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Ectopic mineralization disorders of the extracellular matrix of connective tissue: Molecular genetics and pathomechanisms of aberrant calcification

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Abstract

Ectopic mineralization of connective tissues is a complex process leading to deposition of calcium phosphate complexes in the extracellular matrix, particularly affecting the skin and the arterial blood vessels and common in age-associated disorders. A number of initiating and contributing metabolic and environmental factors are linked to aberrant mineralization in these diseases, making the identification of precise pathomechanistic pathways exceedingly difficult. However, there has been significant recent progress in understanding the ectopic mineralization processes through study of heritable single-gene disorders, which have allowed identification of discreet pathways and contributing factors leading to aberrant connective tissue mineralization. These studies have provided support for the concept of an intricate mineralization/anti-mineralization network present in peripheral connective tissues, providing a perspective to development of pharmacologic approaches to limit the phenotypic consequences of ectopic mineralization. This overview summarizes the current knowledge of ectopic heritable mineralization disorders, with accompanying animal models, focusing on pseudoxanthoma elasticum and generalized arterial calcification of infancy, two autosomal recessive diseases manifesting with extensive connective tissue mineralization in the skin and the cardiovascular system.

Keywords

Ectopic mineralization; Heritable connective tissue diseases; Pathomechanisms of mineralization disorders; Pseudoxanthoma elasticum; Generalized arterial calcification of infancy

1. Introduction

A number of clinical conditions, including aging, cancer, diabetes and autoimmune diseases, have been linked to ectopic mineralization, *i.e.*, aberrant deposition of calcium phosphate complexes in connective tissues. Two general mechanisms of ectopic calcification in the extracellular matrix have been recognized: (a) *metastatic calcification* is a result of elevated levels of serum phosphate and/or calcium, and (b) *dystrophic calcification* occurs in

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diseased tissue as a result of an insult under normal calcium and phosphate homeostasis. Both metastatic and dystrophic forms of calcification have been clinically encountered in a number of “acquired” diseases affecting the skin and the vascular connective tissues (Budoff et al., 2007; Chander and Gordon, 2012). For example, metastatic calcinosis in the skin can manifest with nodular deposits of calcium phosphate, noted in patients with chronic renal failure, which results in hyperphosphatemia. Calciphylaxis is associated with extremely high mortality rate as a result of calcification of skin, subcutis and vascular connective tissues (Ng and Peng, 2011). Localized dystrophic calcification of skin is noted in a number of inflammatory lesions, such as in acne and chronic ulcers, while more widespread calcinosis cutis, *i.e.*, deposition of calcium and phosphate in larger lesions in the skin, can be noted in patients with systemic sclerosis, systemic lupus erythematosus and dermatomyositis, *i.e.*, inflammatory autoimmune connective tissue disorders (Daoussis et al., 2012). Collectively, aberrant mineralization can result from a number of initiating and contributing metabolic and environmental factors, which makes the identification of precise pathomechanistic pathways in these “acquired” disorders exceedingly difficult.

Significant progress in our understanding of the ectopic mineralization processes in general has been made through the study of Mendelian single-gene disorders with phenotypic similarities with the “acquired” forms of metastatic and dystrophic calcification (Table 1). Such studies, with accompanying animal models, have allowed identification of discrete pathways and contributing factors leading to aberrant connective tissue mineralization, and they have provided evidence in support of an intricate mineralization/anti-mineralization network present in peripheral connective tissues (Li and Uitto, 2013; Rutsch et al., 2011). Two of such conditions, pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI), serve as examples of heritable disorders of ectopic mineralization in the skin and the vascular connective tissues.

