Therapeutic options in the treatment of ectopic calcification

PhD Thesis

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List of Abbreviations

4-PBA – sodium phenylbutyrate
hABCC6 – human ATP-binding cassette sub-family C member 6
ACDC – Arterial calcification due to deficiency of CD73
ACP – amorphous calcium phosphate
AD – autosomal dominant
AHSG – α2-HS-glycoprotein or fetuin
ANKH – Progressive ankylosis protein homolog
AMP – adenosine-monophosphate
APS – adenosine-phosphosulphate
AR – autosomal recessive
ATP – adenosine-triphosphate
bFcRn – bovine neonatal Fc receptor
BSA – bovine serum albumin
CMD – craniometaphyseal dysplasia
CNV – choroidal neovascularization
CTAD – anticoagulant mixture of 0.11 M citrate, 15 M theophylline, 3.7 M adenosine, and 0.198 M dipyridamole
CTX – cardiotoxin from Naja pallida venom
DC – dystrophic calcification
DCC – dystrophic cardiac calcification
DPBS – Dulbecco’s phosphate-buffered saline, with 0.9 mM CaCl₂ and 0.5 mM MgCl₂
EC – extracellular
ECM – extracellular matrix
ENPP1 – Ectonucleotide Pyrophosphatase/Phosphodiesterase 1
FA – Freund’s adjuvant
FOP – Fibrodysplasia ossificans progressiva
GACI – Generalized Arterial Calcification of Infancy
HAP – hydroxyapatite, \( \text{Ca}_3(\text{PO}_4)_2(\text{OH}) \)
HAT – hypoxanthine-aminopterin-thymidine medium
HEK – Human Embryonic Kidney 293 cells
HGPS – Hutchinson–Gilford progeria syndrome
HO – heterotopic ossification
HTVI – hydrodynamic tail vein injection
IP – intraperitoneal
K₂H₂P₂O₇ – dipotassium pyrophosphate
K₃EDTA – K₃-ethylenediaminetetraacetic acid
KI – knock-in
KO – knockout
MDCKII – Madin-Darby Canine Kidney II cells
MGP – matrix Gla protein
mAb – monoclonal antibody
µ-FTIR – μ-Fourier-transform infrared microspectroscopy
Na₄P₂O₇ – tetrasiom pyrophosphate, Na₄P₂O₇
Na₂H₂P₂O₇ – disodium pyrophosphate, or sodium acid pyrophosphate, Na₂H₂P₂O₇
NHO – neurogenic heterotopic ossification
pAb – polyclonal antibody
Pᵢ – inorganic phosphate
POH – progressive osseous heteroplasia
PPᵢ – inorganic pyrophosphate
PXE – pseudoxanthoma elasticum, previously known as Grönblad–Strandberg syndrome
SSc – systemic sclerosis
TBI – traumatic brain injury
TC – tumoral calcinosis
TIC – trauma-induced calcification
TNAP – tissue-nonspecific alkaline phosphatase
ttw – tiptoe walking mouse, previously known as twy
wt – wild type
Introduction

This work aims to give a brief introduction to the mechanisms underlying physiological and pathological calcification in living organisms, focusing on rare genetic diseases and the perspectives their research offers. The pathomechanism and potential therapies of *pseudoxanthoma elasticum* (PXE) are discussed in detail, including a personalized approach based on individual patient mutations, and a more generalized option aimed at compensating low pyrophosphate levels observed in patients. These are followed by examples of how the knowledge gained may be applied to other, more prevalent ectopic mineralization diseases: heterotopic ossification induced by trauma and scleroderma.

Chapter 1. Mineralization in sickness and health

“One might ask whether Lot’s wife, in turning back to watch fire and brimstone consume everything she valued, experienced an acute imbalance between phosphate and pyrophosphate that led to her sudden and tragic transformation into a pillar of salt.”

*Michael A. Levine* [1]

Mineralization occurs at several locations in our bodies as part of a physiological process, however when it happens in tissue types that normally do not calcify, or in excessive amounts, we refer to this as ectopic calcification. The phenomenon of biomineralization is an age-old mystery: what makes calcification of our bones possible, and, once mineralized, what is preventing them from dissolving?

The high concentration of phosphate and calcium ions - building blocks of the mineral hydroxyapatite (HAP) - in our extracellular fluid suggests that we may have been asking the wrong question: instead of what could enable mineralization, we should seek the answer to what prevents calcification of soft tissue in our bodies in general. The main inorganic constituents of our skeleton are present in the extracellular fluid at such high concentrations that - in accordance with the basic laws of thermodynamics - these should spontaneously precipitate, forming insoluble mineral deposits of hydroxyapatite [2]. This has been described as ‘Lot’s wife’s problem’ by *Neuman* 40 years ago [3],
suggesting an analogy that compared our living under the constant threat of mineralization to the story of the biblical Lot's wife, who, looking back at the burning city of Sodom, turned into pillar of salt. Yet the fact remains that unaware of what the solubility equation dictates, we do continue to live on, without homeostatic ion concentrations turning us to stone.

In the light of our continued existence as living, breathing organisms, one must assume our bodies are equipped with the necessary anticalcification mechanisms to counteract the basic laws of thermodynamics. Gaining a more complex understanding of the process of biomineralization had at least partially resolved this apparent paradox. Calcium and phosphate are far from being the only inorganic components of serum; the presence of physiological saline and many other ions can substantially influence the solubility of calcium salts. Neuman's problem named after Lot's wife has been resolved by the work of Fleisch and Bisaz, who described a mystery inhibitor present in human plasma which can protect collagen fibrils from calcification. This inhibitor turned out to be inorganic pyrophosphate (PPi), a very simple, ubiquitous molecule capable of inhibiting HAP precipitation in our bodies [4] [5].

It is also worth noting that when calcium and phosphate do precipitate, they initially form amorphous calcium phosphate (ACP) [6] [7], not crystalline hydroxyapatite. Precipitation of ACP requires much higher concentrations [8], and is reversible under physiological conditions. Even if solubility is momentarily exceeded, binding to serum proteins like α2-HS-glycoprotein (AHSG, also known as fetuin) can keep these colloidal mineral particles in solution [9]. If they do persist, with time, these various [Ca^{2+}] x [P] salts are eventually converted to the more stable, insoluble hydroxyapatite that is the major component of our bones. This may explain how spontaneous soft tissue calcification is prevented, while the mineral content of our skeleton exhibits relatively long-term stability in vivo.

Even though mineralization - be it ectopic or physiological - can happen spontaneously, in the in vivo environment it is often not a passive process. Many of our cells have the ability to shape their immediate microenvironment in a way that fundamentally influences calcification. Osteoblast cells in our bones, or vascular smooth muscle cells that undergo osteochondrogenic differentiation can actively facilitate
mineralization of the arteries [10], [11], while osteoclasts have the ability to dissolve hydroxyapatite and break down the organic component of the bone matrix during the process of remodeling. They achieve this by altering local concentrations of calcification-enhancing or inhibiting mediators: as an example, tissue-nonspecific alkaline phosphatase (TNAP) is an ectoenzyme expressed by cells of bone and calcifying cartilage, which can break down calcification-inhibiting pyrophosphate to form two phosphates [12].

Nevertheless, as a general rule, systemic high concentrations of calcium, or phosphate (sometimes expressed as an elevated Ca x P product) favor ectopic mineralization, while low levels have been documented in rickets. However, calcium, phosphate and their product is far from the complete picture, and attributing mineralization or lack thereof solely to their balance would be an oversimplification. A plethora of human diseases have symptoms of excessive calcification, many of which affect people who have normal Ca x P product; as an example diabetes, or even aging itself may be accompanied by soft tissue calcification, without detectable elevation in Ca x P product [8]. Traditionally, ectopic calcification diseases have been categorized as either metastatic calcification or dystrophic calcification.

Metastatic calcification occurs in patients having elevated calcium or phosphate levels (high Ca x P) [13]. The term ‘metastatic’ does not necessarily indicate a connection to malignant tumors, it merely refers to the calcification symptoms typically appearing in otherwise healthy tissues or organs, while the cause may originate somewhere else in the body. Examples include hyperparathyroidism, and end-stage renal disease, which can cause calcification in many different types of soft tissue, including the blood vessels, lungs, and the heart [13],[14]. A rare and often fatal form of metastatic calcification is calciphylaxis, in which circulation in the small arteries is blocked by a combination of fibrosis, calcification in the middle layer of the blood vessels, and thrombosis, resulting in ischemic necrosis of the affected skin and subcutaneous tissue [15].

Vitamin D elevates the levels of phosphate and calcium in the circulation, and excessive dosing with vitamin D has been demonstrated to result in dramatic calcification in experimental animals [16]. In humans, rare cases of vitamin D poisoning
or toxicity from the excessive use of calcium-carbonate containing antacids can also cause substantially elevated serum calcium levels, which in turn can lead to metastatic calcification in the kidneys, arteries, or (in rare cases) the soft tissue surrounding joints. The latter is termed *tumoral calcinosis*, which again has no relation to neoplasia; the term refers to solid depositions of calcium without the presence of any dividing cells. Tumoral calcinosis occurs often in patients who have high serum phosphate (which itself may result from either dietary intake or predisposing genetic conditions), and presents as globular masses of calcified material around joints, but not inside the joint capsule [17].

Metastatic calcification is typically slow-progressing (with the exception of calciphylaxis), and in some cases may remain entirely asymptomatic. Treatment is aimed at normalizing the underlying metabolic imbalance.

**Dystrophic calcification** on the other hand can often be quick to develop, and is a consequence of devitalized or necrotic tissue's natural propensity for mineralization. Simple calculations on the solubility of calcium-phosphate precipitates assume homogeneous nucleation in a water-based solution. However, the *in vivo* environment introduces more complexity, as heterogeneous nucleation, on an organic base, such as collagen or elastin, is favorable at much lower concentrations. Debris resulting from necrosis is an excellent substrate for crystal nucleation, as the phenomenon of dystrophic calcification indicates. Synthetic materials or devitalized tissue found in implants, such as prosthetic aorta and valve replacements are no exception [18]. This remains the case even if cells are excluded from the microenvironment, illustrating that while osteogenic cells may facilitate mineralization, their presence is not absolutely necessary, as in some cases calcification can occur in their absence [19].

A classic example of dystrophic calcification is vascular calcification, which may refer to medial or intimal mineralization, the former most commonly observed in diabetes and renal failure, and the latter being a hallmark symptom of *atherosclerosis*. It is important to note that intima and media calcification are not mutually exclusive, and it is possible to observe both in a patient. As the average life expectancy increases, so does the probability of developing one or more of the chronic diseases that have...
calcification symptoms, and the number of affected individuals worldwide is expected to increase.

Mineralization of atherosclerotic plaques is common in affected patients and was thought to directly contribute to mortality by increasing the incidence of plaque rupture and thrombosis. The presence of coronary artery calcification is generally considered a marker of poor prognosis, even though long-term lipid lowering (statin) treatment is known to increase calcification [20],[21], despite being effective at reducing cardiovascular disease-related mortality. A possible mechanism behind this statin-related ‘calcium paradox’ is that microcalcifications may in fact stabilize these plaques, and improve survival.

On the other hand, media calcification which is also known as Mönckeberg's sclerosis typically does not cause luminal narrowing of the affected arteries [22]. The mineralization in this case affects the elastic fibers of the media, which reduces the elasticity of affected arteries, as typically seen in the rare genetic disease pseudoxanthoma elasticum (PXE). Dystrophic calcification symptoms however are not restricted to the circulation, and in fact may appear in several tissue types.

Traumatic injuries can induce dystrophic calcification in skeletal muscle or connective tissue. In some cases, this may eventually progress to form persistent, histologically mature extraskeletal bone. This is termed heterotopic ossification (HO). It typically affects skeletal muscle, and is initially triggered by major trauma (combat-related injuries, accidents, burns or complications of orthopedic surgery). The incidence of HO increases when the trauma to the skeletal muscle occurs simultaneously with an injury of the central nervous system (spinal cord or brain). In addition to this acquired form, in rare cases HO can also develop as a consequence of a genetic condition [23]. Once mature, extraskeletal bone will not be reabsorbed, it remains stable in size, or may even grow, restricting movement and becoming a significant burden to patients.

Dystrophic calcification is also the most common cause of calcinosis cutis, ectopic mineralization developing in the skin. Calcinosis cutis affects 25-40% of systemic sclerosis patients [24], but it can also occur in dermatomyositis and systemic lupus erythematosus (SLE). Debris originating from necrotic cells destroyed by autoimmunity
serve as the substrate on which the precipitation of calcium is initiated. Calcinosis universalis is a more widespread form, which presents as extensive calcifications in the skin, connective tissue and muscle [25]. Little is known about the pathological processes resulting in cutaneous calcinosis of systemic sclerosis patients, and consequently, therapeutic options are limited.

Chapter 2. Lessons learned from rare diseases

*Exceptio probat regulam in casibus non exceptis.*

To gain a better understanding of the driving forces and inhibitors of mineralization in our bodies, it is worth taking a look at what happens when homeostatic conditions are not met. Investigating the significance of any metabolite or protein implicated in the physiological process of calcification is perhaps the easiest in the context of disease, since the symptoms present in the absence of a molecule will give away a lot about its function. Research of rare calcification diseases with a known genetic cause has shed some light on the most important players in the complex network regulating physiological calcification, and hopefully will get us one step closer to finding a cure for these.

A plethora of rare genetic diseases have a phenotype that includes calcification symptoms, for the sake of brevity and simplicity this work will focus on those that present with ectopic calcification as the sole or most pronounced symptom (see Table1). In each case, disease-specific ectopic calcification symptoms are summarized, and, if possible, their link to the identified causal mutation(s) are explored in brief. The rare ectopic calcification diseases with particular relevance to this work (pseudoxanthoma elasticum, generalized arterial calcification of infancy, and craniometaphyseal dysplasia) will be presented in more detail.

---

1 In accordance with Henry Fowler’s favored interpretation, the presence of an exception applying to a specific case establishes that a general rule, from which the current case is exempt, does exist.
<table>
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<tr>
<th>Disease</th>
<th>Cause and (MOI)</th>
<th>Ectopic calcification phenotype</th>
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<tr>
<td>Pseudoxanthoma elasticum (PXE)</td>
<td>ABCC6 deficiency* (AR)</td>
<td>Mineralization of elastic fibres in skin, eyes, and arteries [26].</td>
</tr>
<tr>
<td>Generalized arterial calcification of infancy (GACI)</td>
<td>ENPP1 deficiency* (AR)</td>
<td>Early onset widespread mineralization of arteries, and to a lesser extent joints [27].</td>
</tr>
<tr>
<td>Arterial calcification due to deficiency of CD73 (ACDC)</td>
<td>CD73 deficiency (AR)</td>
<td>Adult onset mineralization of arteries in the lower extremities, and small joints of hands and feet [28].</td>
</tr>
<tr>
<td>Craniometaphyseal dysplasia (CMD)</td>
<td>ANKH deficiency, (AD) or GJA1 deficiency (AR)</td>
<td>Progressive diffuse hyperostosis of cranial bones [29].</td>
</tr>
<tr>
<td>Hutchinson–Gilford progeria syndrome (HGPS)</td>
<td>Progerin, a mutant form of lamin A (AD)</td>
<td>Premature aging, atherosclerosis and calcification of blood vessels and the aortic valve [30].</td>
</tr>
<tr>
<td>Keutel syndrome (KTLS)</td>
<td>MGP (matrix Gla protein) deficiency (AR)</td>
<td>Facial abnormalities, cartilage and arterial calcification and hypertension [31].</td>
</tr>
<tr>
<td>Hyperphosphatemic familial tumoral calcinosis (HFTC)</td>
<td>FGF23 deficiency (AR) or GalNAcT3 deficiency (AR), or KLOTHO deficiency (AR)</td>
<td>Periarticular calcifications and high serum phosphate levels [17].</td>
</tr>
<tr>
<td>Normophosphatemic familial tumoral calcinosis (NFTC)</td>
<td>SAMD9 deficiency (AR)</td>
<td>Calcifying ulcerative lesions of the skin and mucosa [32].</td>
</tr>
<tr>
<td>Fibrodysplasia ossicinans progressiva (FOP)</td>
<td>ACVR1 gain of function (AD)</td>
<td>Progressive heterotopic endochondral ossification of skeletal muscle, fascia, tendons, and ligaments [23].</td>
</tr>
<tr>
<td>Progressive osseous heteroplasia (POH)</td>
<td>GNAS gene loss of function (AD)</td>
<td>Progressive heterotopic intramembranous ossification of dermal and connective tissue [23].</td>
</tr>
<tr>
<td>Idiopathic basal ganglia calcification (IBGC)</td>
<td>PIT-2, XPR1 or PDGF(R)B deficiency (AD)</td>
<td>Progressive symmetrical brain calcifications affecting the basal ganglia.</td>
</tr>
</tbody>
</table>

Table 1. **Ectopic calcification diseases with their causative genetic mutations and characteristic phenotypes**, modified from [33]. Mode of inheritance (MOI) is indicated in brackets: AR - autosomal recessive inheritance, AD - autosomal dominant inheritance. Asterisk (*) was added to indicate the considerable overlap between the observed phenotypes -- and the causal mutations -- of PXE and GACI [34].
Pseudoxanthoma elasticum

Pseudoxanthoma elasticum (PXE, OMIM:264800) is a rare, recessively inherited ectopic mineralization disease. Symptoms generally manifest in children or young adults, typically starting with the formation of yellowish papules (pseudoxanthomas) in the skin of flexural regions and the side of the neck. These are caused by fragmentation and progressive dystrophic calcification of elastic fibers in the dermis; in advanced cases redundant folds of lax skin may develop (see Figure 1).

