernier E, Tang E, Le Saux O, Aranyi T, van de Wetering K, Varadi A. Oral administration of pyrophosphate inhibits connective tissue calcification. EMBO Mol Med. 2017;9:1463-1470.
111. Russell RG. Bisphosphonates: the first 40 years. Bone. 2011;49:2-19.
112. Jung A, Bisaz S, Fleisch H. The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. Calcif Tissue Res. 1973;11:269-80
113. Ebetino FH, Hogan AM, Sun S, Tsoumpra MK, Duan X, Triffitt JT, Kwaasi AA, Dunfold JE, Barnett BL, Oppermann U, Lundy MW, Boyde A, Kashemirov BA, McKenna CE, Russell RG . The relationship between the chemistry and biological activity of the bisphosphonates. Bone. 2011;49:20-33.
114. Orriss IR, Key ML, Colston KW, Arnett TR. Inhibition of osteoblast function in vitro by aminobisphosphonates. J Cell Biochem. 2009;106:109-118.
115. Idris AI, Rojas J, Greig IR, van't Hof RJ, Ralston SH. Aminobisphosphonates cause osteoblast apoptosis and inhibit bone nodule formation in vitro. Calcif Tissue Int. 2008;82:191-201.
116. Li Q, Kingman J, Sundberg JP, Levine MA, Uitto J. Dual effects of bisphosphonates on ectopic skin and vascular soft tissue mineralization versus bone microarchitecture in a mouse model of Generalized Arterial Calcification of Infancy. J Invest Dermatol 2016;136:275-283.
117. Li Q, Kingman J, Sundberg JP, Levine MA, Uitto J. Etidronate prevents, but does not reverse, ectopic mineralization in a mouse model of pseudoxanthoma elasticum (Abcc6(-/-)). Oncotarget. 2018;9:30721-30730.
118. Ishida K, Ashizawa N, Matsumoto K, Kobashi S, Kurita N, Shigematsu T, Iwanaga T. Novel bisphosphonate compound FYB-931 preferentially inhibits aortic calcification in vitamin D3-treated rats. J Bone Miner Metab. 2019;37:796-804.
119. Varela I, Pereira S, Ugalde AP, Navarro CL, Suarez MF, Cau P, Cadinanos J, Osorio FG, Foray N, Cobo J, de Carlos F, Levy N, Freije JM, Lopex-Otin, C. Combined treatment with statins and aminobisphosphonates extends longevity in a mouse model of human premature aging. Nat Med. 2008;14:767-772.
120. Oshima J, Hisama FM, Martin GM. An encouraging progress report on the treatment of progeria and its implications for atherogenesis. Circulation. 2014;130:4-6.
121. Gordon LB, Kleinman ME, Massaro J, D'Agostino RB, Sr., Shappell H, Gerhard-Herman M, Smoot LB, Gordon CM, Cleveland RH, Nazarian A, Snyder BD, Ullrich NJ, Silvera VM, Liang MG, Quinn N, Miller DT, Huh, SY, Dowton AA, Littlefield K, Greer MM, Kieran MW. Clinical trial of the protein farnesylation inhibitors lonafarnib, pravastatin, and zoledronic acid in children with Hutchinson-Gilford Progeria Syndrome. Circulation. 2016;134:114-125.