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Vascular Calcification Revisited: A New Perspective for Phosphate Transport

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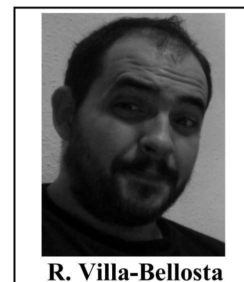

Abstract: Elevated serum phosphorus has emerged as a key risk factor for pathologic calcification of cardiovascular structures, or vascular calcification (VC). To prevent the formation of calcium-phosphate deposits (CPD), the body uses adenosine-5'-triphosphate (ATP) to synthesize inhibitors of calcification, including proteins and inhibitors of low molecular weight. Extracellular pyrophosphate (PPi) is a potent inhibitor of VC, which is produced during extracellular hydrolysis of ATP. Loss of function in the enzymes and transporters that are involved in the cycle of extracellular ATP, including Pi transporters, leads to excessive deposition of calcium-phosphate salts. Treatment of hyperphosphatemia with Pi-binders and Injection of exogenous PPi are the effective treatments to prevent CPD in the aortic wall. The role of sodium phosphate cotransporters in ectopic calcification is contradictory and not well defined, but their important role in the control of intracellular Pi levels and the synthesis of ATP make them an important target to study.

Keywords: ATP, calcium, phosphate, pyrophosphate, vascular calcification,.

INTRODUCTION

Ectopic calcification, the deposition of calcium crystals on soft-tissues, is one of the most important factors determining patients' morbidity and mortality around the world. Pathologic calcification of cardiovascular structures, or vascular calcification (VC), is associated with a number of diseases and is a common consequence of aging [1]. Calcium-phosphate deposition (CPD), in the form of hydroxyapatite or whitlockite, is the hallmark of VC and can occur in blood vessels, myocardium, and cardiac valves [1, 2]. In blood vessels, calcified deposits are found in distinct layers of the aortic wall and are associated with specific pathologies. Intimal calcification occurs in atherosclerotic lesions, whereas medial calcification (so-called "Monckeberg's medial sclerosis") occurs in the medial layer of the aortic wall and is associated with the elastic lamin [3-5].

Despite major advances in recent years, our understanding of the calcification pathogenesis is far from complete. Different mechanisms on the pathogenesis of vascular calcification have been proposed, including 1) loss of inhibitions, 2) osteocondrogenis differentiation of vascular cells, 3) apoptosis, 4) calcium and phosphorus homeostasis, 5) circulating nucleation complexes/paracrine factors and 6) matrix degradation [2, 6].

During the past decade, elevated serum phosphorus (in the form of inorganic phosphate, Pi) has emerged as a key risk factor for vascular calcification in the general population and in patients with chronic kidney disease (see Fig. **1**) [6]. In this case, different factors play an important role in the control of phosphate (Pi) and calcium (Ca) homeostasis, including Pi/Ca excretion and absorption by the kidneys, intestines, and bone. An increased absorption or decreased excretion of phosphate can induce a relative small elevation in serum Pi, which has been correlated with the presence of calcified vessel due to an increase in the CPD formation and saturation in the inhibition [7, 8]. Several diseases have been correlated with the dysregulation of Pi homeostasis, including hyperparathyroidism, vitamin D (hyper-and hypovitaminosis), chronic renal disease, osteoporosis, and diabetes mellitus. Treatment of hyperphosphatemia with phosphate binders is associated with slow progression of cardiovascular calcification in hemodialysis patients (see Fig. **1**) [9, 10].

In vitro experiments show CPD in vascular smooth muscle cells (VSMCs) incubated with high Pi [11]. This observation was interpreted logically as the consequence of an increase in the intracellular Pi [12]. However, old and new studies [7, 8, 13-16] show that the formation of CPD is a passive physicochemical process that does not require any cellular activity, suggesting an important role of Ca/Pi homeostasis.

There are two major consequences regarding the fate of VSMCs in phosphate-induced vascular calcification. The first involves apoptosis-dependent matrix mineralization, which has been detected both in cultured human VSMCs [17, 18] and in arteries from pediatric dialysis patients [19]. The second consequences invokes a profound transition to a bone-forming phenotype, that results in the loss of VSMCs markers (SM α-actin, SM22α) and the expression of osteochondrogenic markers (Runx2/Cbfa1; BMP-2) [20-22]. Recent studies show that calcium-phosphate deposits can induce both the transition to a bone-forming phenotype and