![Fig. 1. Skin manifestations of PXE. Lax skin (A), pseudoxanthomas (B), and their combination with severe loss of skin elasticity (C) at the side of the neck of three PXE patients homozygous for the mutation c.3775del (p.W1259fs) [35].](image)

Ophthalmological symptoms tend to manifest after the appearance of skin lesions. These stem from PXE’s effect on the Bruch’s membrane, an elastin-rich layer of the choroid which separates the small choroidal blood vessels from the retina. In PXE patients, the Bruch’s membrane initially develops spots resembling orange skin (*peau d’orange*), then angioid streaks, which correspond to cracks in this barrier (see Figure 2). These themselves cause no symptoms, but they allow blood vessels to penetrate the retina, resulting in choroidal neovascularizations (CNVs). These have serious consequences, as such newly formed vessels are fragile and break easily. Frequent retinal hemorrhages cause central vision loss, which can develop very rapidly in PXE patients. Among PXE patients older than 50 years, 37% were found to have some amount of visual impairment, and 15% of them were legally blind [36]. The initial trigger of this pathological process is the calcification and thickening of Bruch’s membrane, and
the end result is damage to the macula and severely reduced visual acuity [37]. Visual acuity generally declines with increasing age, but in PXE patients this occurs at an accelerated rate. Severe ophthalmological symptoms of PXE can be managed by intravitreal anti-VEGF injections, which can prevent further neovascularization events.

Fig. 2. Ophthalmological manifestations of PXE. (A) Peau d'orange (A), angioid streaks (B) subretinal hemorrhage (C) and scarring (D) indicated by white arrows in fundoscopy images of affected patients [38].

The dystrophic calcification does not spare the elastic fibers of blood vessels either, and vascular calcification of the media layer of arteries occurs frequently in PXE patients. It most commonly affects the medium-sized arteries of the lower extremities, and consequently, many patients experience intermittent claudication (aching or painful cramping in the legs following exercise). This is due to insufficient circulation of the affected muscles, and is quickly relieved by rest. The sclerosis of the media in these
arteries typically does not result in luminal narrowing, but it reduces the elasticity of the vessel wall, increases arterial stiffness, making these patients prone to vascular disease, and especially peripheral arterial disease [39] [40]. Gastrointestinal bleeding has also been documented to occur in some patients [41]. Mönckeberg’s sclerosis has a unique railroad-track appearance on radiographs (see Figure 3A), with continuous depositions of calcified material in the vessel wall.

The symptoms of PXE are thought to be a direct consequence of fragmentation and ectopic calcification affecting the elastic fibers in the skin, the media layer of the arteries and the Bruch’s membrane in the eye. The prevalence of the disease is estimated to be 1:50,000 [26]. Patients typically have a normal life span, but the disease has substantial adverse effects that have a negative impact on their quality of life.

PXE is caused by mutations in the ABCC6 gene [42]. ABCC6, also known as multidrug resistance-associated protein 6 (MRP6) belongs to the ABCC family of multidrug resistance transporters, which consists of 13 members. Apart from the lack of an N-terminal TMD0 domain in ABCC4, ABCC5, ABCC7 (CFTR), ABCC proteins share a similar membrane topology [43],[44] (see the schematic representation in Figure 20A), verified by the cryo-EM structures of family members CFTR, Abcc1 and ABCC8/SUR1 [45],[46],[47]. ABCC6 belongs to the long type ABCC transporters as it has a TMD0 transmembrane domain in addition to the TMD1 and TMD2 domains and two nucleotide binding (ABC) domains.

The transporter is expressed in the liver, in the basolateral membrane of hepatocytes [48],[49],[50] and to a lesser extent in the kidneys, but the characteristic symptoms of PXE do not affect these organs. Establishing the connection between a systemic calcification phenotype and a protein in the unaffected liver had not been an easy feat [26], yet the explanation is relatively simple to understand: calcification occurs because in the absence of ABCC6, hepatocytes fail to release a metabolite that travels in the circulation to prevent calcification elsewhere in the body. This elusive metabolite turned out to be ATP, even though the physiological substrate of ABCC6 continues to evade discovery to this day. Despite having clear evidence supporting nucleoside triphosphate (mainly ATP) release from ABCC6 expressing cells, and no ATP release from ABCC6 negative cells [45], it is still not known whether ABCC6 itself transports
nucleoside triphosphates, or another ABCC6-dependent mechanism facilitates nucleoside triphosphate release. ATP in the circulation is cleaved to form AMP and the mineralization inhibitor PP\textsubscript{i} by the ubiquitously expressed ENPP1 enzyme (see Figure 3B). As a consequence of their ABCC6 mutations, PXE patients have reduced levels of PP\textsubscript{i} in their circulation, and the same is true for Abcc6\textsuperscript{-/-} mouse models of the disease [26]. The role of PP\textsubscript{i} in PXE and mineralization disorders in general will be discussed further in the introductory Chapters, as well as the Aims section.

Fig. 3. *Ectopic calcification in PXE, and the molecular pathway behind.* CT scan image reveals mineralization in the affected femoral and subpopliteal arteries of a PXE patient (A) [40]. Calcification in PXE is due to mutations that decrease extracellular ATP release via ABCC6 (B): the cleavage of this ATP by ENPP1 within the liver vasculature gives rise to AMP and the mineralization inhibitor PP\textsubscript{i} in the circulation. Decreased ABCC6 function results in reduced extracellular ATP and PP\textsubscript{i} levels, and the precipitation of mineralized deposits.
Abcc6<sup>-/-</sup> mice were generated on a C57/Bl6 background, and found to have normal lifespan and fertility. However they do develop late-onset spontaneous calcification symptoms affecting their blood vessels, first appearing in the kidney cortex at the age of 6 months. Later on these become apparent in other tissue types too, including the aorta and in the Bruch's membrane [51]. Skin symptoms typical in human patients do not appear in Abcc6<sup>-/-</sup> mice, but they develop histologically apparent calcification in the connective tissue surrounding their whiskers (vibrissae) [52].

Many PXE patients harbor at least one missense ABCC6 allele [43],[49], and some of these encode transport-capable proteins that have folding/trafficking issues, resulting in aberrant subcellular localization. Treatment with chemical chaperones, like 4-phenylbutyrate (4-PBA) may enhance the stability and augment trafficking of such mutant proteins, correcting localization and thereby restoring physiological function, provided the protein is, in fact, transport-capable.

4-PBA is an FDA approved drug in the treatment of urea cycle disorders, and it has been demonstrated to have chemical chaperone effect in another ABCC transporter-related disease: it can restore the function of mutant CFTR proteins which cause cystic fibrosis. Previous in vitro and in vivo [34] experiments by our group have indicated that 4-PBA therapy may achieve this in the case of ABCC6 mutations. As PBA can help these proteins reach the basolateral membrane, it may also restore their calcification-preventing function. We aimed to investigate this by analyzing six PXE-causing missense ABCC6 mutations (see Aims I.A.2).

We have established that in the case of these missense variants, subcellular localization is key, however, how much of the produced protein actually appears in the basolateral membrane is not always easy to determine. Specific antibodies are essential tools in research, and several well-characterized antibodies developed for the specific detection of ABCC6 exist, however, they all react with intracellular epitopes [48],[53],[54]. All ABCC-subfamily members have characteristically short extracellular (EC) loops, and this has precluded the generation of antibodies against these epitopes, even though these could be valuable reagents to determine the subcellular localization of mutants. We attempted to create a mAb capable of this, using transgenic animals with a uniquely augmented immune response (see Aims I.A.1).
From a clinical perspective, PXE symptoms are very variable and the disease course is difficult to predict. Patients are known to have decreased plasma PP$_i$ levels compared to healthy controls [55], however, individual differences exist, and whether lower plasma PP$_i$ levels would be reflected by a more severe manifestation of disease is not known. Furthermore, whether differences in the plasma PP$_i$ of patients are due to their ABCC6 genotype, with mutations that result in a complete lack of ABCC6 expression causing a steeper decline in plasma PP$_i$ is also unknown. Section II.1 of Aims details our approach to answering these questions.

**Generalized arterial calcification in infancy**

As opposed to PXE, GACI (OMIM:208000) presents much earlier: typically shortly after birth or in utero. The ectopic calcification phenotype is also much more severe (see Figure 4): blood vessels have extensive mineralized deposits in their walls, and their lumen is obscured by intimal hyperproliferation, causing circulation problems, heart failure, and extremely high blood pressure, the complications of which result in the early death of about half of all affected infants in the first six months of life [56],[57]. In addition to vascular symptoms, some patients also develop periarticular calcifications, and hypophosphatemic rickets secondary to altered phosphate metabolism: survivors of GACI have increased urinary excretion and decreased levels of serum phosphate.
Fig. 4. *Typical symptoms of GACI.* Echocardiograms showing aortic calcifications (arrows) in the walls of the aortic arch and descending aorta (A), aortic root (indicated by arrowhead) and left (LCA) and right (RCA) coronary arteries (indicated by arrows) (B), on a CT scan image showing the abdominal aorta and celiac trunk (C), periarticular calcifications of the joints in the hand from a 1-week-old GACI patient (D). Periarticular calcifications (shown by arrows) of both shoulders on a chest x-ray image (E), and cross-section of the aorta displaying media calcification (arrows) and severe hyperproliferation of the intima (arrowheads) (F) [56].
GACI is an autosomal recessive disease caused by mutations in the ENPP1 gene [27]. ENPP1 (phosphodiesterase nucleotide pyrophosphatase) is a type II transmembrane protein; an ectonucleotidase enzyme expressed in many tissue and organ types, including the liver. It cleaves extracellular nucleoside triphosphates, preferentially ATP released from cells (by ABCC6-dependent or independent pathways) to AMP and PP$_i$, which then acts as a potent antagonist of biomineralization. The complete lack of this endogenous calcification inhibitor is responsible for the severe ectopic mineralization symptoms observed in GACI (see Figure 5).

Fig. 5. Disease mechanism of GACI. Due to mutations affecting ENPP1, generation of mineralization inhibiting extracellular PP$_i$ from ATP is not possible, and severe ectopic calcification characteristic of the disease results.
ENPP1 is ubiquitously expressed, and this includes cells in calcifying tissue, like osteoblasts, chondrocytes and matrix vesicles originating from them. The phosphodiesterase nucleotide pyrophosphatase family itself has three members; ENPP1 (also known as PC-1), ENPP2 (also known as ataxin) and ENPP3 (B10), however, ENPP1 is the only one capable of increasing extracellular PPi levels [58]. Its unique activity is reflected by extracellular pyrophosphate being virtually absent in the circulation of humans (and mice) suffering from ENPP1 deficiency [59],[60].

Mouse models of GACI have contributed substantially to our understanding of the disease. These include the *tiptoe walking* (*ttw*) mice (originally called *twy* [61]), named after the unusual gait they develop due to periarticular calcification and subsequent fusion of peripheral joints. *ttw* mice are natural mutants that carry a Glycine 568 to STOP mutation, resulting in truncated Enpp1 protein [62]. As a consequence, they have severely depleted PPi levels and develop generalized ectopic calcification at a very young age, similar to GACI patients. The calcification phenotype of the human patients and the animal model are remarkably similar, yet some notable differences exist: the earliest and most pronounced symptom affecting *ttw* mice is periarticular calcification progressing to ankylosis; and instead vascular symptoms dominate in humans. In GACI patients, extensive and often lethal vascular calcification is first to develop, accompanied by intimal hyperproliferation and extreme high blood pressure, which are not the typical symptoms in *ttw* mice [63]. While these animals do develop severe vascular calcification affecting the arterial media, this is not the cause of their high mortality. Instead, the most prominent phenotype is joint calcification and ankylosis resulting in restricted movement, weight loss and general failure to thrive.

It is also worth noting that 75% of GACI patients have causal mutations in their *ENPP1* gene [56], but not all of them; and Nitschke *et al.* found that 14 of 92 GACI patients harbored no mutation in their *ENPP1*, but instead had monoallelic or biallelic mutations in their *ABCC6* gene [34]. Careful observation of the clinical presentation of GACI patients revealed PXE-like skin changes and ocular symptoms in 3 cases, suggesting that the phenotype of these two diseases have some overlapping features. The two diseases share an intertwined pathomechanism, since ENPP1 is in the same biochemical pathway, downstream of ABCC6. Its deficiency generally results in a more dramatic
decrease of circulating PP\textsubscript{i}, since it is the only enzyme capable of cleaving nucleoside triphosphates to generate extracellular PP\textsubscript{i}. On the other hand, mutations of ABCC6 would not result in such severe reduction of circulating PP\textsubscript{i}, since ENPP1 activity persists, and this enzyme may gain nucleoside triphosphate substrates from non-ABCC6 dependent sources.

The correlation between the observed decrease in the plasma PP\textsubscript{i} levels and the severity of symptoms in PXE and GACI suggest that low plasma PP\textsubscript{i} might have a causal role in the development of ectopic calcification, and that the lower the observed PP\textsubscript{i} levels, the more severe symptoms we should expect. While this may be true in general terms (GACI patients show a more drastic reduction in PP\textsubscript{i} than PXE patients, and the resulting phenotype is more severe), the rule may not be without exceptions: the severity of ectopic calcification in PXE varies widely between patients, and so does PP\textsubscript{i} concentration in plasma, however, a correlation between the two is yet to be established.

GACI is very rare, and probably underdiagnosed: a 2014 review by Ferreira et al. [64] mentions only about 200 documented cases described in the literature. The observed mortality decreases significantly after the critical first few months [56], with several patients surviving to young adult age (and hopefully beyond). This may be attributable to adaptation; renal phosphate wasting to compensate for the imbalance in the PP\textsubscript{i}/P\textsubscript{i} ratio due to their extreme low PP\textsubscript{i} levels, or possibly the success of experimental treatment by various non-hydrolyzable PP\textsubscript{i} alanolgues.

**Arterial calcification due to CD73 deficiency**

Arterial calcification due to deficiency of CD73, ACDC for short, or by another name, Calcification of joints and arteries (CALJA, OMIM: 211800) is another recessive Mendelian disease with a cause relating to extracellular PP\textsubscript{i} metabolism. Patients develop calcification affecting the media layer of arteries in their legs, and the periarticular regions of their hands and feet [65]. CD73 is an ectoenzyme encoded by the NT5E gene, which cleaves adenosine-monophosphate (AMP) to adenosine and P\textsubscript{i}. The resulting adenosine is an inhibitor of tissue-nonspecific alkaline phosphatase (TNAP), which, in turn, would catalyze the breakdown of PP\textsubscript{i} to two P\textsubscript{i} (see Figure 6).
Fig. 6. **ACDC**. (A) Radiograph displaying characteristic bilateral vascular calcification affecting the femoral and popliteal arteries of a 54-year old female ACDC patient [65]. (B) shows the proposed mechanism of how CD73 deficiency contributes to the calcification symptoms observed in ACDC patients by increasing the extracellular $P_i/PP_i$ ratio.