2. Heritable Disorders with Connective Tissue Mineralization

2.1 Pseudoxanthoma elasticum (PXE) – the prototype of systemic mineralization disorders

PXE, a multisystem ectopic mineralization disorder, manifests with profound deposition of calcium phosphate complexes in the extracellular matrix of connective tissues in a number of organs. Clinically, PXE presents with late-onset, followed by slow, yet progressive, development of clinical manifestations in the skin, the eyes, and the cardiovascular system (Neldner, 1988) (Figure 1). The early skin manifestations consist of small yellowish papules, which progressively coalesce into larger plaques of inelastic and leathery skin. Histopathology of the skin lesions reveals early accumulation of pleiomorphic elastic structures which progressively become mineralized. The skin findings by themselves are primarily of cosmetic concern, but they signify the potential for development of serious eye problems and cardiovascular involvement (Georgalas et al., 2011; Leftheriotis et al., 2013). The characteristic eye lesions consist of angioid streaks which reflect the breakage of mineralized Bruch’s membrane, an elastin rich sheath between the pigmented retina and choroid of the eye (Figure 1). The angioid streaks are associated with breakage of capillaries resulting in bleeding to the eye, scarring, and neovascularization, which cause progressive loss of visual acuity and can lead to blindness if left untreated. The cardiovascular system is affected in PXE by progressive mineralization of medium sized arterial blood vessels, clinically resulting in hypertension, intermittent claudication, occasional bleeding from the intestinal arteries, and rarely, premature myocardial infarcts and stroke.

The inheritance of PXE is autosomal recessive with underlying mutations in most cases in the *ABCC6* gene encoding a putative transmembrane efflux transporter protein, ABCC6 (Uitto et al., 2010). The *ABCC6* gene is expressed primarily in the liver but is essentially undetectable in tissues directly affected by mineralization (Belinsky and Kruh, 1999). These

observations, coupled with studies utilizing a murine model, *Abcc6*$^{-/-}$ mouse, with targeted ablation of the corresponding mouse gene (Gorgels et al., 2005; Klement et al., 2005), have formed the basis to consider PXE as a metabolic disorder with the primary molecular defect in the liver (Jiang et al., 2009). It has been postulated that ABCC6, which is localized to the basolateral surface of hepatocytes (Pomozi et al., 2013), serves physiologically as a pump that is postulated to transport critical anti-mineralization factors from the liver into the circulation. In the absence of functional ABCC6 transporter activity, the serum levels, and consequently the concentrations of such factors in the peripheral tissues, are reduced, allowing slow, yet progressive mineralization to ensue (Figure 1). It should be noted, however, that the precise nature of the molecules transported by ABCC6 is currently unknown (Uitto et al., 2013).

The estimated prevalence of PXE is ~1:50,000, implying that there are ~7,000 patients in the United States and as many as 150,000 globally. Approximately 90% of the PXE patients have mutations in the *ABCC6* gene, and well over 300 distinct loss-of-function mutations have been encountered; these include recurrent p.R1141* and g.del23-29 which account for ~40% of all mutations (Pfendner et al., 2007). The presence of PXE-like cutaneous findings in unusual, genetically distinct clinical conditions has also revealed the contribution of additional mutated genes. One such condition is vitamin K-dependent coagulation factor deficiency with bleeding disorder. Some of these affected individuals develop loose and lax skin with clinical features of PXE. In these patients, no mutations in the *ABCC6* gene were found, but instead, mutations were disclosed in the *GGCX* gene encoding vitamin K-dependent γ-glutamyl carboxylase (Li et al., 2009b; Vanakker et al., 2007). This enzyme catalyzes γ-carboxylation of glutamic acid residues in so-called Gla proteins, which include a number of coagulation factors and matrix Gla protein (MGP), the latter being a powerful anti-mineralization factor when in its fully carboxylated form (Berkner, 2005; Schurgers et al., 2013). As a consequence of inactivating mutations in the *GGCX* gene, MGP in these patients remains uncarboxylated and thus inactive, resulting in aberrant mineralization of the peripheral connective tissues in the skin and the arterial blood vessels.

Because GGCX is a vitamin K-dependent enzyme, it has been suggested that ectopic mineralization in PXE might result from vitamin K deficiency and that ABCC6 could serve as a pump to transport vitamin K or some of its derivatives from hepatocytes to the circulation (Borst et al., 2008). This hypothesis has been tested by feeding the *Abcc6*$^{-/-}$ knock-out mice with vitamin K1 or K2 or by intravenous injection of vitamin K-glutathione conjugate. None of these treatments counteracted the mineralization of connective tissues in this mouse model of PXE (Brampton et al., 2011; Gorgels et al., 2011; Jiang et al., 2011). It has been demonstrated, however, that feeding *Abcc6*$^{-/-}$mice with warfarin, an anticoagulant that interferes with the vitamin K cycle by preventing the reduction of the oxidized form of vitamin K (epoxide) to its reduced form (hydroquinone), dramatically increased the accumulation of mineral deposits in soft connective tissues in comparison to *Abcc6*$^{-/-}$mice kept on control diet (Li et al., 2013c). While these results do not unequivocally establish a role for vitamin K deficiency in the development of mineral deposits in PXE, they suggest that reduction in the γ-glutamyl carboxylation of MGP by warfarin can result severe ectopic mineralization, with apparent clinical implication that patients with PXE on warfarin therapy may be at risk of worsening their disease.