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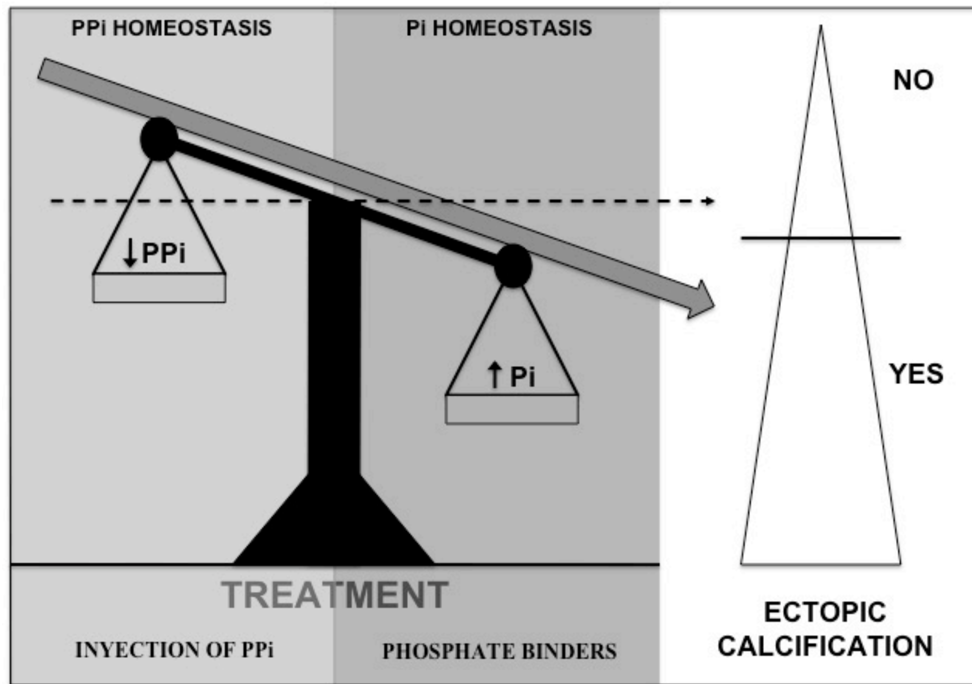

Fig. (1). Schematic representation of a model for ectopic calcification in clinical perspective. Te deposition of calcium-phosphate salts in soft tissues depends of equilibrium between the concentration of inorganic phosphate (Pi) and the synthesis of inhibitors, including pyrophosphate (PPi). A lack of synthesis of CPD inhibitors or an elevated Pi concentration in serum (hyperphosphatemia) induces excessive accumulation of calcium-phosphate crystals. Treatment with Pi-binders (to reduce the concentration of Pi) and injection of exogenous PPi (to increase the amount of PPi) are the effective treatments to prevent ectopic calcification, including vascular calcification.

apoptosis in VSMCs [7, 23, 24], suggesting that the active mechanisms described could be in response to the ectopic calcification [25].

To prevent the passive deposition of ectopic calcium-phosphate crystals, the body uses adenosine-5'-triphosphate (ATP) to synthesize inhibitors of calcification, including proteins and inhibitors of low molecular weight [2, 26]. Recent work has provided evidence for an important role played by the purinergic system, and in particular its links to the extracellular pyrophosphate metabolism [27]. Extracellular pyrophosphate (PPi) is an endogenous inhibitor of VC, both *in vitro* [7, 8] and in vivo [14, 28-30], produced during extracellular hydrolysis of ATP [7, 31]. Moreover, according to a new study [32], ATP is also a direct inhibitor of CPD, with a physicochemical mechanism similar to pyrophosphate, bisphosphonates (non-hydrolysable analogous of pyrophosphate) and polyphosphates [8, 32-35]. The currently known enzymes involved in extracellular ATP/PPi metabolism include members of the eNTPDase family, ENPP family, alkaline phosphatase and ecto-5'-nucleotidase, which all have a broad tissue distribution [27]. Transporters involved in extracellular ATP/PPi metabolism include equilibrative nucleoside transporter [36], Phosphate Transporters [37, 38] and pump/channel that released ATP extracellularly [39].

The role of sodium phosphate cotransporters in ectopic calcification is contradictory [8, 11, 12, 40], but their important role in the control of intracellular Pi levels and the synthesis of ATP make them an important target to study. However, understanding the role of enzymes and transporters involved in the extracellular ATP/PPi metabolism could provide potential future therapeutic targets to prevent ectopic calcification. The purpose of this manuscript is to analyze the contribution of phosphate and extracellular pyrophosphate homeostasis during vascular calcification, including the formation/deposition of hydroxyapatite and the synthesis of inhibitors, with special mention of the contribution of phosphate transport during this process.

1. ON BIOLOGICAL CALCIFICATION

Biological mineralization, or biomineralization, is the formation and deposition of inorganic minerals (biominerals) within or outside the cells of a various organisms. Biomineralization in specific sites of hard tissues (such as in bone, antlers, or dentine) is considered a physiological process; however, the accumulation of biominerals in soft tissues (such as in blood vessels, extracellular matrix of articular cartilaginous tissues of the joints, internal organs, and muscles) is considered ectopic biomineralization, or pathological calcification. Under normal conditions, the soft tissues are not mineralized, but due to aging and other pathological conditions, soft tissues become calcified, which leads to morbidity and mortality.

The main biomineral found in mineralized vertebrate connective tissue are calcium-phosphate salts. In an aqueous system of calcium and phosphate, there are several known non-ion-substituted calcium phosphates; however, not all have been found in biological tissues (see Table **1**). The phosphate ion is a polyatomic ion that consists of one central phosphorus atom surrounded by four oxygen atoms in a tetrahedral arrangement. In biological systems, it is found as a

Table 1. Existing Calcium Phosphates.