Modified from [65].

In mouse models of CD73 deficiency, plasma $PP_i$ is reduced, and $P_i$ is elevated, resulting in an altered $P_i/PP_i$ ratio that favors ectopic mineralization. These animals exhibit similar joint calcification symptoms to those observed in ACDC patients, but lack the characteristic arterial calcification symptoms in their lower extremities [66].

**Craniometaphyseal dysplasia**

Craniometaphyseal dysplasia (CMD) is a rare genetic calcification disorder affecting the skeleton; it causes hyperostosis of the craniofacial bones and flaring metaphyses of the long bones (affected femurs are described as ‘club-shaped’). This latter aspect of the phenotype is also referred to as Erlenmayer flask deformity, as the typical concave curve at the distal metaphysis of the femur is absent (see Figure 7).

Symptoms become apparent in early infancy, and they progress throughout the patients’ lives [67]. The thickening of craniofacial bones may eventually obstruct the
foramina and compress facial nerves, resulting in headaches, hearing and visual impairment, and facial palsy.

Fig. 7. **CMD phenotype.** Thickened craniofacial bones of 3-year-old CMD patient (A) and metaphysis of the femur exhibiting characteristic widening resembling Erlenmeyer flask shape (B) [29].

Curiously, CMD can be a dominant or a recessive disease (this distinction is sometimes indicated with the abbreviations CMDD or CMDR), with different causal mutations behind their similar symptoms. The dominant form of CMD (OMIM 123000) is caused by inactivating heterozygous point mutations which cluster mostly in the C terminus of the progressive ankylosis gene (ANKH)\(^2\). This form is more common, and generally has milder symptoms. The recessive form on the other hand (OMIM 218400) is caused by mutations in the *GJA1* gene encoding a gap junction connexin, CX43 [69]. The phenotype tends to be more severe, but this form of CMD is mercifully rare compared to the dominant form. Different mutations affecting CX43 are implicated in a plethora of rare genetic diseases affecting bone, and the precise mechanism of how these result in the specific symptoms observed is still largely a mystery.

\(^2\) There is a single consanguineous family described in the literature, where a recessive ANKH mutation is identified as the cause of a distinct calcification phenotype, with concurrent severe mental retardation. The mutation identified was L244S [68]. It results in a non-functional transporter that has normal subcellular localization.
The AD form of CMD is one of the calcification diseases addressed in more detail in this work: Ank is the mouse ortholog of ANKH, and ank/ank mice lacking the functional transporter can be considered an appropriate, albeit somewhat imperfect model of the human disease CMD. The protein Ank was hypothesized to be a PP\textsubscript{i} transporter which controls calcification by shuttling pyrophosphate created inside the cells to the surrounding extracellular space. This was based on the observation that Ank expressing cells release PP\textsubscript{i} into their growth media, while Ank negative cells fail to do so [70]. While this observation stands true, recent work by Szeri et al. has revealed that PP\textsubscript{i} is in fact not the substrate transported by ANKH. Instead, it releases citrate and nucleoside triphosphates, including ATP from cells, which ATP in turn is cleaved by ENPP1 on the cell surface to give rise to the calcification-inhibiting pyrophosphate detected in the culture media [71].

Since Ank function in ank/ank mice is completely lost, the resulting calcification phenotype is dramatic: intraarticular HAP deposits form and grow, eventually culminating in joint fusions in limbs and axial skeleton. Digits are affected first: at the age of weaning, mice are unable to grasp bars of the cage top due to calcification of the metacarpophalangeal joints, their mobility is progressively lost until complete immobilization is reached by the age of six months [72].

CMD patients do not share these symptoms, as joint stiffness due to articular calcification is not a hallmark of the disease, and ank/ank mice also lack some characteristic phenotypic features of CMD, like the flaring metaphyses of long bones. To address these discrepancies, a knock-in mouse model was created, carrying a human CMDD-associated point mutation, Phe377del. These ank\textsuperscript{K/K} animals develop CMD-like skeletal abnormalities with an early onset (1st week of life), but later on the severe joint calcification symptoms similar to ank/ank mice also appear. Heterozygous Ank\textsuperscript{+K/K} mice have a milder intermediate phenotype, with young animals appearing healthy. As they age, they develop variable degrees of skeletal symptoms reminiscent of CMD. ank\textsuperscript{K/K} mice represent a more accurate model of the human disease, even though CMD in humans has AD inheritance; this may be due to species-specific differences, which can substantially impact the observed phenotype [73].
Hutchinson–Gilford progeria (HGPS)

Hutchinson–Gilford progeria syndrome (OMIM:176670) is an autosomal dominant (or in rare cases recessive) disease characterised by premature aging, short stature, low body weight, hair loss, and severe cardiovascular and cerebrovascular disease [30] [74]. Most cases arise as de novo mutations as all affected patients die at a very young age due to vascular complications.

The disease is caused by a mutation in the LMNA gene, resulting in a truncated lamin A protein called progerin. Knock-in mice expressing progerin (Lmna^{G609G/+} and Lmna^{G609G/G609}), which are animal models of HGPS, have been shown to have reduced ATP and pyrophosphate levels in their plasma, and injecting them with PPi, daily could ameliorate their vascular calcification symptoms [75]. ATP treatment alone could not improve aortic calcification or survival of Lmna^{G609G/+} mice, but combined with TNAP and ectonucleoside triphosphate diphosphohydrolase (eNTPD) inhibitors, it could elevate extracellular PPi levels, reducing calcification and improving longevity [76].

Keutel-syndrome (KS)

Keutel syndrome (OMIM: 245150) is extremely rare, and is caused by recessively inherited mutations in the MGP gene encoding matrix Gla protein. MGP is an extracellular matrix protein that inhibits calcification. MGP deficiency in Keutel syndrome causes progressive cartilage calcification, hearing loss, facial abnormalities, arterial calcification and hypertension [27]. KS is incurable, but some of its symptoms can be managed with treatment.

Familial tumoral calcinosis (TC)

As previously mentioned in the introductory chapter, tumoral calcinosis is a condition characterized by subcutaneous depositions of (often large) masses of solid calcified material in periarticular regions (see Figure 8). The calcified deposits may develop in areas affected by repetitive minor trauma, and they contain ACP and HAP [77]. The hereditary, familial forms of TC have a genetic predisposing cause in the background, and they may arise in the context of high, or normal serum phosphate [78].
Fig. 8. **Clinical manifestations of HFTC and NFTC.** A) CT image of a calcified deposit near the hip (indicated by arrow). B) Calcified tumor in the skin near the wrist in a young NFTC patient. Images taken from [78].

Hyperphosphatemic familial tumoral calcinosis (HFTC) is caused fibroblast growth factor 23 (FGF23) deficiency or resistance: recessive mutations of FGF23 gene (OMIM:617993), or GALNT3 gene (OMIM: 211900), or KL (OMIM:617994). FGF23 lowers phosphate concentrations in blood by decreasing renal reabsorption, and as a consequence, mutations that lead to inactivated FGF23 cause elevated phosphate levels. However, the same end result may arise without any mutation in FGF23.

GLANT3 encodes N-acetylglactosaminytransferase 3 (GalNAct3), which catalyzes glycosylation of FGF23 in the Golgi. This posttranslational modification increases the stability of FGF23, so inactivating mutations of GalNAct3 would again lead to decreased FGF23.

Another causal mechanism of HFTC is an inactivating mutation in the gene KL, encoding KLOTHO. KLOTHO acts as a coreceptor of FGF23 in the proximal tubule of the kidney, and as such, it is essential to its blood phosphate regulating effect [17].

Conversely, normophosphatemic familial tumoral calcinosis (NFTC) does not cause elevated serum phosphate, and appears to be even more rare. Generally, NFTC patients develop an inflammatory rash of affected skin and mucosa, which then progress to painful, ulcerating calcifying lesions. Affected patients harbor an autosomal recessive mutation in the SAMD9 gene [32]. The physiological function of SAMD9 remains elusive, as is how its deficiency contributes to the pathology of NFTC. Whether local or circulating PPi concentrations are altered in this disease is also not known.
**FOP and POH**

Fibrodysplasia ossificans progressiva (FOP, OMIM: 135100) and progressive osseous heteroplasia (POH, OMIM: 166350) are extremely rare, devastating diseases that cause progressive extraskeletal bone formation for which there is currently no treatment. They both show autosomal dominant inheritance.

FOP results from an activating mutation (e.g., R206H) of ACVR1, a bone morphogenic protein (BMP) receptor, and causes heterotopic ossification in muscles, tendons, and ligaments. The prevalence of FOP is estimated to be ~1:2,000,000, making it an ultra-rare disease with approximately 700 cases described so far. The first apparent phenotypic presentation is the congenital malformation of the great toes [79], while calcification symptoms typically manifest later during the first decade of life. The initial trigger is inflammation brought on by infection, or trauma, which can be as mild as small accidental injuries sustained during everyday life. Patients experience flares of disease, resulting in the formation of persistent ectopic bone that severely limits movement, eventually resulting in permanent immobility (see Figure 9). FOP is progressive and incurable; treatment consists of avoiding injuries and efforts to mitigate the damage caused by disease flare-ups [80].

![Fig. 9. The phenotype of FOP.](image)

Extensive heterotopic bone formed on a patient's back (A), and characteristic great toe malformations (B), which are apparent before the presentation of any other symptom. Images were taken from [81].
A subset of suspected FOP patients presented with atypical disease manifestations: without great toe malformations, markedly different patterns of HO and no mutations in their \textit{ACVR1} gene. They turned out to have a distinct disease that is even more rare, and was named progressive osseous heteroplasia. POH causes cutaneous ossification starting in the dermis, which progresses to involve deeper layers of connective tissue and muscle during childhood [82],[83] (see Figure 10). Skin calcification symptoms may be already present at birth, and inflammation does not appear to play a role in their development. Causal mutations are in the \textit{GNAS} gene encoding the alpha subunit (G,\textsubscript{\alpha}) of a heterotrimeric G-protein, that has a crucial role in cellular signalling, however, it is presently unclear what role G,\textsubscript{\alpha} deficiency plays in the observed clinical manifestation of POH [84].

![Fig. 10. The phenotype of POH. Cutaneous heterotopic ossification in the arm of a 2.5 year old girl (A) and radiograph of the same limb at 8 years of age (B) [83].]({})

Serum calcium and phosphate levels are normal in both diseases, whether plasma PP, is altered in either FOP or POH is unknown.
Idiopathic basal ganglia calcification (IBGC)

Idiopathic basal ganglia calcification, also known as Fahr disease, or primary familial brain calcification (PFBC), is a genetically heterogeneous syndrome associated with several mutations. Causes include mutations in the SLC20A2 gene, encoding PiT-2, a sodium-dependent phosphate transporter [85], mutations of XPR1, encoding a phosphate exporter [86],[87], or deficiency of platelet-derived growth factor β (or its receptor) [88]. The latter results in blood-brain barrier dysfunction, while the former two cause altered phosphate transport: they all lead to the development of symmetric intracranial calcium depositions, progressive deterioration of motor functions and neuropsychiatric symptoms [89].

PiT-2 imports $P_i$, while XPR1 transports it in the opposite direction: their mutations result in impaired transport function, and extracellular or intracellular nucleation of mineralized deposits, respectively. IBGC is progressive and intractable. Serum phosphate and calcium levels are generally normal in these patients [90]. Pyrophosphate levels in the central nervous system or plasma of these patients have not been determined.
Chapter 3. From PP\textsubscript{i} to bisphosphonates - and back

Several of the rare ectopic mineralization diseases discussed in the previous chapter point out the central role extracellular inorganic pyrophosphate plays in the regulation of calcification. PXE, GACI, ACDC, CMD and HGPS are linked directly to alterations in PP\textsubscript{i} metabolism, while others have no identified relation to systemic or local PP\textsubscript{i} levels according to our current knowledge.

As previously discussed, the profound anti-calcification effect of inorganic pyrophosphate has been established since the 1960s [4], and a 2016 review by Orriss et al. describes PP, as the body's natural water softener [91], inhibiting biomineralization by preventing the precipitation of calcium and phosphate, and halting the growth of HAP crystals. The balance of extracellular pyrophosphate and phosphate concentrations influences mineralization, and alterations in this ratio can facilitate or prevent calcification (see Figure 11).
Fig. 11. *Extracellular phosphate/pyrophosphate balance and ectopic mineralization.*

Extracellular PP$_i$ is present in micromolar concentrations, yet this is sufficient to counteract the pro-calcification effect of millimolar P$_i$ levels. Conditions that increase P$_i$ or lower PP$_i$ levels favor ectopic mineralization by altering this balance, while reducing P$_i$ or increasing PP$_i$ has the opposite effect. Inactivating mutations affecting *ABCC6*, *ENPP1*, *ANKH* and *NT5E* cause ectopic mineralization diseases PXE, GACI, CMD and ACDC, respectively, through decreased circulating PP$_i$ levels. High phosphate levels arising in the context of kidney failure, or due to HTFC caused by *FGF23* deficiency or resistance, or indeed excess dietary P$_i$ intake or vitamin D poisoning (which increases serum calcium and phosphate levels [92]), can also shift the balanced state of physiological mineralization conditions to excessive precipitation of calcium salts. Figure adapted from [93].

Based on our current understanding of the physiological calcification process, and our knowledge of the pathomechanism of the rare diseases described in Chapter 2, a tentative scheme of the causal mechanisms of ectopic mineralization may be drawn, as shown in Figure 12.
Fig. 12. **Soft tissue calcification and PP, homeostasis.** Local and systemic factors in the regulation of biomineralization: proteins in the membrane transport and hydrolysis of ATP, and the regulators of calcium and phosphate and pyrophosphate concentrations.

ANKH, and ABCC6 (in the liver) increase extracellular levels of ATP, which can be hydrolyzed by ENPP1 to generate AMP and the mineralization inhibitor PP\textsubscript{i}. TNAP in turn catalyzes the breakdown of PP\textsubscript{i} into two inorganic phosphate molecules, which facilitate calcification. AMP is cleaved to adenosine and P\textsubscript{i} by the enzyme CD73. The resulting adenosine inhibits the enzyme TNAP, blocking the hydrolysis of PP\textsubscript{i} to P\textsubscript{i}.

PiT-1 and PiT-2 (also known as SLC20A1 and SLC20A2) are sodium-dependent phosphate transporters with ubiquitous tissue expression patterns; they facilitate the uptake of P\textsubscript{i} by various cell types. PiT-2 and Annexin V can also be found on the surface of matrix vesicles in calcifying tissue, and the uptake of P\textsubscript{i} and Ca\textsuperscript{2+} via these transporters contributes to the nucleation of HAP inside these vesicles during the physiological bone formation process [94]. XPR1 is a phosphate exporter, and its mutations also result in impaired phosphate transport. Nucleation of mineralized deposits is possible inside of...
matrix vesicles as well as on the substrate of extracellular matrix components. HAP - hydroxyapatite, ECM - extracellular matrix. Figure adapted from [95].

It is reasonable to assume that the impact of PP_i extends further than rare heritable disease; and this has prompted investigations into the significance of pyrophosphate in pathologies affecting greater numbers. The most simple approach to this is measuring its concentration in urine or blood plasma samples taken from affected individuals and comparing them to healthy controls.

Determining extracellular PP_i in blood is technically challenging, because platelet activation can induce the release of massive amounts, which causes falsely high PP_i concentrations in the assay [96]. Plasma obtained by simple centrifugation of anticoagulated blood is not platelet-free, and therefore not suitable for reliably measuring extracellular PP_i. Plasma separator blood collection tubes, which work by filtration through an inert gel, have also been found to yield platelet-depleted, but not platelet-free plasma. Platelets would be easy to eliminate by triggering the coagulation cascade, but this inevitably results in their activation, which must be avoided. The solution to this problem is filtration through a 300 kDa molecular weight cut-off (MWCO) membrane filter that can exclude all platelets from the filtered plasma without activating them [97].