2.2 Generalized arterial calcification of infancy (GACI) – a pediatric disorder of vascular mineralization

GACI is a severe autosomal recessive disorder diagnosed with prenatal or perinatal manifestations of ectopic vascular mineralization (Figure 1). In fact, the majority of patients die within the first year of life as a result of vascular complications (Nitschke and Rutsch,

2012b). In the majority of cases GACI is caused by mutations in the *ENPP1* gene, which encodes ectonucleotide pyrophosphatase/ phosphodiesterase 1 (Rutsch et al., 2003). This enzyme hydrolyses ATP to AMP and inorganic pyrophosphate (PP_i); the latter can serve as a powerful anti-mineralization factor (Figure 2).

Traditionally, PXE and GACI have been considered to be clinically two distinct entities, the vascular mineralization in PXE being of late onset with accompanying skin and eye findings but essentially with normal lifespan, while in GACI the vascular mineralization results in early demise of the affected individuals. Recent studies have revealed, however, considerable both phenotypic and genotypic overlap between these two conditions (Nitschke and Rutsch, 2012a). For example, several families diagnosed with GACI have been recently found to have mutations in the *ABCC6* gene, and many of the same mutations have been shown to cause classic PXE in unrelated families (Li et al., 2013b; Nitschke et al., 2012). In a particularly interesting family, one of the siblings died in early infancy with clinical findings consistent with GACI, but another sibling had manifestations of late onset of PXE due to mutations in the *ABCC6* gene (Le Boulanger et al., 2010). Significant clinical overlap between PXE and GACI has also been noted in a number of pediatric cases with early clinical findings of vascular calcification diagnosed as GACI and associated with cutaneous findings characteristic of PXE (Li et al., 2013a; Li et al., 2012; Nitschke et al., 2012). Collectively, these studies indicate that mutations in the *ENPP1* and *ABCC6* genes can result in phenotypically overlapping manifestations both in GACI and PXE, suggesting the possibility that divergent mineralization pathways due to underlying mutations in distinct genes converge to common phenotypic manifestations.

3 Pathomechanisms of Ectopic Mineralization

3.1 Lessons from mouse models

Several mouse models that have been developed to mimic the heritable ectopic mineralization disorders have been extremely helpful in deciphering the pathomechanisms of the mineralization process (Table 1). For example, the *Abcc6*$^{-/-}$mice, developed by target ablation of the corresponding mouse gene, recapitulate the genetic, histopathologic and ultrastructural features of PXE (Gorgels et al., 2005; Klement et al., 2005). In particular, these mice develop ectopic mineralization of the skin, eyes, and the vascular arterial blood vessels similar to that in patients with PXE. Characterization of the mineral deposits in connective tissues in *Abcc6*$^{-/-}$mice has shown that they consist of calcium and phosphorus co-localizing in the lesions, as determined by energy dispersive X-ray analysis, and the ratio of calcium and phosphorus progressively increases with maturation of the lesions reaching a value of ~2.0, comparable to endochondral bone (Kavukcuoglu et al., 2012). Increased mineralization is also reflected by increased mineral-to-matrix ratio as demonstrated by Fourier transform infrared imaging spectroscopy. The overall mineralization process is characterized by initial deposition of amorphous calcium and phosphate complexes followed by progressive maturation to hydroxyapatite.

Recently, a novel mouse model for GACI has also been developed as part of a ENU mutagenesis program at The Jackson Laboratory. Phenotypically these mice were noted to have stiffening of the joints with aging, and consequently, the mutation was named “ages with stiffened joints” (*asj*) (Harris et al., 2012). These mice were found by histopathologic examination to have extensive mineralization of a number of tissues, including arterial blood vessels. The underlying mutation was found to be a p.V246D missense mutation in the *Enpp1* gene, which resulted in absence of the ENPP1 protein in the liver, and the lack of enzymatic activity resulted in reduced PP_i levels in the plasma (Li et al., 2013d). The progress of mineralization was shown to be highly dependent on the mineral composition of the diet, with significant shortening of the lifespan on a diet enriched in phosphorus and low

in magnesium, the majority of mice dying within the first six weeks of age. Collectively, the *asj* mouse serves as an animal model for GACI, and together with information on *Enpp1*$^{-/-}$ knockout mice (Mackenzie et al., 2012), confirms the critical role of the pyrophosphate as an anti-mineralization factor under normal physiologic conditions.