Abbreviation	Compound	Formula	Molar Ca/P ratio	Presence in calcified tissues
MCPA	monocalcium phosphate anhydrous	$Ca(H_2PO_4)_2$	0,5	NO
MCPM	monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2H_20$	0,5	NO
DCPA	dicalcium phosphate anhydrous	$CaHPO_4$	1	NO
DCPD	dicalcium phosphate dihidrate	$CaHPO_42H_20$	1	YES
β-TCP	β-tricalcium phosphate	β-$Ca_3(PO_4)_2$	1,5	NO
OCP	octocalcium phosphate	$Ca_8H_2(P0_4)_65H_20$	1,33	YES
ACP	amorphous calcium phosphates	$Ca_9(PO_4)_6nH_20$	1,5	YES
HA	hydroxyapatite	$Ca_{10}(PO_4)_6(OH)$	1,67	YES

free phosphate ion in solution and is called inorganic phosphate (Pi), to distinguish it from phosphates bound with different biological molecules. Aqueous Pi exists in four forms (see Fig. **2**) according to it triprotic equilibrium: 1) trihydrogen phosphate (H_3P0_4), 2) dihydrogen phosphate ion ($H_2PO_4^-$), 3) hydrogen phosphate ion (HPO_4^{2-}), and 4) phosphate ion (PO_4^{2-}). Inorganic phosphate is quite strong with respect to the first dissociation (pK_{a1}=2.1), moderately weak with respect to the second (pK_{a2}=6.9), and very weak with respect to the third (pK_{a3}=12.4).

In the presence of calcium, various phosphates are obtained by charge neutralizing these different inorganic phosphate ions [41]: 1) monocalcium phosphate anhydrous (MCPA), 2) dicalcium phosphate anhydrous (DCPA), and 3) β-tricalcium phosphate (β-TCP). MCPA and DCPA are hydrated to form monocalcium phosphate monohydrate (MCPM) and dicalcium phosphate dihydrate (DCPD), respectively (see Fig. **2**). DCPD, also called Brushite, is often found in calcified tissues [42-44], whereas MCPA, MCPM, DCPA, and β-TCP never appear in calcifications. The Mg-substituted β-TCP form, called whitlockite, is found in some calcified tissues [45, 46], such as in the aorta [47, 48]; whitlockite is not formed under physiological condition unless magnesium ions are present. The final product of the calcium and phosphate salts reaction in neutral or basic solutions is crystalline hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)$), the main component of bone [49]. The process of normal calcification occurs during the growth of hydroxyapatite; however, precipitation of biological hydroxyapatite in aberrant is also possible; which may lead to "HA deposition disease."

There are two precursors of HA: amorphous calcium phosphate (ACP; $Ca_9(PO_4)_6nH_2O$) and octocalcium phosphate (OCP; $Ca_8H_2(PO_4)_65H_2O$). ACP consists mainly of roughly spherical $Ca_9(PO_4)_6$ clusters (called Posner`s clusters) that appear to be energetically favored compared to ($Ca_3(PO_4)$ and $Ca_6(PO_4)_4$ clusters [50]. The structure of HA, which was first reported in 1964 [51], can be interpreted as an aggregation of Posner´s clusters [52]. OCP is often found as an unstable transient intermediate during the precipitation of the thermodynamically more stable HA [53]. Ion-substituted ACPs are found in soft-tissue pathological calcifications [43], whereas OCP, one of the stable components of human dental and urinary calculi [53], has been strongly suggested as a precursor to hydroxyapatite, which is found in natural and prosthetic heart valve [54] (although OCP has not been observed in vascular calcification).

The deposition of these calcium-phosphate salts, both *in vitro* and *in vivo,* takes place on extracellular matrix proteins, such as elastin and collagen [7, 55]. According to the charge neutralization theory of calcification, the high glycine content of these matrix proteins favors the formation of beta-turns that are known to interact with calcium ions [5]. In bone and connective tissues, these salts are predominantly deposited on the type I collagen and elastic fibers, respectively. Elastin, the main component of the elastic fibers in the medial layer of the aortic wall, is synthesized early in life and its expression and organization affects arterial aging. A recent study showed that a mouse model of elastin haploinsufficiency had a significant reduction in arterial calcification [56]. Moreover, the products derived from elastin degradation accelerate the phosphate-induced mineralization *in vitro* and *in vivo* via up-regulation of alkaline phosphatase [57].

2. ON ECTOPIC CALCIFICATION INHIBITORS

Extracellular fluids (such as serum, urine and synovial fluids) in vertebrates are supersaturated with phosphate and calcium, resulting in a tendency for spontaneous calcium phosphate precipitation. The synthesis of CPD inhibitors is therefore essential for survival. In the extracellular fluids, there are a range of endogenous low and high molecular weight inhibitors, including 1) low molecular weight (such as, pyrophosphate and citrate) and 2) small and medium-sized proteins (such as, Matrix Gla Proteins, Fetuin A, and osteopontin) [2].