The challenges however do not end here, as the pyrophosphate concentration in the filtered sample has to be determined. The traditional, more cumbersome but extremely sensitive radioactive method [98], which allows the detection of as little as 20 pmol PP_i is replaced in many laboratories by less sensitive but more convenient fluorescence- or luminescence-based assays. Plasma concentrations of extracellular pyrophosphate are in the micromolar range, which makes any assay sensitive enough to yield reliable and reproducible results at such low concentrations a feasible alternative.

The luminescence-based PP_i assay our group uses to determine PP_i content of plasma samples relies on two enzyme reactions: (1) ATP sulfurylase enzyme converting the pyrophosphate in samples to ATP in the presence of excess adenosine-phosphosulphate (APS); and (2) a luciferin/luciferase reaction generating luminescent signal from the ATP arising in reaction (1) [99], [55]. This method is
sufficiently sensitive to determine PP$_i$ concentrations of human and mouse plasma samples, and can be performed in a 96-well plate system which makes it relatively high-throughput. Figure 13 displays the enzyme reactions of the luminescent PP$_i$ determination method.

![Diagram](image)

**Fig. 13. Luminescence-based pyrophosphate determination.** PP$_i$ content of samples is converted to ATP by ATP sulfurylase enzyme in the presence of excess adenosine-phosphosulphate (APS). ATP is then detected by a coupled luciferin/luciferase reaction (A). The luminescent signal of a PP$_i$ standard solution demonstrates the sensitivity of this assay method (B) [99].

Different assay techniques yield numerically different PP$_i$ concentration values which are difficult to compare if they were not obtained by the same plasma preparation and measurement method. Plasma PP$_i$ determination is not an approved diagnostic test, and will have to be refined and standardized before it can become one. Even the relatively simple filtration and luminescent PP$_i$-determination techniques are not available in most laboratories that carry out routine blood tests, and as a consequence, PP$_i$ is seldom if ever measured.

In spite of these obstacles, plasma PP$_i$ levels are probably worth investigating in any disease involving soft tissue, and especially vascular calcification. Pyrophosphate levels have been measured in end-stage renal disease patients undergoing dialysis, who
are particularly prone to vascular calcification in the form of Mönckeberg’s arteriosclerosis, and were found to be significantly decreased [100]. This may be the case in other calcification diseases too, and if so, this would indicate these patients may benefit from PP$i$ supplementation.

Bisphosphonates (BPs), which are non-hydrolyzable analogs of PP$i$ that were originally developed to treat osteoporosis, have been experimented with in the treatment of ectopic calcification diseases with varying degrees of success [101], [56], [102], [103], [104]. PP$i$ is hydrolyzed by ubiquitous phosphatases present in the intestine, and as a consequence, its bioavailability was thought to be negligible [105]; therefore delivering it orally to patients was considered unfeasible [91]. Some BPs in contrast have similar anticalcification effects, but do not break down in vivo, and this is due to their different structure: the two PO$_3$ (phosphonate) groups are linked through a carbon, in the place of which pyrophosphate would contain an oxygen (see Figure 14) [106].

![Fig. 14. The structure of etidronate (A), a first-generation bisphosphonate, and pyrophosphate (B).](image)

Various bisphosphonates have been used as experimental treatments to counteract the severe calcification symptoms of GACI [56], however, a 2021 paper on the natural history of the disease by Ferreira et al. found no survival benefit of BPs after matching cases for the age at treatment initiation in their analysis [57]. The time of starting treatment is of crucial importance, because GACI patients experience an extremely high risk of mortality in their first few months of life (critical period). Failure to account for this may make the analysis susceptible to survival bias, and overestimation
of therapeutic effect: those who survive longer during the critical period are more likely to receive treatment, and many of those who do receive BP treatment may have a reduced risk of mortality not due to treatment effect, but because they are older.

In addition, prolonged exposure to bisphosphonates may sometimes result in severe side effects. The cause of BPs’ toxicity is that they interfere with bone metabolism; their effects are not limited to direct physicochemical blocking of HAP crystal growth - they also have an inhibitory effect on osteoclasts. Low concentrations of BPs have an antiapoptotic effect on osteocytes and osteoblasts, while higher concentrations taken up by osteoclast cells inhibits their bone resorptive activity and can induce their apoptosis [107]. As a result, BP treatment is not without risks, and in very rare cases may cause dramatic side effects like osteonecrosis or osteopetrosis [108], [109].

BPs have also been tested in the treatment of PXE. The variable phenotype and disease progression rate in this disease complicates the interpretation of clinical trial results. The effect of etidronate has been tested on the progression of arterial calcification in the legs and CNVs, in randomized controlled trials including 74 and 71 patients, which found some reduction in the femoral calcium burden detected by CT scans compared to the controls [101], but no benefit in the case of CNV events [110].

Since PPi, the natural calcification inhibitor, was thought to have no bioavailability [91],[106], its effectiveness in PXE has never been tested. Our group challenged this assumption through preclinical models and PPi absorption studies, detailed in the following chapters. Pyrophosphate salts are generally safe to consume and are used as a food additive (E450(a) indicates the presence of a sodium salt of PPi); they can be found in processed meat and dairy products as well as toothpaste.

**Chapter 4. Beyond rare diseases**

Although we have gathered considerable knowledge about heritable calcification diseases, and have increasing numbers of the causative gene mutations identified, many unanswered questions remain, and we have a long way to go before a cure for these could be found.
Nevertheless it is important to stress that ectopic calcification itself is much more prevalent than the rare genetic diseases discussed in Chapter 2; it is an accompanying symptom (with major implications for the quality of life) in several pathologies that affect greater numbers. Many of these are considered to be heavily influenced by environmental factors, and do not have a known causative genetic component (although some associations between certain alleles and the likelihood of developing a disease have been observed). Calcification in these cases is a secondary consequence brought on by currently unknown metabolic or physiologic changes induced by the disease.

An example of these is **systemic sclerosis (SSc)**, a progressive connective tissue disease affecting the circulation, and resulting in damage to multiple organs including the skin. Roughly a quarter of patients develop calcinosis [111], dystrophic calcification affecting skin and subcutaneous soft tissue, most frequently in their hands. SSc is considered rare, but its prevalence of 1:6,500 [112] makes it more common than the previously discussed Mendelian diseases. It has two subtypes: a limited cutaneous (lc) and diffuse cutaneous (dc) SSc form. The diffuse form is generally accompanied by more severe pulmonary, renal and heart involvement than the limited form, in which the characteristic skin changes are restricted to the acral regions. Cutaneous calcinosis may occur in both forms, and these deposits consist predominantly of HAP [113]. Figure 15 displays a rather severe case of calcinosis affecting both hands of a patient.
Skin calcinosis in SSc is not just an aesthetic problem: calcifications are very painful, can limit movement, and may cause ulceration of the skin above, serving as a potential entry site for recurrent infections [115]. Currently there is no universally effective treatment for SSc, or calcinosis in SSc [116]. The underlying mechanism of the latter is poorly understood; precisely how and why SSc leads to ectopic calcification in a subset of these patients remains to be elucidated [95]. Animal models of SSc-related calcinosis are lacking, which makes any related research challenging. Whether a systemic or local decrease of the mineralization inhibitor PPi contributes to pathogenesis is an interesting question with potential therapeutic implications, and this will be discussed in more detail in this work.

Yet another example mentioned in Chapter 1 is trauma-induced dystrophic calcification, which is prevalent, and may result in the formation of heterotopic bone. The risk of developing HO is increased in patients who have suffered forearm (20%) or femoral shaft fractures (52%), severe burns (60%), or traumatic brain- or spinal cord injuries (11–20%). Following combat-related blast injuries and amputations, the frequency of HO is even higher, approximately 65% [117]. No specific therapy of HO
exists; non-steroid anti-inflammatory drugs or radiation therapy may be applied as prophylaxis, but once formed, HO can only be treated by physical therapy, or surgical removal of heterotopic bone. The latter in some cases proves ineffective due to a high chance of regrowth, especially if the excised bone tissue was not fully matured, or its removal was incomplete [118].

The driving forces of HO in humans are poorly understood, but animal models may help us gain some insight. Recent work by Moore-Lotridge et al. has confirmed that extracellular deposition of nanohydroxyapatite crystals, if their clearance is impaired, can serve as precursors of HO formation in skeletal muscle in a mouse model [119]. This suggests that if extracellular nanohydroxyapatite deposition is inhibited, this may prevent the formation of HO altogether. It also raises the question of whether PPi deficiency contributes to the formation of HO, since Moore et al. obtained these results in Abcc6⁻/⁻ and Abcc6⁺/⁻ mice, which have decreased levels of circulating pyrophosphate.
Aims

The aims of this thesis are centered around 3 main topics: (I) therapeutic approaches in the treatment of PXE, (II) analysis of plasma PP, levels in calcification diseases, and (III) PP, as a therapeutic intervention in NHO, a non-PXE ectopic calcification disease.

I. Therapeutic approaches in the treatment of PXE

A. Personalized approach involving ABCC6 mutation-specific conformational therapy

1. **ABCC6 specific mAb development**
   Since previous work by our group has established that in the case of missense mutants, the subcellular localization of ABCC6 often has a crucial role in PXE pathology [120], [121], tools that can facilitate the determination of the localization of these variants are needed. We aimed to generate a mouse monoclonal antibody that can detect ABCC6 and its mutant variants on the intact cell surface, confirming physiological localization in the plasma membrane.

2. **Investigating the subcellular localization of PXE-causing ABCC6 mutations in mouse liver**
   We aimed to establish the *in vivo* subcellular localization of 6 PXE-causing missense ABCC6 variants (R518Q, G992R, T1130M, R1314Q, L1335P and E400K) found in a large cohort of PXE patients from Angers, France, by transiently expressing them in hepatocytes of Abcc6<sup>−/−</sup> mice *via* the hydrodynamic tail vein injection HTVI method previously described in [120], [121]. In addition to analyzing their localization in the mouse liver, we also tested whether treating these animals with the chemical chaperone 4-PBA could augment the proteins’ trafficking to the plasma membrane.
B. Oral PP, supplementation as a potential therapy in PXE

Pyrophosphate is known to be a potent endogenous inhibitor of ectopic mineralization, and under the general assumption that its poor oral absorption limits its therapeutic potential, bisphosphonates, its nonhydrolyzable analogues, have been applied as experimental therapy in various ectopic calcification diseases [106]. BPs however are not without side effects, as doses which inhibit ectopic calcification can also impair physiological mineralization.

The negligible bioavailability of orally given pyrophosphate was never categorically proven, chiefly because reliable methods of measuring PP\textsubscript{i} in plasma were lacking. We aimed to investigate whether orally given PP\textsubscript{i} could be absorbed from any part of the GI system, and whether it could counteract ectopic mineralization in mouse models of PXE and GACI.

1. Effect of oral PP\textsubscript{i} treatment on quick-developing induced calcification (DCC) in Abcc6\textsuperscript{-/-} mice.
2. Effect of oral PP\textsubscript{i} treatment on spontaneously developing calcification observed in Abcc6\textsuperscript{-/-} mice.
3. Effect of oral PP\textsubscript{i} treatment on spontaneously developing calcification observed in ttw mice, animal models of a closely related calcification disease GACI.
4. Absorption of PP\textsubscript{i} in mice: kinetics, dose-dependence and absorption from different parts of the gastrointestinal system. Optimization of oral PP\textsubscript{i} treatment; identifying compounds yielding the highest plasma concentrations.
5. Absorption of PP\textsubscript{i} in healthy human volunteers: kinetics, dose-dependence.
6. Preclinical uptake studies to identify the optimal salt form and delivery method for long term treatment; absorption studies in mice, as well as PXE and non-PXE human volunteers.

II. Plasma PP\textsubscript{i} levels in ectopic calcification diseases

Decreased plasma PP\textsubscript{i} levels are characteristic of PXE, and since PP\textsubscript{i} acts as a systemic and local inhibitor of mineralization, this may be the case in other ectopic calcification
diseases too. This section details our efforts to determine circulating \( PP_i \) levels in a large PXE cohort, in systemic sclerosis patients, and a mouse model of craniometaphyseal dysplasia.

A. Plasma \( PP_i \) in PXE

We aimed to study plasma \( PP_i \) levels in a cohort of 207 pseudoxanthoma elasticum patients from Utrecht, The Netherlands. It has been already established that plasma \( PP_i \) is decreased in people affected by this disease, but the observed reduction appears variable; some PXE patients have \( PP_i \) levels overlapping with lower values seen in healthy control volunteers.

The severity of disease experienced by PXE patients is also known to vary; and mutations which result in complete loss of \( ABCC6 \) function (no protein detected, or a complete lack of transport function of the mutant protein) would be expected to cause a more dramatic decrease in the released ATP, lower plasma pyrophosphate, and consequently, a more dramatic manifestation of symptoms. However, elucidating genotype-phenotype correlations in PXE proved to be a challenging endeavor [122], and there are many possible explanations for this.

1) The confounding effect of patient age. PXE is a lifelong, slow-developing disease; symptoms initially present in young adult- or childhood, typically starting in the skin, and they progress at a variable rate: patients may experience bouts of worsening disease interspersed by periods of long term non-progression. As affected patients age, eventually they may develop new PXE symptoms, or pre-existing ones may worsen, but the course of the disease is unpredictable: severe cardiovascular symptoms or vision impairment may never occur in some, and currently we have no way of predicting the progression of PXE in any given patient. For this reason, drawing conclusions from a PXE cohort with some inevitable variability in their age is challenging. The rarity of the disease however makes forming a cohort of same-age patients rather difficult.

2) Compound heterozygosity. PXE has numerous causal \( ABCC6 \) mutations, and consequently, many patients are compound heterozygous, carrying a given mutation on one allele, and a different one on the other. Telling apart the effect of these mutations on the phenotype observed poses further difficulty that might make drawing any
conclusions about a genotype-phenotype correlation altogether impossible. In order to 
overcome this, patients’ genotypes can be grouped into categories, so that one can draw 
comparisons between patients with 2 truncating mutations, 2 non-truncating mutations, 
or a ‘mixed’ genotype of a missense mutation and a truncating mutation. Mutations that 
give rise to a premature termination codon or a frameshift are more likely to result in 
completely non-functional protein. This approach has been able to detect increased 
severity of ophthalmological and skin symptoms in patients harboring one or two 
truncating mutations compared to those who have two missense alleles [123]. A more 
recent study has found similar associations, with worse prognosis of ophthalmological 
and arterial calcification symptoms for patients who harbor two truncating mutations 
[124]. This is at odds with previous research describing 15 patients having a highly 
variable phenotype despite harboring the same ABCC6 mutation in homozygous form 
[35], and recent observations by Boraldi and colleagues [125], who did not detect any 
association between disease severity and ABCC6 genotype in a large cohort of 310 Italian 
PXE patients. A possible shortcoming of the variant grouping method is that different 
missense mutations may have very different effects on ABCC6 function; one may result 
in only slight impairment of function, while another substitution may render the protein 
completely incapable of transport. Without relevant research available on the missense 
mutation in question, we may only rely on predictions to determine whether it results in 
a functional transporter or not.

3) There is no correlation. It is within the realm of possibilities that the differences 
in PXE symptom severity are the effect of currently unidentified modifier genes, or 
simply the result of unknown environmental factors. PXE patients harboring the same 
mutation in a homozygous form have been described to have markedly different clinical 
manifestations of disease [35], suggesting a more complex genotype-phenotype 
relationship.

Bearing these possibilities in mind, and considering that ABCC6 is known to 
influence PP, levels, we aimed to investigate whether any association between ABCC6 
genotype and plasma PP, or PP, levels and disease phenotype can be demonstrated in a 
large cohort of PXE patients.
B. Plasma PP\textsubscript{i} in systemic sclerosis (SSc) patients

Approximately 18-49\% of SSc patients develop cutaneous calcification symptoms [116], which cause a significant burden for those affected. Currently, there is no universally effective therapy for calcinosis in SSc, although experimental treatments exist: surgical excision, bisphosphonates, phosphate binders, and minocycline, a tetracycline derivative with calcium chelating properties, are used for treating calcinosis, with varying degrees of success [111].