3.2 Pro-mineralization factors

Several factors can modify the mineralization process, including the availability of the nucleation sites in extracellular elastic structures and/or collagen fibers, the tissue-specific micro environment favoring mineralization, including the presence of local and systemic anti-mineralization factors, and the local P_i/PP_i ratio. Specifically, PP_i is a powerful inhibitor of mineralization and changes in the P_i/PP_i ratio could either facilitate or inhibit mineralization reactions, depending on the precise balance of these ions in the micro environment (Thouverey et al., 2009). The rate of mineralization may also relate to the matrix-vesicle (MV) nucleated processes in these diseases, as MVs have been shown to play a role in skeletal mineralization (Golub, 2010). If the pathological mineralization of connective tissues in the ectopic mineralization diseases is mechanistically similar to that of skeletal calcification, MVs may play a role by serving as a nucleation site.

3.3 Anti-mineralization factors

A number of anti-mineralization factors, either systemic or local, have also been identified to be associated with the mineralization processes; these include fetuin-A, MGP, progressive ankylosis protein homolog (Ank), and tissue non-specific alkaline phosphatase (TNAP) (Figure 2) (Heiss et al., 2003; Luo et al., 1997; Mornet, 2000; Wang et al., 2005). The critical role of fetuin-A (also known as the α_2-Heremans-Schmid glycoprotein; AHSG) as a systemic inhibitor of mineralization has been documented by development of severe calcification in various organs of *Ahsg*$^{-/-}$mice (Jahnen-Dechent et al., 1997). In these mice, the serum calcium and phosphate homeostasis is not changed, but decreased inhibitory activity of fetuin-A in serum of these animals allows precipitation of calcium phosphate in vascular tissues. Attesting to the anti-mineralization activity of fetuin-A is also demonstration that overexpression of full-length fetuin-A cDNA in the liver of *Abcc6*$^{-/-}$mice reduced soft tissue mineralization in these mice (Jiang et al., 2010). Physiologically, fetuin-A inhibits mineralization by formation of soluble colloidal spheres with calcium and phosphate, so-called “calciprotein particles” (Heiss et al., 2003). These particles ordinarily become progressively more crystalline and insoluble in timeand temperature-dependent manner, but incorporation of fetuin-A facilitates solubilization of these particles, removing them away from the site of mineralization.

MGP is another powerful anti-mineralization factor expressed abundantly, for example, in smooth muscle cells of the arterial walls. The critical role of MGP in the mineralization process was initially demonstrated by development of *Mgp*$^{-/-}$mice which die within a few months of age due to mineralization of elastic fibers and with subsequent rupture of aorta and elastic arteries (Luo et al., 1997). In the lesional skin of patients with PXE, as well as in the *Abcc6*$^{-/-}$ mice, there is a preponderance of the uncarboxylated form of MGP, suggesting that inactivation of this physiological anti-mineralization factor may contribute to formation of calcium phosphate deposits (Gheduzzi et al., 2007; Li et al., 2007b).

4. Mineralization/Anti-mineralization Networks in the Skin and Vascular Connective Tissues

In addition to PXE and GACI, there are a number of other heritable single-gene disorders demonstrating ectopic connective tissue mineralization with phenotypic similarities with the acquired forms of metastatic and dystrophic calcification (Table 1). Among them, a group of

diseases, familial tumoral calcinosis (FTC), affects primarily skin and the subcutaneous tissues (Sprecher, 2010). The hereditary counterpart of acquired metastatic mineralization is the hyperphosphatemic variant of FTC (HFTC) characterized by progressive deposition of calcium phosphate in skin and periarticular spaces. HFTC is associated with marked hyperphosphatemia as a result of mutations in three genes that are involved in the regulation of phosphate excretion in the kidney; these mutated genes include *FGF23*, *GALNT3* and *KL* (Figure 2) (Benet-Pages et al., 2005; Ichikawa et al., 2007; Topaz et al., 2004). The second form of FTC, known as the normophosphatemic type (NFTC), manifests with extensive mineralization of the skin and subcutaneous tissues with preceding inflammation manifesting mostly in mucosal tissues. NFTC phenotype results from mutations in *SAMD9*, a gene with currently unknown function (Topaz et al., 2006). The corresponding protein, SAMD9, has been suggested to serve as a tumor suppressor gene, but the relevance of this observation to the mineralization phenotype remains unclear (Li et al., 2007a).