Extracellular pyrophosphate (PPi) is a potent physicochemical inhibitor of hydroxyapatite crystal formation and growth [14, 58-60]. Recent studies have shown that endogenous production of PPi and daily injections of exogenous PPi prevent medial vascular calcification in rats and mice (see Fig. **1**) [28-30]. In hemodialysis patients, plasma PPi is reduced by 32% after standard hemodialysis [61, 62]; in a mouse model of progeria, PPi in plasma was decreased 4-fold [30]. In both cases, this decrease in PPi leads to exces-

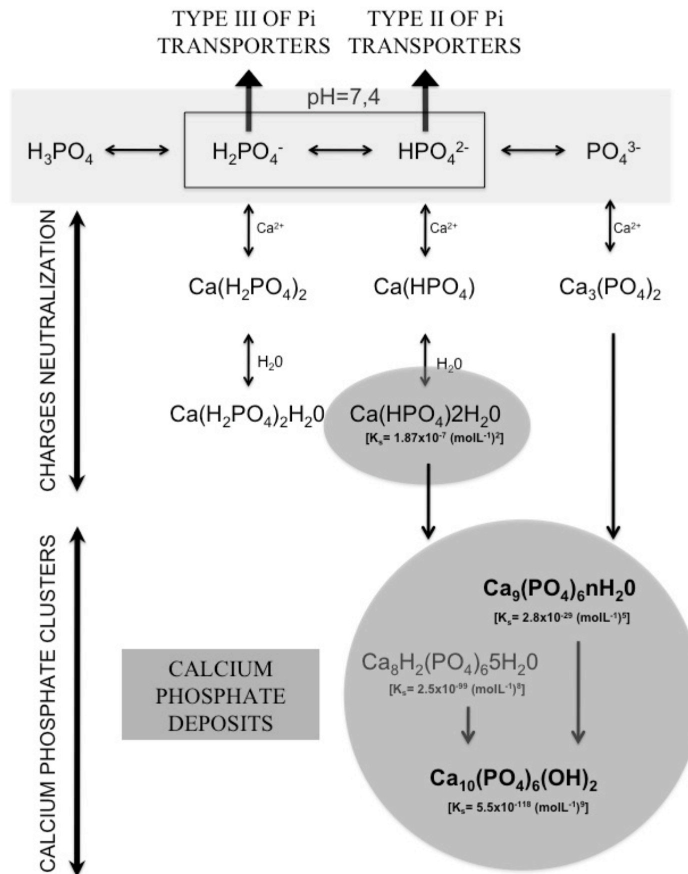

Fig. (2). Schematic representation of calcium-phosphate crystals formation. In the grey box, the four species of inorganic phosphate are represented according to it triprotic equilibrium. The hydroxyapatite formation ($Ca_{10}(PO_4)_6(OH)_2$) can be divided into two steps. In the presence of calcium, various phosphates, and its hydrated forms, are obtained by charge neutralizing these different inorganic phosphate ions (step 1): monocalcium phosphate anhydrous ($Ca(H_2PO_4)_2$), dicalcium phosphate anhydrous ($Ca(HPO_4)$), β-tricalcium phosphate ($Ca_3(PO_4)_2$), monocalcium phosphate monohydrate ($Ca(H_2PO_4)2H_2O$) and dicalcium phosphate dihydrate ($Ca(HPO_4)2H_2O$). Hydroxyapatite and its two precursors (amorphous calcium phosphate ($Ca_9(PO_4)_6nH_2O$); octocalcium phosphate ($Ca_8H_2(PO_4)_65H_2O$)), are obtained by aggregation of different calcium-phosphate (CaPi) clusters (step 2). In the grey circles are the salts found in ectopic calcifications, also known as "calcium phosphate deposits".

sive vascular calcification in the medial layer of the aortic wall (see Fig. **1**).

PPi is degraded to Pi enzymatically by tissue non-specific alkaline phosphatase (TNAP) in extracellular fluids (see Fig. **3**). Over-expression of TNAP in cells is sufficient to cause medial vascular calcification in aortic rings *ex vivo*; and addition of alkaline phosphatase to culture media is sufficient to cause matrix calcification [63, 64]. Moreover, TNAP activity is increased in models of medial vascular calcification, such as in uremic rats or in a mouse model of Hutchinson-Gilford Progeria Syndrome [30, 65]. A recent study showed that *in vivo* over-expression of TNAP increases skeletal mineralization [66]; however, other studies have shown that phosphatase inhibitors can prevent vascular smooth muscle calcification *in vitro* [67, 68] (see Fig. **1**) and that the ablation of phosphatase function produces a loss of skeletal mineralization [69].

The major generator of endogenous extracellular PPi in cartilage (and in a variety of other tissues) is the enzyme ecto-nucleotide pyrophosphatase/phosphodiesterase (eNPP), which hydrolyzes extracellular ATP to generate PPi and AMP (see Fig. **3**). Members of the eNPP family include NPP1, NPP2, and NPP3; they exist both as membrane proteins, with an extracellular active site, and as soluble proteins in body fluids (such as, PC-1, autotaxin, and B10). In vascular smooth muscle cells and in the aorta, eNPP1 is the main source of extracellular PPi [31, 32, 64]. Mutations in eNPP1 result in generalized arterial calcification of infancy (GACI), which is characterized by calcification of the internal elastic lamina of large and medium-sized arteries [70, 71]. Moreover, eNPP1-null mice develop ectopic artery calcification [72-74].