Whether SSc patients have altered concentrations of the systemic mineralization inhibitor pyrophosphate in their circulation was previously unknown. It is unclear whether PP\textsubscript{i} in the circulation can have any effect on calcinosis localized in the skin. We aimed to investigate if SSc patients with calcinosis have altered plasma PP\textsubscript{i} concentrations compared to age- and sex-matched healthy control subjects, or SSc patients not affected by calcinosis.

C. Plasma PP\textsubscript{i} in CMD

It is currently unknown whether PP\textsubscript{i} levels are altered in CMD patients. Observations of decreased PP\textsubscript{i} levels in the context of Ank or ANKH deficiency mainly come from in vitro studies of cell lines overexpressing the wt or mutant forms of the transporter. Plasma PP\textsubscript{i} levels in mouse models of CMD were also previously unknown. We aimed to measure these in ank\textsuperscript{LysI} mice, which carry the CMD-causing Phe377del mutation, and compare the results to wt mice of the background strain.

III. PP\textsubscript{i} as a therapeutic intervention in a non-PXE ectopic calcification disease

Heterotopic ossification is a relatively common complication of major trauma, presently without specific therapy. We aimed to test whether circulating PP\textsubscript{i} levels are altered in a mouse model of neurogenic HO, and to test the efficacy of PP\textsubscript{i} treatment in the prevention of trauma-induced calcification in the same animal model: CD1 mice subjected to combined trauma of a head injury and injection of cardiotoxin in the hamstring muscle.
Materials and Methods

Study Approval - Patients and healthy volunteers

Oral uptake study involving healthy human volunteers (female and male) was approved by the National Review Board of the Ministry of Health, Hungary (ETT TUKEB). The actual permit based on the above approval has been issued by National Public Health and Medical Officer Service (NNK; authorization number: 16412-5/2021).

The cross-sectional study of plasma PP, in the Utrecht cohort of PXE patients was approved by the medical ethical review board of the University Medical Center Utrecht (IRB#18-767, 16-622).

In the case of systemic sclerosis patients, the study protocol was approved by the Ethics Committee of the Hungarian National Public Health Centre (No: 16985-9/2020).

Informed consent was obtained from each volunteer prior to the study and experiments conformed to the principles of Declaration of Helsinki what is indicated in the above document. All patient samples were handled in an anonymized form also approved by the above document.

Study Approval - Animals

All animal protocols were approved by the Food Chain Safety and Animal Health Directorate of the Government Office of Pest County, Hungary (XIV-I-001/707-4/2012, PE/EA/280-7/2019, PE/EA/1096-7/2020 and PE/EA/748-2/2021). Mice were housed in IVC-type cages at an approved animal facility at the Research Centre for Natural Sciences, and were kept under routine laboratory conditions with a 12-hour light-dark cycle and ad libitum access to water and chow.

Long-term PP$_i$ treatment of mouse models of the diseases PXE ($Abcc6^{-/-}$) and GACI ($ttw$) was carried out in accordance with ethical permit PE/EA/1096-7/2020. $Abcc6^{-/-}$ mice generated on 129/Ola background were backcrossed to C57BL/6j strain > 10 times [51]. DCC induction by cryoinjury of $Abcc6^{-/-}$ mice was performed as previously described
in accordance with permit XIV-I-001/707-4/2012. C57BL/6J mice used in experiments designated as wild type of $\text{Abcc6}^{-/-}$ line were derived from mice purchased from The Jackson Laboratories. Wild type CD1 mice, and ttw mice were obtained from the breeding of heterozygous ($\text{Enpp1}^{+/-}$) mice [62].

Absorption of different PP$_i$ compounds was tested and compared in order to identify compounds providing superior absorption properties in 3-month-old fasting female C57/Bl6 mice, following the administration of the tested PP$_i$ forms via gastric gavage under general anesthesia, in accordance with ethical permit PE/EA/748-2/2021.

Mice transgenic for bFcRn carry 5 copies of the bovine neonatal Fc receptor (bFcRn) alpha chain on a BALB/c genetic background [126]. Experiments on bFcRn transgenic mice were carried out in strict accordance with the recommendations of the Guide of the Institutional Animal Care and Ethics Committee at ImmunoGenes Ltd, in accordance with the ethical approval PEI/001/2196-2/2013 issued by the Food Chain Safety and Animal Health Directorate of the Government Office of Pest County, Hungary.

Trauma-induced calcification following brain injury was examined using wt CD1 mice obtained from the breeding of heterozygous ($\text{Enpp1}^{+/-}$) mice, in accordance with ethical permit PE/EA/280-7/2019.

Table 2 summarizes the genotype, age and sex of the mice used in each of the animal experiments.

<table>
<thead>
<tr>
<th>Experiment name</th>
<th>mouse genotype</th>
<th>age (weeks)</th>
<th>sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral PP$_i$, treatment effect on vibrissae calcification</td>
<td>$\text{Abcc6}^{-/-}$ [51]</td>
<td>22*</td>
<td>m and fm</td>
</tr>
<tr>
<td>Oral PP$_i$, treatment effect on vibrissae calcification*</td>
<td>ttw [61]</td>
<td>4</td>
<td>m and fm</td>
</tr>
<tr>
<td>Oral PP$_i$, treatment effect on kidney and arterial calcification*</td>
<td>ttw</td>
<td>8</td>
<td>m and fm</td>
</tr>
<tr>
<td>PP$_i$ uptake following gastric gavage</td>
<td>$\text{Abcc6}^{-/-}$, C57/Bl6J</td>
<td>12-15</td>
<td>m and fm</td>
</tr>
<tr>
<td>In vivo hABCC6 expression by HTVI</td>
<td>$\text{Abcc6}^{-/-}$</td>
<td>12</td>
<td>m and fm</td>
</tr>
</tbody>
</table>
Table 2. **Genotype, age and sex of mice included in animal experiments.** In the case of long-term oral treatments given via drinking water, treatment was initiated at 3 weeks of age, and in the table the age of mice at the termination of experiment is shown. As an exception, in the case of ttw mice (merked with an asterisk) some treatment groups received oral PP\(_i\), *in utero*, by treating the mothers of ttw offspring during gestation.

### Anaesthesia

During surgical procedures or treatments causing pain or discomfort (including gastric gavage, blood collection and trauma-induction), general anesthesia was applied by intraperitoneal injection of a cocktail of tiletamine/zolazepam (30 mg/kg, Zoletil, Virbac, France), xylazine (12.5 mg/kg, Primazin, Alfasan, The Netherlands) and butorphanol (3 mg/kg, Butomidor, Richter Pharma, Austria). Depth of anesthesia and well-being of mice was closely monitored during all procedures.

Post-operative pain management, if necessary, was carried out with subcutaneous injections of 5 mg/kg butorphanol (Butomidor, Richter Pharma, Austria).

### Cell culture

All cell lines were grown in DMEM supplemented with L-glutamine, penicillin/streptomycin and 10% FBS. Hybridoma cells were also grown under similar conditions, following HAT selection [127].
HEK cells expressing rat Abcc6 and hABCC6 [128] were grown under continuous puromycin selection (2 µg/ml) in order to maintain their high levels of ABCC6 expression.

When confluent growth was reached, cells were washed in PBS, then harvested by scraping and kept on ice for a maximum of 1 h until further use. Then they were either mixed with the adjuvant for immunization or used for flow cytometry.

Immunization of mice and hybridoma production

Bovine FcRn transgenic mice were immunized by intraperitoneal injections of a combination of 5x10⁵ HEK cells expressing high levels of hABCC6 and adjuvant. The first injection contained Complete Freund's adjuvant (CFA), and for subsequent booster shots Incomplete Freund's adjuvant (IFA) was used. For the testing of immune sera, required blood samples were collected by retro orbital puncture.

Based on the reactivity of their immune sera tested on ABCC6 expressing Madin-Darby Canine Kidney II (MDCKII) and Human Embryonic Kidney 293 (HEK293) cells, and additionally, mouse liver samples, two mice were selected for hybridoma generation. The animals were euthanized by cervical dislocation, and their spleens were harvested under aseptic circumstances. Hybridomas were generated by fusion of splenocytes with SP2/0-Ag14 mouse myeloma cell line [127]. Of the more than 1500 hybridomas tested, mEChC6 was selected as best candidate, and its isotype was determined by RapidYield mmAb isotyping kit (Crystal Chem, Elk Grove Village, IL), in accordance with manufacturer's instructions. This mAb was further characterized as described in the next sections.

Screening on MDCKII cells

Serum samples of immunized mice, and later, hybridoma microculture supernatants were screened to identify ones producing antibodies which react with an EC epitope of ABCC6. For this, 1.5x10⁴ MDCKII cells were seeded on 96-well plates (Greiner Bio-One, Kremsmünster, Austria) in normal growth medium. Testing was performed before cells reached confluence, following this protocol: medium was aspirated, and plates were
washed in cold DPBS (Sigma-Aldrich, St- Louis, MO) supplemented with 0.9 mM CaCl₂ and 0.5 mM MgCl₂, then incubated in blocking solution containing 2.5% bovine serum in DPBS on ice, for 30 minutes. Tested sera were added at 1:100, 1:500 and 1:1000 dilution to wells, and cells were incubated with them for 60 min. For supernatant samples, the tested dilution was 1:2. After washing with DPBS, goat anti-mouse secondary antibody (Invitrogen, Waltham, MA) was added, diluted 1:500 in blocking solution for 60 min; the cells were then washed in cold DPBS. DAPI staining of nuclei (1:500, 10 min) was used to reveal cells that lost membrane integrity during the labeling process. Plates were immediately imaged by an ImageXpress Micro XLS automated microscope.

Screening by flow cytometry

Hybridoma microcultures were screened to identify ones producing antibodies which react with an EC epitope of ABCC6. HEK cells were harvested by scraping and filtered through a 100 µm cell strainer to avoid clumps. 2x10⁵ hABCC6 expressing and hABCC6 negative cells were used for the testing of each supernatant sample. HEK cells were fixed in a 4% PFA solution for 10 min at room temperature, then incubated in 1% BSA solution in PBS to minimize aspecific binding on their surface. Test supernatant samples were added to cells at a dilution of 1:2, followed by labeling with goat anti-mouse eFluor660 secondary antibody (Invitrogen, Waltham, MA). Flow cytometry measurements of supernatant samples were performed using a BD FACSCalibur cytometer.

For further testing, mEChC6 was conjugated to Alexa Fluor 488 (Invitrogen, Waltham, MA) according to manufacturer's instructions. M6II-7 (Monosan, Uden, The Netherlands), used here as a control antibody, is a well-characterized rat mAb reacting with an intracellular epitope of ABCC6 [48]. In the case of this antibody, intracellular labeling necessitated an additional permeabilization step with 0.1% Triton-X for 10 minutes, before adding primary Ab (1:50). Cells were washed, then incubated with a goat anti-rat (Invitrogen, Waltham, MA) secondary antibody (1:250) and mAb mEChC6-A488 (diluted 1:100) for 30 minutes at room temperature; flow cytometry was performed with an Attune NxT flow cytometer.
HTVI and immunohistochemistry

hABCC6 expressing liver samples were obtained from 12 week-old Abcc6−/− female mice by hydrodynamic tail vein injection (HTVI) of hABCC6 in pLIVE plasmid as described [120]. Briefly, wt and mutant ABCC6 variants were cloned into pLIVE expression plasmid (Mirus Bio, Madison, WI). The plasmid dose the animals received was 70 µg/mouse in a volume of sterile saline calculated as mouse weight in grams divided by 10 (e.g.: for a mouse weighing 18 g, 18/10 = 1.8 ml would be the total injected volume). The full amount was injected in one of the lateral tail veins in less than 5 seconds, achieving the necessary hydrodynamic shock that creates a temporary disruption in the membrane of hepatocytes, allowing the construct to enter some of the cells. Using this method, 5-10% of all hepatocytes would express ABCC6.

Four days later, the animals were euthanized by an overdose of the anesthetic, then liver lobes harvested from mice expressing hABCC6 were snap-frozen in 2-methylbutane cooled by liquid nitrogen and immunohistochemistry was performed as described in [120]. Briefly, from 2 liver lobes of each mouse 7 µm thick cryosections were made, and fixed to glass slides in cold methanol for 10 minutes, then washed in PBS 3 times. Slides were incubated for 1 hour at room temperature in a blocking solution consisting of PBS supplemented with 2.5% bovine serum and 0.1% Triton-X 100.

Primary antibodies were applied for 1 hour at room temperature diluted in blocking solution, A488-labelled mEChC6 at a concentration of 2.9 µg/ml and M6II-7 at a dilution of 1:100. To detect M6II-7, goat anti-rat secondary antibody (Invitrogen, Waltham, MA) was applied 1:250, 60' at room temperature. Slides were mounted with coverslips in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and images were acquired using a Zeiss LSM710 inverted confocal microscope with 20x and 63X objective (Carl Zeiss, Jena, Germany). To minimize the cross-talk between imaged channels, sequential image collection was used. Cells are shown as a single confocal section. All images were processed using ZEN software (Carl Zeiss, Jena, Germany).
Immunoblotting and limited proteolysis of hABCC6

2 µg protein samples of hABCC6 expressing Sf9 cell membrane fractions [53] and 10 µg of ABCC1, ABCC2, ABCC3 [129],[130],[131] and rat Abcc6 [54] expressing Sf9 cell membrane fractions were loaded on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. ABCC6 variants were detected with pAb HB6 (1:2000) [53], mAb mEChC6 (1:2000) and pAb K14 (1:2000, a kind gift of Bruno Stieger, Zurich). The mAbs MRp6 [132], M2-14 and M3-II-9 [133] (kind gifts from Rik J. Scheper, Amsterdam) were used for the detection of ABCC1, ABCC2 and ABCC3, respectively. HRP-conjugated anti-rabbit (Sigma-Aldrich, St- Louis, MO, diluted, 1:15000) and anti-mouse (Jackson ImmunoResearch, Ely, UK, diluted 1:10000) secondary antibodies and WesternBright ECL (Advansta, San Jose, CA) were used to develop chemiluminescent signals.

Limited proteolysis of hABCC6 was performed as described in [134]. Briefly, 80 µg of isolated Sf9-wtABCC6 membranes were digested by 0.1 - 8 µg trypsin (type XIII, Sigma-Aldrich, St- Louis, MO) in 200 µl of reaction buffer (50 mM MOPS, 50 mM KCl, 0.5 mM EGTA, 2 mM dithiothreitol, pH 7.0) for 10 min at 4ºC. Reaction was stopped by adding excess soybean trypsin inhibitor (type 1-S, Sigma-Aldrich, St- Louis, MO). Thereafter membranes were washed with reaction buffer, dissolved in loading buffer and loaded on 10% polyacrylamide gels, then subsequently blotted and probed with specific Abs as described above.

In vivo labeling of hABCC6

hABCC6 was expressed in vivo by HTVI of the pLIVE expression plasmid as described earlier [120]. 3 days later, A488 conjugated mmAb mEChC6 was injected in the lateral tail vein at 75 µg/animal in 100 µl sterile PBS [135]. 24 hours after mAb injection the mice were euthanized and potential unbound antibodies remaining in their circulation were washed out by perfusion with saline. Liver samples were processed and labelled with control mAb M6II-7 as described above.
4-PBA administration

Mice were treated with 4-phenylbutyrate (Santa Cruz Biotechnology, Dallas, TX) according to the protocol previously described in [120] one day before HTVI, and daily thereafter until euthanasia. The dose given was 100 mg/kg/day in IP injections, and 6.25 mg/ml in drinking water. Control animals received saline injections and normal drinking water.

DCC induction by cryoinjury

For the induction of dystrophic cardiac calcification in Abcc6−/− mice, a freeze-thaw injury was inflicted on the heart muscle [121]. The animals were under general anesthesia (tiletamine/zolazepam 30 mg/kg, xylazine 12.5 mg/kg and butorphanol 3 mg/kg) for the entire duration of the procedure.