In addition to PXE and GACI, another recently described clinical entity, arterial calcification due to CD73 deficiency (ACDC), also known as calcification of joints and arteries (CALJA; OMM211800), displays prominent vascular involvement (Markello et al., 2011; St Hilaire et al., 2011). This disease is caused by mutations in the *NT5E* gene, encoding an enzyme that breaks down AMP to adenosine and inorganic phosphate (P_i) (Figure 2). As a consequence of this enzyme deficiency in ACDC, the activity of tissue non-specific alkaline phosphatase (TNAP) is increased leading to accelerated conversion of PP_i to P_i, a pro-calcifying factor. Thus, the mineralization phenotypes in these heritable disorders in complex gene/protein systems attest to the presence of a complex mineralization/anti-mineralization network in part revolving around the P_i/PP_i stoichiometry. This information is critical for understanding the pathomechanisms of different mineralization disorders, both heritable and acquired, providing a perspective to development of pharmacologic approaches to limit the phenotypic consequences and complications of ectopic mineralization.

5. Pharmacologic Perspective

No treatment for systemic manifestations of PXE and GACI is currently available. For patients with PXE, intravitreous injection of vascular endothelial growth factor antagonists can be offered to prevent neovascularization, and considerable improvement has been reported in visual acuity in these patients (Finger et al., 2011; Zebardast and Adelman, 2012). However, the manifestations in the skin and the cardiovascular system become progressively evident as a result of systemic mineralization with advancing age. For patients with GACI, limited success has been reported by utilization of bisphosphonates, stable pyrophosphate analogs in which a carbon substitutes for oxygen and two R-groups replace the phosphate residues (Edouard et al., 2011; Rutsch et al., 2008). These drugs have two principal biological activities, (a) the anti-osteoclastic activity, and (b) inhibition of hydroxyapatite formation mimicking that of PP_i. Bisphosphonates are currently being used for treatment of Paget's disease and osteoporosis, disorders in which osteoclasts are overly active (Ralston, 2013; Warriner and Saag, 2013). Depending on the precise structure of the bisphosphonates, the ratio of these two activities, *viz.* anti-mineralization *vs.* anti-osteoclastic, need to be balanced for treatment of GACI where the primary aim is to inhibit mineralization with minimized effects on osteoclasts.

Quite recently, the possibility of supplementing the diet with magnesium as a way of preventing systemic mineralization has been brought to a clinical trial in patients with PXE. This notion was based on early animal experiments which demonstrated that feeding *Abcc6*$^{-/-}$mice with a diet supplemented with magnesium at 5 times higher levels than in the standard rodent diet completely prevented the connective tissue mineralization (LaRusso et

al., 2009; Li et al., 2009a). Conversely, reduction of the magnesium content to about 20% of that in control diet profoundly accelerated the mineralization processes (Jiang and Uitto, 2012). The mechanism by which magnesium inhibits mineralization relates to formation of mineral complexes in which magnesium replaces calcium and the excess calcium is excreted in the urine. Magnesium phosphate complexes are more soluble under physiological conditions as compared to those consisting of calcium phosphate, and consequently, less mineralization occurs, potentially providing a novel treatment for patients with ectopic mineralization disorders. In fact, a double-blinded clinical trial on patients with PXE with magnesium supplementation of their diet is currently underway (http://clinicaltrials.gov/show/NCT01525875). It is noteworthy that the mouse studies indicated magnesium being effective only in preventing the mineralization process, but not in reversing the existing mineral deposits. Thus, the magnesium supplementation, if shown by the clinical trials to be effective, should be instituted as soon as the diagnosis has been established.