A direct competitor of the substrate for eNPP is the family of ectonucleoside triphosphate diphosphohydrolase (known as eNTPD, also called Apyrase). Members of the apyrase family, including eNTPD1 or CD39, can hydrolyze ATP and ADP with varying preferences for the individual type of nucleotide. eNTPD1 is the major ecto-enzyme expressed in the rat aorta which hydrolyzes 90% of ATP [64] and releases small amount of ADP due to its high affinity for ADP (in an ATP/ADP ratio of 1:0.8) [32]. CD39 has the most evidence linking it directly to the regulation of signaling through purine receptors, but has not been linked to ectopic calcification directly. A recent study indirectly linked

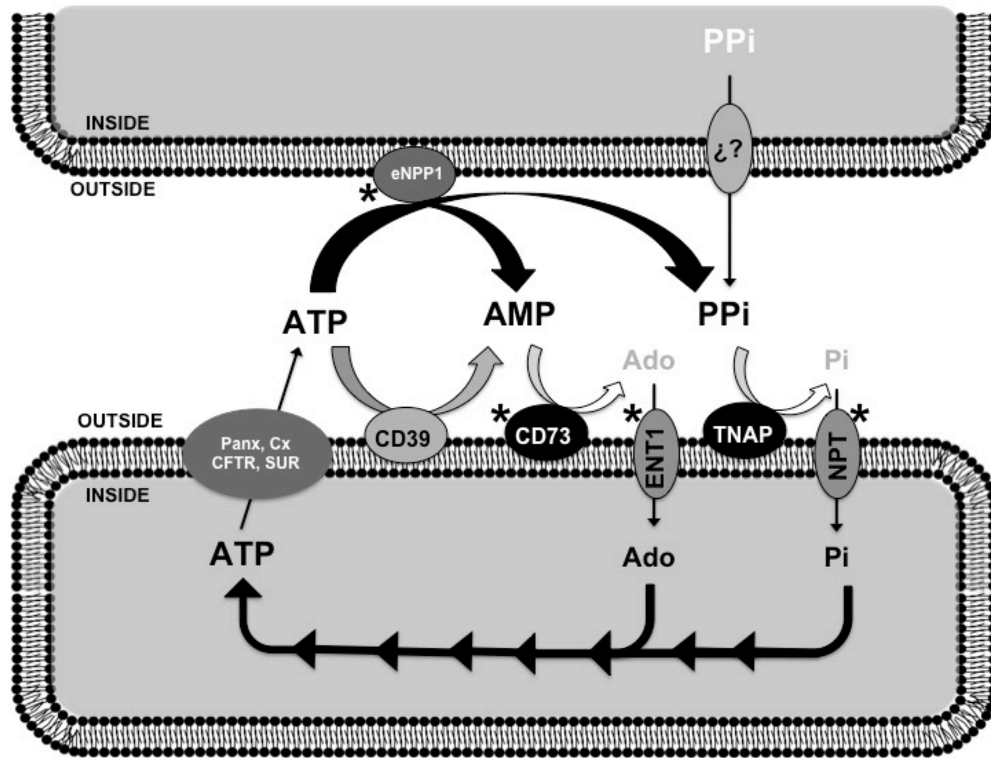

Fig. (3). Schematic representation of the ectoenzymes and transporters involved in the extracellular ATP metabolism. Extracellular pyrophosphate (PPi) is the major endogenous inhibitor of ectopic calcification, that is degraded by tissue non-specific alkaline phosphatase (TNAP) and produced during extracellular hydrolysis of adenosine-5'-triphosphate (ATP), via ectonucleotide pyrophosphatase phosphodiesterase 1 (eNPP1). A direct competitor of the substrate for eNPP1 is the ectonucleoside triphosphate diphosphohydrolase (CD39) that hydrolyze ATP or ADP releasing inorganic phosphate (Pi) and adenosine-5'-diphosphate (ADP) or adenosine-5'-monophosphate (AMP) respectively. The last step in the degradation of extracellular ATP is the membrane-bound ecto-5`nucletotidase (CD73) that preferentially binds AMP and converts it to adenosine (Ado) and Pi. To generate ATP by mitochondria or another metabolic pathway, Ado and Pi are recovered from the extracellular space by equilibrative nucleoside transporter 1 (ENT1) and sodium-phosphate transporter (NPT), respectively. To close the cycle of extracellular ATP metabolism, ATP is released by cells via exocytotic mechanisms and via multiple types of membrane channels, including connexin hemichannels (Cx), pannexin (Panx), cystic fibrosis transmembrane conductance regulator (CFTR), and the sulfonylurea receptor (SUR). (*) Indicates the ectoenzymes and transporters involved in extracellular ATP metabolism that induce ectopic calcification by loss of its function.