Following the shaving and disinfection of the abdominal area, the skin and the peritoneum were opened with a midline incision of ~ 2 cm, revealing the liver and the intestines. A plastic half-tube was inserted in the abdominal cavity, holding the internal organs at a safe distance for the next phase of the surgery. At the end of the tube, the beating heart was revealed through the diaphragm. A blunt steel rod cooled in liquid nitrogen was inserted through the tube and touched to the heart of the animal through the diaphragm for 10 seconds.

After the removal of the rod and the plastic tube, the wound on the peritoneum and the skin was closed with absorbable sutures. Animals were returned to their cages and were allowed to recover under close observation. Post-operative pain management was applied as needed. If performed by a skilled operator, the survival rate of the procedure is >90%.

Determination of tissue calcium content

*Colorimetry*

Hearts of Abcc6−/− mice that had undergone cryoinjury and the tissue blocks harboring the vibrissae of ttw mice were finely minced, then the calcium content was dissolved in
0.15 N HCl for 48 h. Total tissue calcium content was determined by complexometry using the Cresolphthalein Complexone Method (Stanbio Calcium CPC LiquiColor kit, Boerme, TX, USA) following the manufacturer’s instructions.

**Alizarin Red staining of vibrissae sections**

Calcification of the vibrissae of Abcc6<sup>-/-</sup> mice was quantified by histochemistry as described [52]. Tissue blocks with the hair capsules (vibrissae) were removed, paraffin-embedded, sectioned, and stained with Alizarin Red S (Sigma-Aldrich, St- Louis, MO) solution (pH>4.2) for 5 minutes to visualize calcium deposits. The extent of calcification was quantified by morphometry utilizing image analysis software FIJI [136]. The extent of calcification around the vibrissae was quantified by two investigators in a blinded fashion.

**Alizarin Red staining of whole arteries**

Determination of calcification of arteries in the hind limbs of ttw mice was performed by Alizarin Red staining as described in [137]. Briefly, 55 day-old ttw mice were euthanized by injection of an excessive amount of anesthetic (tiletamine/zolazepam 30 mg/kg, xylazine 12.5 mg/kg and butorphanol 3mg/kg). After perfusion with 10 ml of saline, the large arteries between the heart and the distal part of hind limbs were dissected, and fixed by immersion in 95% ethanol at room temperature for 48h. Fixed arteries were stained with 0.003% in 1% KOH for 48 h before mounting between two glass slides and microscopic imaging.

**Detection of calcification in kidneys**

Kidney tissue sections of 4 µm were stained by the Yasue procedure as described [138]. Sections were perpendicular to interlobar arteries and 500 µm away from renal hilum. A morphometric analysis was performed (7–13 fields) by using FIJI software [136]. Results are expressed as the ratio of the calcified area indexed to the whole kidney tissue area.
Quantification of calcified areas

Images of tissue blocks containing vibrissae were combined using Hugin-Panorama photo stitcher (Free Software Foundation, Inc., Boston, MA USA), and calcified areas were quantified using Fiji [136].

Individual images of the arteries were combined using Hugin-Panorama photo stitcher (Free Software Foundation, Inc., Boston, MA USA). The resulting images were then processed using ImageMagick (https://www.imagemagick.org). After they were color inverted, the maximum of the red and blue channels were subtracted from the green channel. Subsequently, the red and blue channels were discarded, and the remaining grayscale images were blurred using a sigma value of 4 pixels. To remove any remaining low frequency background noise, a high-pass filter with a cut-off wavelength of 500 pixels was applied to each image. Finally, pixel values below 5% of the maximum possible brightness were annulled. Every pixel with a non-zero value in this final image was detected as calcified, the number of which were then and normalized to the total area of the artery.

Plasma preparation for PPi measurements - mouse samples

Mice were anesthetized by intraperitoneal injection of an anesthetic cocktail containing tiletamine/zolazepam 30 mg/kg, xylazine 12.5 mg/kg and butorphanol 3mg/kg. Needles and syringes used for blood collection were flushed with a drop of 1000 U enoxaparin sodium (Clexane, Sanofi-Aventis) to prevent coagulation during blood draw. Following a midline incision, skin and muscle tissue were gently pulled back to reveal the ribcage underneath. With a 21G needle (inner diameter ~0.5 mm) attached to a 1ml syringe, about 1 ml blood was slowly drawn by cardiac puncture without opening the chest cavity.

After removing the needle, blood was added to 50 µl CTAD anticoagulant (BD, Franklin Lakes, Nj; containing 0.11 M buffered trisodium citrate solution, 15 M theophylline, 3.7 M adenosine, 0.198 M dipyridamole), gently mixed by inverting the tube a couple of times, and kept on ice until centrifugation at 1000 g for 10 minutes at 4°C. The upper fraction containing plasma was gently aspirated without disturbing the
buffy coat layer, and transferred to the lower compartment of Centrisart I 300,000 MWCO filters (Sartorius, Frankfurt, Germany). The inserts with the PES membrane filter were placed back in the tubes, and the filters were subsequently centrifuged at 2200 g, for 30 minutes at 4°C. Following filtration, the plasma accumulated in the upper compartment was transferred to precooled 1.5 ml eppendorf tubes, promptly frozen at -70°C and kept there until analysis.

Plasma preparation for PP$_i$ measurements - human samples

Blood collection from human volunteers was performed using a 21G needle attached to a 2.5 ml syringe from the medial cubital vein, or a similarly placed peripheral venous catheter. Alternatively, CTAD vacutainer tubes (BD, Franklin Lakes, NJ) with appropriate adapters were also found to be suitable, yielding similar PP$_i$ values, provided the tubes are filled completely during the blood draw process. In the case of some systemic sclerosis patients, difficulties in venous blood sampling necessitated the use of 22G butterfly needles (inner diameter ~0.4 mm) instead; this did not influence the quality of the obtained samples in any way.

The addition of heparin in this case was not necessary, instead the sole anticoagulant used was CTAD, with 50 µl of CTAD added to ~1.2 ml blood, then gently mixed by inverting. The remaining steps of the plasma preparation process were carried out as described in the previous section (Plasma preparation for PP$_i$, measurements from mice). This protocol was used for sampling in the case of all healthy volunteers partaking in the determination of baseline fasting PP$_i$ levels as well as oral pyrophosphate absorption studies, and systemic sclerosis patients from Hungary. In the case of the Utrecht healthy control and PXE cohort, the CTAD anticoagulant was supplemented with K$_2$EDTA to match an already existing protocol for blood sampling (50µl 15% K$_2$EDTA added to a full 4.5 ml CTAD vacutainer tube).

Oral administration of PP$_i$ - mice

Sodium pyrophosphate tetrabasic decahydrate (Na$_4$P$_2$O$_7$, BioXtra quality) was purchased from Sigma-Aldrich (St-Louis, MO), and used in animal experiments. In the case of
long-term oral treatment studies, Na₄P₂O₇ was given as a 10 mM, 1 mM or 0.3 mM solution in different treatment groups. The PP₇ concentration in drinking water samples was found to be stable for at least 4 days. PP₇-containing drinking water was changed every second day.

Disodium pyrophosphate (Na₂H₂P₂O₇) used for oral treatment of mice was purchased from Sigma, and K₂H₂P₂O₇ was obtained from ERAS Labo (Grenoble, France). Mice were given 10 mM solution as their drinking water from the day of the DCC induction procedure until the termination of the experiment.

In PP₇ absorption tests, pyrophosphate dosing was carried out with the mice under general anesthesia (tiletamine/zolazepam 30 mg/kg, xylazine 12.5 mg/kg and butorphanol 3 mg/kg) during the whole duration of the procedure, and the following blood draw by cardiac puncture.

In the initial absorption studies confirming elevation of circulating PP₇ after oral delivery, PP₇ treatment was carried out by applying a paper filter soaked in 50 mM Na₄P₂O₇ solution to the oral mucosa, which was left in place until blood draw was performed.

Absorption kinetics was first tested after gastric gavage of 200 µl 50 mM Na₄P₂O₇ solution followed by blood draw at 0 - 30 minutes post-treatment.

In addition to 50 mM PP₇ solution, the absorption of additional concentrations of 1, 10, 25 and 100 mM Na₄P₂O₇ were also tested by gastric gavage of 200 µl, followed by blood collection after 15 minutes.

Absorption was determined in different parts of the gastrointestinal tract using 50 mM Na₄P₂O₇ solution. Each set of experiments was performed on different animals, since the amount of plasma required for PP₇ measurement necessitates terminal blood draw in mice. Oral absorption was tested by pipetting 100 µl PP₇ solution in the animal's mouth after the ligation of the esophagus. Absorption from the stomach was tested by gastric delivery, carried out with a curved gavage needle attached to a 1 ml syringe. 200 µl of PP₇ was injected after the ligation of the pylorus in these animals. Intestinal absorption was tested by injecting 200 µl of PP₇ solution in the small intestine of mice. In each case, blood was collected 15 minutes after PP₇ dosing, as described under the section 'Plasma preparation for PP₇ measurement from mice'.

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Different PP\textsubscript{i} compounds tested for best absorption properties (Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, K\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, (NH\textsubscript{4})\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, monoarginine-H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, monolysine-H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} and bisethanolamine-H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} were each given in a dose equal to 39 mg/kg PP\textsubscript{i} in a volume of 5 µl/g weight, to 3-month-old female C57/Bl6 mice. (NH\textsubscript{4})\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, monoarginine-H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, monolysine-H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} and bisethanolamine-H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} by were synthesized by Theratrophix (Sunnyvale, CA) specifically for this project. K\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} (GMP quality) was synthesized by ERAS Labo (Grenoble, France), and Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7} (food grade) was purchased from Fosfa (Breclav, Czech Republic), in 3 month-old female C57/Bl6 mice.

Animals were fasted for 4 hours prior to all absorption experiments.

PP\textsubscript{i} uptake study in humans

\textit{Na}\textsubscript{4}P\textsubscript{2}O\textsubscript{7}

For human absorption studies, Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}, tetrakisodium pyrophosphate anhydrous, Code 118 was purchased from ICL Food Specialist (St. Louis, Missouri, USA). Nine or ten healthy volunteers (age 24–69, fasting) ingested a tetraksodium pyrophosphate solution containing 40 mg/kg or 67 mg/kg or 98 mg/kg (43, 72, 110 mM, pH 8.0, respectively). The ingested amounts of tetraksodium pyrophosphate correspond to 13–33% of the maximal tolerable daily intake published by the World Health Organization, WHO (http://www.inchem.org/documents/jecfa/jeceval/jec_2259.htm). The duration of ingestion was less than one minute.

Blood samples were collected before ingestion (0 min) and 30, 60, 120, 240 and 360 minutes after from the median cubital vein. Drinking water was allowed but volunteers were asked not to eat until the last time point of blood sampling. Samples were processed as described under the section ‘Plasma preparation for PP\textsubscript{i} measurement from humans’.

\textit{Na}\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}

Disodium pyrophosphate, Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, food grade, was obtained from FOSFA (Břeclav, Czech Republic).\textsuperscript{3} The product used in our absorption studies was SAPP28. 1000 mg or

\textsuperscript{3} https://web.fosfa.cz/en/products/products-according-to-name/sodium-phosphates/
500 mg Na$_2$H$_2$P$_2$O$_7$ powder was loaded into 00 size gelatin (from Molar Chemicals, Halásztelek, Hungary) or cellulose (from Capsuline, Davie, FL) capsules.

Volunteers ingested a dose of 50 mg/kg Na$_2$H$_2$P$_2$O$_7$, which corresponds to a 39 mg/kg PP$_i$ dose. Eg.: in the case of a volunteer weighing 50 kg, two 1000 mg capsules and one 500 mg capsule would be given.

0' blood samples were taken to determine baseline fasting PP$_i$ levels in each volunteer, then the total capsulated PP$_i$ dose was ingested at once with a glass of water. Blood samples were collected before ingestion (0 min) and 30, 60, 120, and 240 minutes after, from the median cubital vein. Drinking water was allowed but volunteers were asked not to eat until the last time point of blood sampling.

K$_2$H$_2$P$_2$O$_7$

Dipotassium pyrophosphate, K$_2$H$_2$P$_2$O$_7$, GMP quality, was obtained from ERAS Labo (Grenoble, France). 1000 mg or 500 mg K$_2$H$_2$P$_2$O$_7$ powder was loaded into 00 size gelatin (from Molar Chemicals, Halásztelek, Hungary) or cellulose (from Capsuline, Davie, FL) capsules.

Volunteers ingested a dose of 50 mg/kg K$_2$H$_2$P$_2$O$_7$, which corresponds to a 39 mg/kg PP$_i$ dose. Eg.: in the case of a volunteer weighing 50 kg, two 1000 mg capsules and one 500 mg capsule would be given.

0' blood samples were taken to determine baseline fasting PP$_i$ levels in each volunteer, then the total capsulated PP$_i$ dose was ingested at once with a glass of water. Blood samples were collected before ingestion (0 min) and 30, 60, 120, and 240 minutes after, from the median cubital vein. Some volunteers were also sampled at an additional time point (15'). Drinking water was allowed but volunteers were asked not to eat until the last time point of blood sampling. Samples were processed as described under the section ‘Plasma preparation for PP$_i$ measurement from humans’.

Determination of plasma PP$_i$

Determination of PP$_i$ concentration in plasma was performed by the ATPS method, as described in [55]. Plasma PP$_i$ concentrations were determined using ATP sulfurylase to
convert PP\textsubscript{i} contained in samples to ATP in the presence of excess adenosine 5’ phosphosulfate (APS; Sigma-Aldrich, St. Louis, MO).

Briefly, to 10 μl of plasma sample, 70 μl of assay mix containing 32 mU ATP sulfurylase (New England Biolabs, Ipswich, MA or ProSpec, Ness-Ziona, Israel), 16 μmol/L APS, 80 μmol/L MgCl\textsubscript{2} and 50 mmol/L HEPES (pH 7.4) was added. The mixture was incubated for 30 minutes at 37 ºC, after which ATP sulfurylase was inactivated by incubation at 90 ºC for 10 minutes.

Generated ATP was quantified using the ATP-monitoring reagent BacTiter-Glo (Promega, Madison, WI). 20 μl of BacTiter-Glo reagent was added to 20 μl of sample. Bioluminescence was subsequently determined in a microplate reader (EnSpire Multimode Reader; PerkinElmer, Waltham, MA). Each sample was assayed as 3 parallel measurements, and PP\textsubscript{i} concentrations were determined by comparing sample wells to standard PP\textsubscript{i} solution containing 0 – 2.5 μM Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}.

TBI and muscle injury by CTX in mice

**TBI by modified Marmarou method**

Female wt CD1 mice weighing 24-32 g were subjected to traumatic brain injury (TBI) under general anaesthesia. TBI was induced using the modified Marmarou method described in [139], using a weight-drop device consisting of a brass weight (19 mm diameter, 95 g) ending in a small steel cap (2 × 10 mm) to restrict the zone of contact to the top of the mouse’s head, suspended by a fishing line.

During the process, the weight is dropped vertically through a guiding tube (20 mm diameter × 1.5 m length), under which the anesthetized animal is placed. The mouse is supported by a stage of thin aluminium foil, which breaks upon impact, allowing the animal to drop and land on a sponge cushion placed 10 centimetres below. This setup minimizes additional injuries, since placing the animals on a solid support would result in further trauma as the impact would crush the head of the mouse against the support underneath. The line suspending the weight prevents it from landing on top of the animal after it fell on the sponge cushion under the stage. The referenced article provides a more detailed description and supplementary video of the process.
Cardiotoxin (CTX) purified from *Naja pallida* snake venom was obtained from Latoxan (Portes lés Valence, France). CTX was dissolved in sterile saline, and was injected in the hamstring muscle of mice using a 26G needle at a dose of 0.12-0.16 mg/kg immediately after TBI.

Following TBI and CTX treatment, mice were returned to their cages and were monitored closely during recovery. The modified Marmarou method has been described to have 5% mortality in the case of single impact injuries, and we have observed a similar mortality rate. In those cases, death was caused by fractures or intracranial bleeding. The severity of the procedure necessitates close observation of the mice afterwards. Animals that exhibited signs of paralysis were immediately terminated by injecting a lethal amount of anesthetic to prevent prolonged suffering. In most cases, mice showed a complete recovery without any apparent symptoms of severe lasting damage to the central nervous system.