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Abbreviations

PXE	pseudoxanthoma elasticum
GACI	generalized arterial calcification of infancy
MGP	matrix Gla protein

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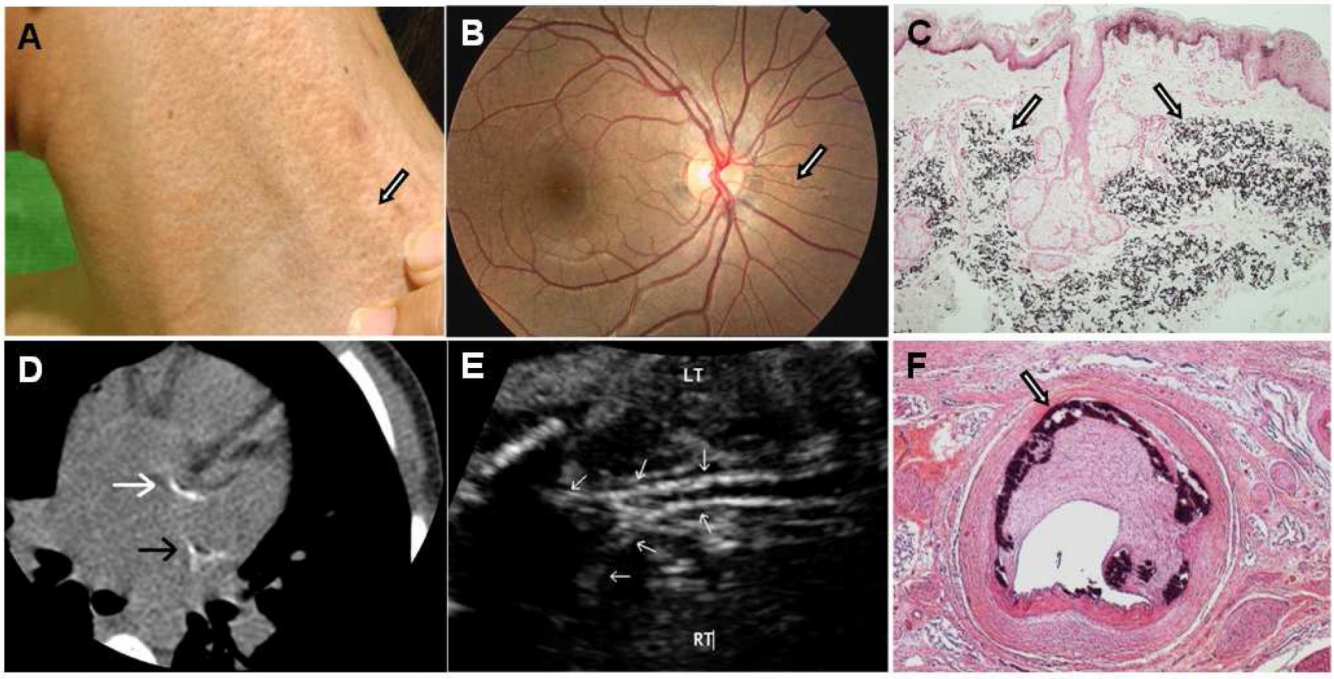

Figure 1. Clinical and histopathologic features of pseudoxanthoma elasticum (A–C) and generalized arterial calcification of infancy (D–E)

(A) Characteristic skin lesions consisting of yellowish papules coalescing into an inelastic plaque of skin on the side of the neck (arrow). (B) Fundoscopic examination reveals angioid streaks (arrow). (C) Histopathology of skin demonstrates accumulation of pleiomorphic elastic structures which are mineralized in the middermis (arrows; von Kossa stain). (D) Computed tomography angiogram of the heart reveals abnormal calcification of the left circumflex artery (white arrow) and left main coronary artery (black arrow) in a 2 year-old patient. (E) Prenatal ultrasound demonstrates extensive calcification of the aortic bifurcation (arrows). (F) Calcification of mesenteric artery in a newborn (arrow; von Kossa stain).