CD39 with vascular calcifications [30]. Smooth muscle cells derivate from aortas of a new mouse model of Hutchinton-Gilford Progeria syndrome, characterized by excessive vascular calcification, shown a high expression and activity of eNTPD1. These data suggests that CD39 could potentially limit the availability of ATP for PPi conversion by eNPP1. The expression and regulation of this enzyme in the context of ectopic calcification should be further explored.

Another ecto-enzyme involved in extracellular ATP metabolism is the membrane-bound ecto-5`nucletotidase (NT5E, called CD73). This enzyme preferentially binds AMP and converts it to adenosine (Ado) and Pi (see Fig. **3**). Mutations in CD73 induce medial arterial calcification of the lower extremity arteries with periarticular calcification [75]. Serum calcium and phosphate levels are normal; however, cells from these patients showed high levels of TNAP activity that are significantly reduced by adenosine supplementation or by CD73 over-expression, suggesting that adenosine inhibits TNAP activity. Nevertheless, the increase in TNAP activity in CD73-deficient cells could be a compensatory mechanism used to regulate hydrolysis of AMP to Ado and Pi. TNAP is a non-specific ecto-phosphomonoesterase with broad substrate specificity [76]. It releases Pi from a variety of organic compounds, including adenosine 5`-tri, -di-, and monophosphates, as does AMP [77]. In addition, a recent study showed that TNAP rapidly hydrolyzes extracellular AMP in primary somatosensory neurons lacking of other AMP nucleotidases, such as CD73 and prostatic acid phosphatase [78].

Like TNAP, CD73 is a GPI-anchored ectonucleotidase. In both enzymes, a soluble form cleaved from GPI-anchor has previously been described [27]. Both enzymes can catalyze the final step of extracellular nucleotide degradation [77, 78], but CD73 is the major enzyme responsible for the formation of extracellular adenosine from released adenine nucleotides. This last step in the degradation of extracellular nucleotides is very important for cell viability, as only the transport of nucleosides (such as adenosine) has been demonstrated, not nucleotides (such as AMP) across the cell membranes. Adenosine should be recovered from the extracellular space to generate ATP by mitochondria or another metabolic pathway. There are two known types of nucleoside transporters involved in the transport of adenosine into cells: concentrative nucleoside transporters (CNTs; SLC28)

and equilibrative nucleoside transporters (ENTs; SLC29). In a recent study, the loss of the equilibrative nucleoside transporter 1 (ENT1, SLC29A1) in mice could explain the diffuse idiopathic skeletal hypertosis (DISH) in humans, which is characterized by the ectopic calcification of spinal tissues [36]. In this study, the authors found a significant reduction in the expression of eNPP1, ANK, and TNAP in the intervertebral discs from ENT1$^{-/-}$ null mice compared to wild-type mice. This is the first report of a role for ENT1 in regulating the calcification of soft tissues and closes the cycle of extracellular ATP metabolism (see Fig. **3**).

The extracellular ATP metabolism cycle begins with the transport of ATP to the extracellular space (see Fig. **3**). ATP release by cells occurs through at least two mechanisms. Multiple types of membrane channels have been shown to mediate ATP release, including connexin hemichannels (Cx), pannexin (Panx), cystic fibrosis transmembrane conductance regulator (CFTR), multidrug resistance gene product *mdr* (P-glycoprotein), and the sulfonylurea receptor (SUR) [79-83]; the last three proteins mentioned use the energy of ATP hydrolysis to facilitate the movement of a large array molecules across the cell plasma membrane, such that they are members of a class of integral membrane proteins so-called ABC transporters. In addition to ATP released through these channels, cells can release ATP via exocytotic mechanisms [84].

In 2000, two new genes were identified that play an important role in the control of tissue calcification and arthritis [85-87]; however, the molecular mechanisms remain, in part, unknown (see Table **2**). First, it was reported that mutations in ABCC6 cause *Pseudoxanthoma ellasticum* (PXE), a heritable disorder of connective tissue characterized by testicular microlithiasis [88] and calcification of the elastic fibers in skin, arteries, and retina [85, 86]. ABCC6 (ATP-binding cassette sub-family C member 6) is a member of the super family of ABC transporters, also known as multidrug resistance-associated protein 6 (MRP6). The MRP family is composed of several related pumps that are able to transport various molecules across extra- and intra-cellular membranes including glutathione-S-conjugates, bilirubin glucuronide, glycocholic acid, and cyclic nucleotides [89, 90]. ABCC6 may acts as a pump that releases endogenous low molecular weight inhibitors of calcium phosphate deposits in fluids outside cells, such as ATP or citrate; however, this has not been demonstrated.