**µCT**

Trauma induced calcifications were quantified by µ-CT. Measurements were performed using a NanoX-CT (Mediso, Budapest, Hungary) cone-beam µ-CT imaging system. Circular CT scans were acquired of two samples at a time with an 8W power X-ray source with 55 kV tube voltage, 1.36 magnification, 900 ms exposure time, 1:1 binning and 360 projections in 7 minutes.

For reconstruction, we used back projection with a Butterworth filter, with the isotropic voxel size set to 70 µm. A semi-automatic segmentation procedure was carried out using the open-source 3D Slicer software[^140] to partition calcified tissue which has a signal intensity within the range induced by bone structure (maximum) and soft tissue (minimum). Areas containing calcified tissue were localized manually. A local intensity threshold was applied to these areas to select the actual region of interest, excluding the similar intensity noise from other regions of the image. The volume of the

segmented areas was measured, and a 3D model was created for visualization with the built-in tools of the software. For visualizing bone structures in the 3D figures, we used the volume rendering module of the software.

µFTIR

Microcalcifications were characterized using Fourier transform infrared microspectroscopy (µ-FTIR). 4-µm tissue sections were deposited on low-emission microscope slides (MirrIR, Keveley Technologies, Tienta Sciences, Indianapolis). FTIR hyperspectral images were recorded with a Spectrum spotlight 400 FTIR imaging system (Perkin Elmer Life Sciences, Courtaboef, France), with a spatial resolution of 6.25 µm and a spectral resolution of 8 cm⁻¹. Each spectral image covering a substantial part of the tissue consisted of about 30,000 spectra.

Gene expression analysis following TBI

Total RNA was extracted from 30 mg of muscle and liver tissues using TRIzol reagent (Thermo Fisher Scientific, MA, USA), and cDNA was synthesized using the SuperScript III First-Strand synthesis kit with random hexamers (Thermo Fisher Scientific, MA, USA). 1 µg of total RNA was used per reaction. The synthesis was performed in accordance with the manufacturer’s instructions.

RT-qPCR was carried out using the Applied Biosystems StepOnePlus RT-PCR system and the Design Wizard StepOne software (Applied Biosciences Inc, CA). Expression level of Abcc6 (Mm00497698_m1) and Enpp1 (Mm01193761_m1) in liver and Runx2 (Mm00501584_m1), Sox9 (Mm00448840_m1), Bmp-2 (Mm01340178_m1) and Bmp-4 (Mm00432087_m1) in muscle was detected using commercially available TaqMan probes (Thermo Fisher Scientific, MA). The expression level of Abcc6 and Enpp1 in the liver was normalized to β₂ microglobulin (Mm00437762_m1). In muscle tissue, the expression levels of four housekeeping genes (Gapdh (Mm99999915_g1), Actin-b (Mm00607939_s1), Hmbs (Mm01143545_m1) and β₂ microglobulin (Mm00437762_m1)) were found unstable between the different treatment groups, and therefore, they were
not used as reference genes. Instead, the obtained expression data was normalized to the total amount of RNA.

PP$_i$ treatment after TBI+CTX

Mice subjected to trauma were treated with Na$_4$P$_2$O$_7$ to determine pyrophosphate's effect on trauma induced calcification. In addition to oral treatment by replacing the drinking water with 1mM PP$_i$ solution, mice received intraperitoneal (IP) injections of 80-100 mg/kg PP$_i$ dissolved in saline. The first IP injection was given immediately after TBI + CTX, or ADR + CTX treatment, except for the delayed treatment group (TBI + CTX+30 min PP$_i$), in which case it was given 30 minutes after TBI + CTX.

IP injections became necessary because the anesthetic mixture used during the TBI+CTX procedure induces long-term narcosis lasting several hours if mice are left undisturbed, during which the animals will not drink. We assume trauma induced calcification would develop quickly after muscle injury, so therapeutic interventions aimed at preventing it should be applied as soon as possible. On the other hand, as opposed to chronic conditions necessitating lifelong oral PP$_i$ treatment, this would not be the case with trauma induced HO; injectable treatments are feasible, and possibly even higher doses of PP$_i$ may be used safely without creating a substantial burden of long-term high salt intake.

Statistical analysis

Mouse calcification data (natural calcification markers in PXE and GACI disease models, trauma induced calcification in neurogenic HO) and plasma PP$_i$ levels in mice and human volunteers were analysed by two-tailed Mann-Whitney non-parametric test. Correlation of plasma PP$_i$ levels with disease markers and age in PXE patients was determined by calculating Pearson correlation coefficients.

In general, values are expressed as mean ± standard error of the mean (SEM), unless otherwise indicated in the figure legend. A $p < 0.05$ was considered statistically
significant, and the actual $p$-values are indicated on the corresponding figures. Statistical analysis was performed using GraphPad Prism version 8.0.1. Animal numbers in individual data sets varied, and are shown in the figures or the corresponding legends (n).

Figures

Figures were created using GraphPad Prism version 8.0.1. and BioRender scientific illustration tools$^5$.

$^5$ Available from https://biorender.com/.
Results

I. Therapeutic approaches in the treatment of PXE

A. Personalized approach involving ABCC6 mutation-specific conformational therapy

1. ABCC6 specific mAb development

Our efforts to raise an EC binding mAb against human ABCC6 led to the generation of a hybridoma that produces mEChC6, a murine (IgG2a) mAb which reacts with a linear extracellular epitope of hABCC6.

We confirmed the specificity of mEChC6 detecting hABCC6 protein expressed in the livers of mice. In this experimental setup, wt hABCC6 was expressed transiently in vivo in the liver of mice by HTVI of a hABCC6 expression plasmid [120],[49],[121]. Our earlier work has shown that HTVI results in the expression of hABCC6 in its native subcellular localization, the basolateral plasma membrane of murine hepatocytes [120],[49],[121]. The expression of the protein is generally achieved in roughly 5-10% of all hepatocytes by this method, and non-expressing cells serve as inherent negative controls. Since mAb mEChC6 is of mouse origin, using it to visualize hABCC6 in mouse tissue samples poses a difficulty: the high background signal resulting from the use of an anti-mouse secondary antibody. To overcome this, we directly labeled mAb mEChC6 with Alexa488 and applied it to 7 µm sections of hABCC6-expressing CD1 mouse livers. This revealed that mEChC6 is indeed hABCC6-specific because it reacts with its target protein with the same efficiency as the well characterised rmAb M6II-7 (see Figure 16B), without any cross-reaction with mouse proteins, including murine Abcc6.
Fig. 16. **Specificity of the new anti-hABCC6 mAb, mEChC6.** Immunohistochemical detection of hABCC6 expressed in mouse liver (A) with mmAb mEChC6 (green) and with mAb M6II-7 (red). Nuclei are visualized with DAPI (blue). Liver cells not expressing the human protein serve as negative control. Western blots of hABCC1, hABCC2, hABCC3, hABCC6 and rat Abcc6 (B). Western blot of hABCC1, hABCC2, hABCC3, hABCC6 and rat Abcc6 probed with mmAb mEChC6 (C). All ABCC proteins were expressed in Sf9 insect cells; 25 µg total protein per lane was loaded in the gels. (D) Coomassie stain of the membrane shown in (C).
We obtained further confirmation of mEChC6’s specificity by testing it in western blot of ABCC proteins expressed in Sf9 insect cell membrane fractions. Our mAb detected a band corresponding to hABCC6 in the appropriate membrane fraction, while showing no detectable reaction with either homologs ABCC1, ABCC2, ABCC3 or the rat ortholog Abcc6 (see Figure 16C).

Extracellular binding of mEChC6 is confirmed by flow cytometry and in vivo mAb labeling experiments (see Figure 17 and 18). In flow cytometry experiments, non-permeabilized HEK cells expressing either hABCC6, rat Abcc6 or no ABCC6 protein were subjected to labeling by mEChC6, of which it only reacted with hABCC6 expressing line. In contrast, control mAb M6II-7 detects no ABCC6 in any of the cell lines if they are not permeabilized, since it reacts with an internal epitope.

Fig. 17. mEChC6 reacts with an EC epitope of hABCC6. Flow cytometry of HEK cells expressing the hABCC6 (hABCC6 HEK, green), the rat Abcc6 protein (rAbcc6 HEK, blue) and the parental line (HEK, red). Fixed but not permeabilized cells were stained with mAbs mEChC6 and M6II-7 (left and middle panels); fixed and permeabilized cells were stained with M6II-7 (right panel).
Fig. 18. **Injected mEChC6 reacts with hABCC6 expressed in mouse liver.** Detection of hABCC6 in mouse liver following intravenous injection of mAb. Fluorescent immunohistochemical images of hABCC6 expressed in mouse liver, detected with A488-conjugated mmAb mEChC6 injected into the tail vein (left panel, green) and with rmAb M6II-7 applied after cryosectioning, detected by A546-conjugated anti-rat secondary Ab (middle panel, red) and merged image (right panel). Nuclei are visualised with DAPI (blue).

To validate these results in vivo, we injected A488-labeled mEChC6 in the tail vein of mice that have previously undergone HTVI of hABCC6-pLIVE plasmid to express wt ABCC6 in a fraction of their hepatocytes. The injected mEChC6-A488 was allowed to bind its target for 24 hours, then the mice were terminated. Any unbound antibody was removed from their circulation by perfusion, and their livers were sectioned and stained with M6II-7 as control. Signal of mEChC6-A488 overlaps with that of M6II-7, confirming the detection of hABCC6 in hepatocytes by the in vivo injected antibody.

To discern the exact position of the mAb’s epitope, western blotting and limited proteolysis experiments were performed. We have narrowed down the potential location of the epitope by performing limited tryptic proteolysis of hABCC6 and detecting the resulting fragments on western blots with the antibodies HB6, mEChC6, and K14 (see Figure 19A). HB6 and K14 are rabbit polyclonal Abs; HB6 reacts with an epitope in the L0 region, while K14 on the other hand detects an epitope in the C-terminal region, as shown in Figure 20A. A band of ~100 kDa appeared on both the
blots developed by HB6 and mEChC6 (indicated by arrowhead), but it was absent on the blot developed by K14. Conversely, a band of ~65 kDa was apparent on the K14 blot but was missing from the HB6 and mEChC6 blots (indicated by arrowhead). The abundance of these signals corresponded to the extent of tryptic digestion. We interpret these two bands as results from the first tryptic cleavage at the linker region, splitting ABCC6 to an N-terminal and a C-terminal half (indicated by a red arrow in [Fig20A]). These results indicate that mEChC6 recognizes an external epitope in the N-terminal half of ABCC6.

Fig. 19. **Mapping of regions interacting with mmAb mEChC6.** (A) limited proteolysis of hABCC6 expressed in Sf9 cells. 8 µg of protein was loaded per lane, digested with the indicated amounts of trypsin for 10 minutes. hABCC6 fragments were detected with mEChC6 and specific Abs with known epitopes in the N-terminal (HB6) and C-terminal (K14) positions of the protein. (B) Western blot detection of wt hABCC6 and ΔABCC6 with pAb HB6 (left) and with mAb mEChC6 (right). Both proteins were expressed in Sf9 cells and 2 µg total protein from the membrane fraction was loaded per lane. (C) Sequence
alignment of the EC segments of human, rat and mouse Abcc6 as well as that of human ABCC1, ABCC2 and ABCC3 in the TMD1 and TMD2 regions.

To further refine the position of the epitope, we have performed western blotting of ΔABCC6, which lacks the N terminal TMD0 and L0 regions, and wt ABCC6 expressed in SF9 insect cells. ΔABCC6 is not detected by HB6, which has its epitope in the L0 region. It is however recognized by mEChC6, which indicates its epitope is harbored by one of the extracellular loops in the TMD1 domain (see Figure 19B).

The length of linear epitopes recognized by Abs range between 4 -12 amino acids, and in many cases even a single conservative amino acid substitution can result in decreased binding [141]. To determine the possible location of mEChC6’s epitope, we compared the EC loops of hABCC6 with the corresponding regions of the rat and mouse Abcc6, ABCC1, ABCC2 and ABCC3 (see Figure 19C).
Fig. 20. **Characterizing mEChC6, a murine mAb binding an EC epitope of hABCC6.**

A schematic model representing hABCC6 (A), displaying the positions of the epitopes of ABCC6 antibodies HB6, M6II-7, and K14. The potential positions of the epitope mhEC6 detects are shown as green loops in the TMD1 and TMD2 domains. ΔABCC6, a truncated protein lacking the TMD0 and L0 regions, is indicated. The main proteolytic cleavage site in the limited proteolysis experiment is marked by a red arrow. (Y) marks an N-glycosylation site, according to [142]. Membrane-bound and EC regions of hABCC6 as predicted by homology modeling based on four cryoEM structures of bovine Abcc1 (B).

Each of these ABCC proteins has more than one conservative amino acid substitution in their EC segments corresponding to aa 338-347 of human ABCC6 (Figure
19C). Based on these alignments, we conclude that the most probable candidate for the linear epitope recognized by mEChC6 is the EC segment of aa 338-347 (FIGDPKPPA).

Personal contribution in the experiments listed: Of the animal experiments, HTVI of pLIVE-ABCC6 constructs, mAb TVI, perfusion and harvesting of tissue samples, cryosectioning and immunofluorescence. Cell culture, including the raising of cells for immunizations, for the specificity testing of sera, and hybridoma supernatants. Flow cytometry and western blotting.

2. Investigating the subcellular localization of PXE-causing ABCC6 mutations in mouse liver

Six missense mutations found in the Angers cohort of PXE patients were cloned into pLIVE expression plasmids, and were expressed in vivo in mouse hepatocytes by HTVI (see Figure 21). Liver samples obtained from the mice revealed one of the analyzed variants (T1130M) localized to the basolateral membrane like the wild type protein, while five demonstrated various degrees of trafficking problems, with a portion of the produced protein reaching the basolateral membrane, and some retained in intracellular compartments. One of these mutations (G992R) was found to be completely retained intracellularly, with virtually no ABCC6 appearing in the basolateral membrane.

Treatment by 4-PBA could rescue the localization of three of the five variants which were found to be mislocalized (R1314Q, L1335P and E1440K). R518Q and G992R on the other hand showed no effect of 4-PBA.
Fig. 21. **Subcellular localization of 6 PXE-causing ABCC6 variants expressed in mouse hepatocytes.** The positions of the mutations in question within ABCC6 (A) and images of their observed localization *in vivo* (B), displayed as pairs of a control (HTVI treatment alone) and a 4-PBA-treated animal in each case. 4-PBA treatment appeared to improve the localization of the mutations R1314Q, L1335P and E1400K. Mutations R518Q, G992R and T1130M remained unaffected. Scale bar = 20 µm.

*Personal contribution in the experiments listed:* HTVI of pLIVE-ABCC6 constructs, harvesting of tissue samples, cryosectioning and immunofluorescence.
B. Oral PP supplementation as a potential therapy in PXE

Since our findings challenged the prevailing notion that the bioavailability, and hence the therapeutic potential, of pyrophosphate was negligible \[91],[106\], the effectiveness of orally given PP, had to be confirmed in multiple experimental models of ectopic calcification. In addition to inhibition of induced calcification by cryoinjury method, efficacy had to be affirmed in the case of spontaneously developing mineralization phenotype markers of \textit{Abcc6}\textsuperscript{−/−} and \textit{ttw} mice, which have more relevance to human disease.

Furthermore, having established that such treatment is effective, it was also necessary to demonstrate \(\text{PP}_i\) is absorbed from the gastrointestinal tract, causing detectable elevation of plasma concentrations in mice, as well as in humans. After identifying the optimal salt form and delivery method for a potential therapeutic application, similar absorption rates had to be verified in PXE patients.