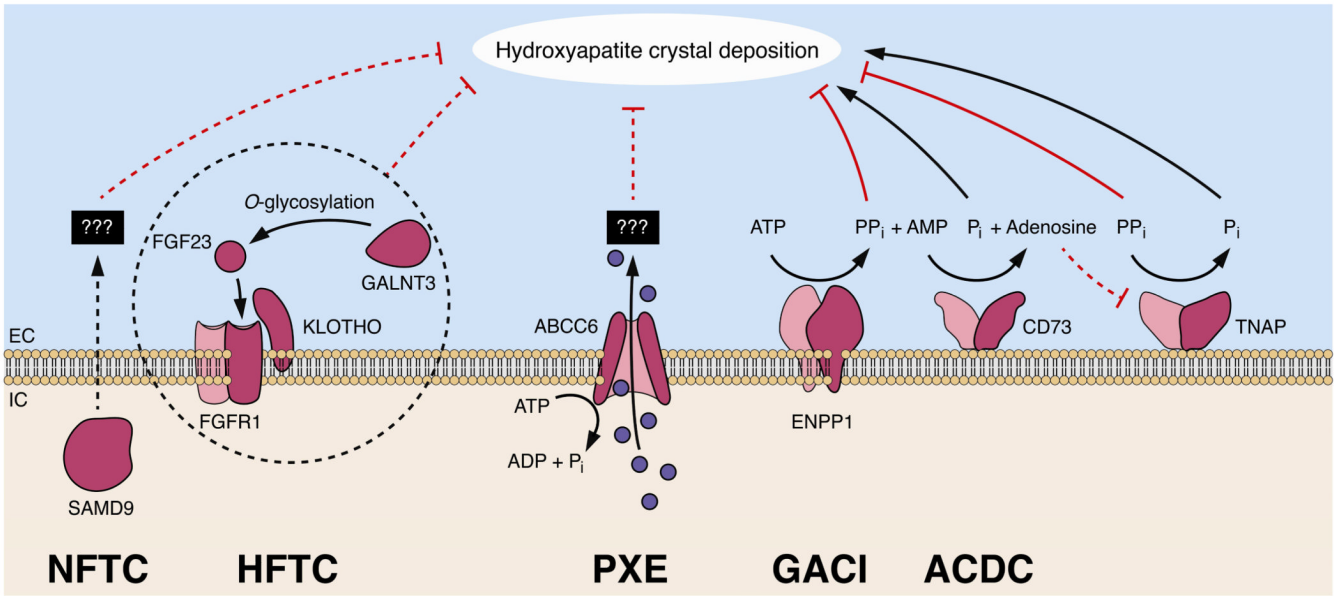

Figure 2. Genetic complexity of the mineralization/anti-mineralization network in connective tissues

Mutations in specific genes can contribute to deposition of hydroxyapatite in heritable ectopic mineralization disorders: NFTC, normophosphatemic familial tumoral calcinosis; HFTC, hyperphosphatemic FTC; PXE, pseudoxanthoma elasticum; GACI, generalized arterial calcification of infancy; ACDC, arterial calcification due to CD73 deficiency. The blue solid circles represent currently unidentified anti-mineralization factors physiologically transported by ABCC6 from intracellular milieu (IC) to the extracellular space (EC). (Adapted from Li and Uitto, 2013, with permission).

Table 1

Diseases with ectopic mineralization phenotypes, genes, and corresponding mouse models

Human Disease	Phenotypic Features	Arterial and cartilage calcification	Mouse Model
Pseudoxanthoma elasticum (PXE)	Mineralization in the skin, eyes and cardiovascular system	*ABCC6*, ATP-binding cassette C subfamily, member 6	*Abcc6*$^{-/-}$,KK/HlJ
Generalized arterial calcification of infancy (GACI)	Arterial calcification, joint and spine ossification	*ENPP1*, Nucleotide pyrophosphatase/ phosphodiesterase 1	*Enpp1*$^{-/-}$, *ttw/ttw, asj*
Arterial calcification due to CD73 deficiency (ACDC)	Vascular and joint calcification	*NT5E*, CD73	*Nt5e*$^{-/-}$
Normophosphatemic familial tumoral calcinosis (NFTC)	Ulcerative mineralization lesions in skin	*SAMD9*, Sterile alpha motif domain containing 9	*Samd9L*$^{-/-}$
Hyperphosphatemic familial tumoral calcinosis (HFTC)	Mineralization masses in skin	*KL*, Klotho, *GALNT3*, ppGaNTase-T3, *FGF23*, Fibroblast growth factor 23	*Klotho*$^{-/-}$, *Galnt3*$^{-/-}$, *Fgf23*$^{-/-}$
Multiple vitamin K-dependent coagulation factor deficiency	Vitamin K-dependent coagulation factor deficiency, PXE-like skin changes	*GGCX*, Vitamin K-dependent gamma-carboxylase	*Ggcx*$^{-/-}$
Keutel syndrome	Arterial and cartilage calcification	*MGP*, Matrix gla protein	*Mgp*$^{-/-}$