The second gen reported is known as progressive ankylosis (ANK) [87]. Mutations in the ANK gene cause a severe form of generalized joint calcification and arthritis when studied in a mouse model. Loss of ANK function cause excessive hydroxyapatite formation in ANK$^{-/-}$ null mice. Cells from the ANK mutant have a reduction in extracellular PPi levels, and overexpression of ANK in tissue cultured cells results in an increase in extracellular PPi [87]. Using several helix prediction programs was proposed for the ANK protein 7-12 membranes-spanning helices and a central channel [87, 91, 92]. Therefore, it seems as though ANK regulates PPi transport from the cytoplasm to the extracellular milieu [87, 91, 93]; however, recent studies showed that ANK could be a channel or regulator of adjacent channels, which release ATP outside the cells [31, 64, 94].

In humans, mutations in the channel core of ANK cause craniometaphyseal dysplasia (CMD) [91, 92], and mutations in the N- and C-terminus of the ANK protein cause chondrocalcinosis (CC) [95-97]. CMD is a rare skeletal condition of abnormal bone formation characterized by an increased density of craniofacial bones and abnormal modeling of the metaphyses of the tubular bones. CC is a disease of articular cartilage that is radiographically characterized by the deposition of calcium pyrophosphate dihydrate crystals in the joint. Like mouse progressive ankylosis, CMD is associated with a decrease in extracellular PPi levels [91, 92] whereas CC is associated with an increase in the amount of PPi in the extracellular space which induce the spontaneous formation of calcium pyrophosphate crystals [95-97].

Recent reports suggest that the Pi/PPi ratio is strictly controlled by a complex interplay of genes that regulate Pi and

Table 2. Existing Genetic Disease involved in extracellular ATP/PPi metabolism that produces ectopic Calcification.

Protein Afected	Role	Genetic Disease	Symbol	Ectopic Calcification	Ref
eNPP1	Synthesis of PPi	Generalizaed Arterial Calcification of Infancy	GACI	Medial Arterial	Rutsch F., 2003.
CD73	Hydrolisis of AMP			Medial Arterial and Periarticular	St Hilaire C., 2011.
ENT1	Ado Transporter	Idiopatic Skeletal Hypertosis	DISH	Spinal Tissues	Warraich S., 2013.
Pit-2	Pi Transporter	Familial Idiopathic basal Ganglia Calcification		Basal Galglia and cortex	LeGeros RZ. 2007.
ANK	¿?	Craniometaphyseal dysplasia	CMD	Craniofacial Bones	Foster BL., 2006.
ANK	¿?	Condrocalcinosis	CC	Articular cartilage	Wang J, 2009.
ABCC6	¿?	Pseudoxanthoma ellasticum	PXE	Elastic fibers in skin, arteries and retine. Testicular calcification.	Gurley KA, 2006. Costello JC, 2011. Williams CJ, 2002.

PPi concentrations [98-100]. ANK could play a key role in this complex process by regulating ATP excretion by different channels, regulating phosphate transport and regulating both eNPP1 and TNAP activities [101-104]. In addition, transfection of eNPP1 in osteoblasts enhances extracellular PPi levels only when wild-type ANK is present [100]; over-expression of wild-type ANK proteins result in down-regulation of TNAP activity in chondrogenic cells [102].

3. ROLE OF PHOSPHATE TRANSPORT IN ECTOPIC CALCIFICATION

Classically, ATP has been considered the major energy source in the cell and is produced by a wide variety of enzymes from adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine (Ado). In mammalian cells, substrate level phosphorylation and oxidative phosphorylation are two major mechanisms of ATP biosynthesis. Metabolic processes that use ATP as an energy source convert it back into its precursors. Therefore, ATP is continuously recycled in the cell. However, over the last few years, a novel role for ATP as a potent extracellular signaling molecule and as a source of extracellular PPi has emerged [105]. Several ectoenzymes use extracellular ATP for this purpose (as mentioned in the previous section) and the final products of extracellular ATP hydrolysis by these enzymes are Ado and Pi [27, 105], which need to be transported into the cell to synthesize new ATP. Therefore, a loss in the transport of Ado (see adobe) or Pi (see below) can result in a decrease in the synthesis of ATP, which can in turn decrease the synthesis of inhibitors and lead to excessive calcium-phosphate deposition.

Cellular Pi levels are controlled by sodium phosphate co-transporters (NPT) [106]. The roles of NPT in human clinical disease and physiology processes have not been defined. Two families of sodium phosphate (NaPi) co-transporters have been principally identified, each with multiple members [107]: Type II (also called SLC34 or NaPi-II) and type III (SLC20 or NaPi-III). Type I (SLC17 or NaPi-I) phosphate transporters mediate the transmembrane transport of organic anions. Originally identified as Pi transporters, the relativity low affinity of NaPi-I family members for phosphate suggested that they transported more readily organic and inorganic anions than phosphate. Unlike Type I, Type II and type III transporters transport phosphate with high affinity ($K_m \approx$ 0,1 or less) but show differences in their affinities for $H_2PO_4^-$ and HPO_4^{2-} ions [108-110].

The SCL34 family [111] comprises three members (also called NaPi-II), which are expressed in the small intestine (NaPi-IIb) and the kidney (NaPi-IIa and NaPi-IIc), two important sites for the control of phosphate homeostasis. NaPi-IIa is expressed predominantly both on renal proximal tubules and on the osteoclast basolateral membrane. Under normal conditions, NaPi-IIa is the NPT responsible for 95% of phosphate reabsorption in the proximal tubule [109]. Expression of NaPi-IIc was found exclusively in kidney and was described as being growth related [112, 113]. Interestingly, mice deficient in NaPi-IIa exhibit nephrocalcinosis and kidney stone formation but these defects are not observed in people with a loss of NaPi-IIa function [114]. Instead, mice deficient in NaPi-IIc exhibit hypercalciuria and hypercalcemia, but not renal calcification or osteomalacia [115-117]. Moreover, expression of NaPi-IIb has been detected in a number of tissues, including small intestine, lung, mammary glands, testis, and liver [111]. The NaPi-IIb knockout is lethal [118], but NaPi-IIb loss-of-function is associated with alveolar calcification in middle age [119, 120], and sometimes calcification in other organs, such as the testis [119] (see Table **3**).

Type III sodium-phosphate co-transporters are represented by Pit-1 and Pit-2, which are members of the SLC20

Table 3. Sodium Phosphate Transporters (NPT). Expression and loss of it function.

Transporter	Expression	Loss of Function	Ref.
Pit-1 (SLC20A1)	VSMCs Chondrocytes	Not produced ectopic calcification Cartilage Calcification	Crouthamel MH, 2013. Cecil DL, 2005.
Pit-2 (SLC20A2)	Brain Liver Heart VSMCs	Brain Calcification ¿? ¿? ¿?	Wang C, 2012.
NaPi-IIa (SLC34A1)	Kidney Bone (osteoclast)	Nephrocalcinosis, stone formation Osteoporosis, tickets	Beck L, 1998.
NaPi-IIb (SLC34A2)	Small Intestine Testis Liver Lung Mammary Glands	¿? Testicular Calcification ¿? Pulmonary microlithiasis ¿?	 Corut A. 2006. Huqun, IS, 2007.
NaPi-IIc (SLC34A3)	Kidney	¿?	

family of solute carriers [121]. Both co-transporters mediate the movement of Pi ions across the cell membrane [40, 122] and are ubiquitously expressed, suggesting a “housekeeping” function. More precise localization studies revealed different levels of Pit-1 and Pit-2 expression. Expression of Pit-1 mRNA is highest in osteoblasts, vascular smooth muscle cells (VSMCs), and bone marrow [12, 40, 123]. Pit-1 plays a critical role in cartilage calcification and regulation of apoptosis and cell proliferation [124-127]. Deletion of Pit-1 expression in the mouse showed it played an essential function in liver development [128]. In a recent study, the targeted deletion of Pit-1 in VSMCs in mouse did not induce aortic calcification due to compensatory regulation by Pit-2 [129]. Moreover, the expression of Pit-2 is highest in VSMCs [40], liver, heart, and brain [121]. An association has recently been found between loss-of-function of Pit-2 and familial idiopathic basal ganglia calcification in humans [38]. In addition, a recent study [130] has shown that knockout of Pit-2 in mice causes calcification in the thalamus, basal ganglia, and cortex, demonstrating that reduced Pit2 expression alone can cause brain calcification (see Table **3**).

CLINICAL PERSPECTIVE

This association between los of function in NPT and ectopic calcification complete the cycle of extracellular ATP metabolism (see Fig. **3**) and suggest that the role of sodium phosphate co-transporters in the initiation and progression of ectopic calcification should be reinterpreted as a key piece in the synthesis of calcification inhibitors. Moreover, in clinical practice, is important to evaluate both phosphate and pyrophosphate homeostasis in order to respond two important questions 1) Does Pi in serum is high? and 2) Does PPi in serum is low?. In the case of positive response the first treatment that we should think in order to designer the correct therapeutic strategic to prevent vascular calcification is, respectively: 1) phosphate binders in order to reduce the amount of Pi in blood; and 2) injections of PPi in order to increase the availability of PPi. Both types of treatment mentioned in this review have been used in clinical trials in patients receiving chronic hemodialysis (for example, www.clinicaltrial.gov identifiers: NCT01755078 and NCT01503021).

LIST OF ABBREVIATIONS

ACP = amorphous calcium phosphate

Ado = adenosine

ADP = adenosine-5’-diphosphate

AMP = adenosine-5’-monophosphate.

ATP = adenosine-5’-triphosphate

β-TCP = β-tricalcium phosphate

CC = chondrocalcinosis.

CMD = craniometaphyseal dysplasia

CPD = Calcium-phosphate deposition

DCPA = dicalcium phosphate anhydrous

DCPD = dicalcium phosphate dehydrate

eNPP = ecto-nucleotide pyrophosphatase/phosphodiesterase

ENT1 = equilibrative nucleoside transporter 1

HA = hydroxyapatite

MCPA = monocalcium phosphate anhydrous

MCPM = monocalcium phosphate monohydrate

NaPi = sodium phosphate co-transporters

OCP = octocalcium phosphate

PPi = Pyrophosphate

TNAP = Tissue non-specific alkaline phosphatase

VC = Vascular calcification

VSMCs = vascular smooth muscle cells

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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DISCLOSURES

None

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