1. **Effect of oral \(\text{PP}_i\) treatment on quick-developing induced calcification (DCC) in \textit{Abcc6}\textsuperscript{−/−} mice**

In \textit{Abcc6}\textsuperscript{−/−} mice, dystrophic cardiac calcification (DCC) develops if the heart muscle is subjected to freeze-thaw injury. This phenomenon is dependent on \textit{Abcc6} deficiency, as \textit{C57/Bl6} mice, which serve as the background stain, do not develop mineralization if subjected to a similar injury.

In mice treated with 10mM \(\text{Na}_4\text{P}_2\text{O}_7\) dissolved in their drinking water, the myocardial calcification detected by Alizarin Red staining was markedly reduced (see Figure 22A). DCC can also be quantified by extracting the total calcium content of tissue, which then can be measured by a simple colorimetric assay using the cresolphthalein complexone (CPC) method. Total calcium content of hearts following DCC in \textit{Abcc6}\textsuperscript{−/−} mice was determined to be significantly lower in the animals treated with 1mM or 10mM \(\text{Na}_4\text{P}_2\text{O}_7\) compared to control mice drinking tap water (see Figure 22B) [143].
Fig. 22. **Dystrophic cardiac calcification in hearts of Abcc6⁻/⁻ mice.** Calcium detected by Alizarin Red staining is revealed as red deposits indicated by arrows (A). Calcium content of Abcc6⁻/⁻ mouse hearts following freeze-thaw injury (B). PP₇ treatment of 1mM or 10mM concentration was started one day before DCC, and continued for 3 days, when the mice were terminated and total heart calcium content was extracted for colorimetric determination. Scale bar = 1mm. Mean ± SEM values are displayed, p-values were calculated by Mann-Whitney test.

2. **Effect of oral PP₇ treatment on spontaneously developing calcification observed in Abcc6⁻/⁻ mice**

In Abcc6 deficient mice, the connective tissue sheath surrounding the whiskers (vibrissae) are affected by gradual progressive mineralization, first appearing at 5-6 weeks of age [52]. This phenotype can only be revealed by µCT imaging or histology, and requires a longer time to develop, as opposed to the induced calcification discussed previously. It is also dependent on the lack of Abcc6 activity, as wild type animals are not affected.

Oral PP₇ supplementation markedly reduced the calcification of Abcc6⁻/⁻ vibrissae revealed by Alizarin Red staining (Figure 23). This observation was true for both the number of sampled vibrissae which had calcification, and the extent of calcification in
them: the total calcified area measured in stacked serial tissue sections from PP$_r$-treated mice was significantly smaller than what we observed in the controls.

![Fig. 23. Vibrissae calcification detected by Alizarin Red staining.](image)

Cross sections of calcifying vibrissae from 22 week-old untreated control (A) and 10mM Na$_4$P$_2$O$_7$-treated Abcc6$^{-/-}$ mice (B) show significant reduction of calcified deposits in the treated animals. Quantifying the calcified areas (C) confirms the inhibition of ectopic mineralization in the vibrissae of PP$_r$-treated mice. The number of animals and vibrissae analyzed in each group are shown below the bars. Scale bar = 1mm. Mean ± SEM values are displayed. $p$-values were calculated by Mann-Whitney test.

3. Effect of oral PP$_r$ treatment on spontaneously developing calcification observed in ttw mice, animal models of a closely related calcification disease GACI

ttw mice, used here as animal models of GACI, develop more prominent ectopic calcification in their vibrissae, at a younger age. In addition to planimetry of calcium-deposits revealed by Alizarin Red-staining (Figure 24), the larger amount of mineral content in tissue samples makes colorimetric quantification of the dissolved calcium feasible, which is a quick and effective method of assessing the ectopic mineralization in these animals.
Just like in human patients, Enpp1 deficiency in mice results in more dramatic calcification. Since the pathologic process begins during fetal life, starting treatment before birth is reasonable, if it can be achieved. Treatment with different doses of PP_i for the entire duration of in utero life (by treating the pregnant mothers) and afterwards until 30 days of age, had significantly reduced the calcium content of vibrissae (see Figure 24 and 25). Similar reduction was observed if treatment was applied only to the mothers during pregnancy, and immediately ceased after birth. In contrast, adding PP_i to the drinking water of young mice after weaning proved to be ineffective. Of the doses tested, the 10 and 1 millimolar solutions appeared to have similar anticalcification effects.

Fig. 24. Calcification of ttw vibrissae. In an untreated control animal (A), calcification is extensive, with almost complete rings of calcification surrounding each individual vibrissa observed. In comparison, in a ttw mouse treated with 10mM PP_i until the age of 30 days (B) mineralization (indicated by arrows) is dramatically reduced. Scale bar = 1mm.
Fig. 25. **Oral PP<i> i</i> treatment reduces the calcification of ttw vibrissae.** The calcium content extracted from the muzzle skin samples of 30 day-old ttw mice shows significant reduction of mineral content when PP<i> i</i> was added to drinking water. PP<i> i</i> treatment given to young mice only after weaning appeared to be ineffective (Group 1), as opposed to early intervention in the other Groups. Similar effectiveness was observed in animals treated with 10 mM and 1 mM solution (Groups 2, 3 and Groups 4 and 5, respectively). Interestingly, treatment applied to pregnant mothers before birth, and stopped afterwards (Groups 3 and 5) proved to be equally effective. Mean ± SEM values are displayed. <i>p</i>-values were calculated by Mann-Whitney test.

The vascular calcification observed in ttw mice has more relevance to human disease, as it is the hallmark phenotype and main cause of mortality in GACI patients. The same phenotype, though less pronounced, can also be observed in PXE. It appears in the smaller and medium-sized arteries, and affects the media layer rich in elastic fibers.
In our experiments, treatment of pregnant mice with as little as 0.3 mM Na$_4$P$_2$O$_7$ significantly reduced ectopic mineralization in their $ttw$ offspring, which was most prominent in the large arteries in the hind legs of their $ttw$ offspring (Figure 26).

Calcified area within the blood vessel wall was quantified and normalized to the total area of the vessel for each animal. The results were compared between the untreated controls and the animals receiving 0.3 mM PPi $in utero$ (see Figure 26C), which revealed a significant reduction of mineralized area in the treated mice.

![Fig. 26. Oral PP$_i$ treatment inhibits vascular calcification.](image)

Severe media sclerosis affecting the large arteries of hind legs in an untreated $ttw$ mouse (A) vs one receiving 0.3 mM Na$_4$P$_2$O$_7$ treatment $in utero$ (B). In the PP$_i$-treated mice, foci of calcification were both less in number and appeared smaller. Quantification of the total calcified area normalized to the total area imaged (C) confirms the effectiveness of PP$_i$ therapy. Mean ± SEM values are displayed. $p$-values were calculated by Mann-Whitney test.

4. Absorption of PPI in mice: kinetics, dose-dependence and absorption from different parts of the gastrointestinal system

The effective inhibition of calcification demonstrated that pyrophosphate is very likely to be absorbed when given orally, however, this remained to be proven experimentally.
The necessary dose, kinetics and site of highest absorption were also to be determined in mice before oral uptake could be tested in human volunteers.

In fasting Abcc6−/− mice, a 200 µl dose of 10 mM Na₄P₂O₇ was sufficient to induce significant elevation of plasma PPᵢ, with more concentrated solutions producing even higher plasma levels (Figure 27A).

The study of absorption kinetics in mice revealed a rapid increase of plasma PPᵢ even 2.5 minutes after dosing, peaking at approximately 10 minutes, followed by an equally swift decrease back to baseline levels (Figure 27B). In addition, PPᵢ appeared to be well absorbed from all the sites tested in the gastrointestinal system of mice (Figure 27C).

**Fig. 27. Absorption in Na₄P₂O₇ in mice.** Absorption from the stomach is dose-dependent (A), and quick, producing the highest elevation of plasma concentration approximately 10 minutes after dosing via gavage (B). Selective dosing in different parts of the gastrointestinal system revealed the absorption of PPᵢ at all sites tested (C). Animal numbers are indicated or above bars on graphs. Dose-dependence experiment (A) was carried out on Abcc6−/− mice, but for the study of kinetics and testing of absorption sites, C57/Bl6 mice were used (hence the different 0 min control levels). In (B) and (C), 50mM PPᵢ solution was used for dosing, in a 200µl volume (excluding dosing in the oral cavity, where 100 µl was the highest volume which was possible to administer). In experiments (A) and (C), all blood samples were collected after 15 minutes. Mean ± SEM values are displayed. p-values were calculated by Mann-Whitney test.
While these results confirmed absorption of Na₄P₂O₇ in mice, oral bioavailability remained to be verified in humans.


In our healthy human volunteers, Na₄P₂O₇ was absorbed from the stomach, which resulted in elevation of plasma pyrophosphate levels in each of our volunteers, albeit with significant individual variability. Figure 28 demonstrates that even the lowest tested dose, 40 mg/kg Na₄P₂O₇, resulted in elevated plasma PP_i, which was sustained for at least 120 minutes post-dose.

Fig. 28. Na₄P₂O₇ in human volunteers. PP_i was significantly elevated in the blood plasma of participants who drank a solution of Na₄P₂O₇ in 40 mg/kg, 67 mg/kg and 98 mg/kg dose for at least 120 minutes post-ingestion. 9-10 healthy volunteers (female and male, aged 22-62 years) were included at each timepoint displayed in the figure. Mean ± SEM values are displayed. p-values were calculated by Mann-Whitney test.
6. **Preclinical uptake studies to identify the optimal salt form and delivery method for long term treatment; absorption studies in mice and human volunteers.**

The high salt load (especially in the case of 98 mg/kg Na₄P₂O₇ dose) resulted in unpleasant taste, and it would also raise the possible concern of undesirable high sodium intake in the long term. Therefore, we also tested the absorption of alternative salt forms, which contain less sodium, or no sodium at all, in mice, and in human volunteers, if the compound was available in a suitable quality for human experiments.

Absorption of Na₃H₂P₂O₇, K₂H₂P₂O₇, (NH₄)₂P₂O₇, monoarginine-H₂P₂O₇, monolysine-H₂P₂O₇, and bisethanolamine-H₂P₂O₇, each given in a dose equal to 39 mg/kg PP, were tested in mice in order to determine if they yielded similar or better absorption profiles as Na₄P₂O₇. Figure 29 panel A shows the chemical structures of compounds synthesized for this study, and panel B displays their absorption characteristics. PP was absorbed in each form, however the two amino acid derivatives, the monoarginine-H₂P₂O₇ and the monolysine-H₂P₂O₇ resulted in the highest peak plasma concentrations (15.6±1.5 and 13.5±1.3 µM, respectively). The potassium and sodium forms showed a similar maximum (9.4±0.9 and 10.4±2.7 µM, respectively), while on the other hand, administration of the bisethanolamine and the (NH₄)₂PP derivatives yielded only 5.3±0.5 and 3.4±0.6 µM peak plasma concentrations, respectively.
Fig. 29. Absorption of sodium-free pyrophosphate compounds and Na$_2$H$_2$P$_2$O$_7$ in mice.
The chemical structure of the pyrophosphate compounds studied (A) are displayed next to the plot of plasma PP$_i$ in mice after dosing with pyrophosphate compounds via gastric gavage (B). The dose applied was 39 mg/kg pyrophosphate in each case. Mean ± SEM are displayed, n≧3 at each data point.

Of the compounds tested in mice, only K$_2$H$_2$P$_2$O$_7$ is available in GMP quality suitable for human studies. When testing absorption in humans, Na$_2$H$_2$P$_2$O$_7$ and K$_2$H$_2$P$_2$O$_7$ were tested enclosed in two different capsule types: gelatin, which breaks down at the acidic pH of the stomach, and cellulose, which dissolves in an alkaline environment, releasing its cargo in the small intestine. Loading of the tested salt forms into capsules eliminated the unpleasantly bitter taste, and also yielded superior absorption compared to Na$_4$P$_2$O$_7$ dissolved in water. Figure 30 displays the absorption of the various PP$_i$ formulations tested in humans. 39 mg/kg Na$_2$H$_2$P$_2$O$_7$ loaded into gelatin (shown in red) or cellulose (shown in green) capsules, and this was compared to the absorption resulting from drinking 40 mg/kg Na$_4$P$_2$O$_7$ dissolved in water (in blue). Gelatin capsules filled with Na$_2$H$_2$P$_2$O$_7$ appeared to yield superior absorption, therefore the absorption of K$_2$H$_2$P$_2$O$_7$ was tested from gelatin capsules only. The absorption of this compound was as good as that of the sodium counterpart in similarly capsulated form.
Fig. 30. **Oral uptake of pyrophosphate in humans from different forms and formulations.** After drinking 40 mg/kg Na$_2$P$_2$O$_7$-water solution, plasma concentrations showed only moderate elevation (A) in healthy volunteers, n=10. In contrast, Na$_2$H$_2$P$_2$O$_7$ loaded in gelatin capsules (39 mg/kg pyrophosphate, n=9) produced much higher plasma concentrations (B). Cellulose capsules (C) filled with Na$_2$H$_2$P$_2$O$_7$ performed worse than gelatin in a similar experimental setup (39 mg/kg pyrophosphate, n=7). A comparison of uptake curves (D) reveals the differences between the tested dosing methods. Error bars represent SD on panels A, B and C, while mean and SEM are displayed on D.

Since the ultimate aim of these oral bioavailability studies is to supplement PP, to those who develop ectopic mineralization due to deficiency, absorption in patients in addition to healthy volunteers was also necessary. As Figure 31 indicates, the absorption of Na$_2$H$_2$P$_2$O$_7$ loaded in gelatin capsules in PXE patients was similar as in healthy volunteers. The peak PP$_i$ concentrations were 5.5 ± 0.9 µM in the non-PXE group and
5.7±1.4 µM in the PXE group. Baseline concentrations, as expected, were different, 1.3±0.1 and 0.5±0.1 µM in the non-PXE and PXE group, respectively.

![Graphs A, B, and C](image.png)

**Fig. 31. Absorption of orally delivered pyrophosphate in PXE patients.** Plasma PP, of PXE patients after taking Na$_2$H$_2$P$_2$O$_7$ in gelatin capsule formula (A) in a 39 mg/kg dose, n=9. Compared to healthy volunteers (B), of uptake curves PXE patients showed similar absorption after taking an oral dose of 39 mg/kg Na$_2$H$_2$P$_2$O$_7$ in gelatin capsules, n=9. The baseline plasma pyrophosphate levels of healthy volunteers and PXE patients involved in the absorption study (C) were significantly different (n= 9, p-value calculated by Mann-Whitney U test). Error bars represent SD on panel A and C, and SEM on panel B.

Absorption was also tested after dosing with gelatin-encapsulated K$_2$H$_2$P$_2$O$_7$, a pyrophosphate salt which is completely sodium free, and found to yield similar peak concentrations as Na$_2$H$_2$P$_2$O$_7$ (see Figure 32A and B).

Inorganic phosphate levels in plasma were also monitored after dosing of healthy volunteers with K$_2$H$_2$P$_2$O$_7$. The normal range of P, in plasma is between 0.81-1.45 mmol/L in healthy adults [144]. As shown in Figure 32C, during our experiments, phosphate concentrations measured in the blood of volunteers never exceeded the upper limit of 1.45 mmol/L after administering PP, suggesting that the hydrolysis of PP will not result in abnormally high phosphate levels.
Fig. 32. **Potassium pyrophosphate $K_2H_2P_2O_7$ shows similar absorption characteristics in humans as $Na_2H_2P_2O_7$, and plasma inorganic phosphate remains in the normal range.**

Healthy volunteers taking $K_2H_2P_2O_7$ in gelatin capsules (39 mg/kg pyrophosphate dose, mean ± SD, n=6) had high plasma PP$_i$ concentrations, with similar individual differences as observed in the case of $Na_2H_2P_2O_7$ (A). Individual plasma PP$_i$ curves with comparisons of absorption in the same six healthy volunteers (female and male, aged 25-64 years) from $K_2H_2P_2O_7$ (solid line) versus $Na_2H_2P_2O_7$ (dashed line), salt form in gelatin capsules (B), mean ± SD, n=6. Plasma inorganic phosphate (P$_i$) levels of volunteers after dosing with $K_2H_2P_2O_7$ remain within the normal range of 0.81-1.45 mmol/L, indicated by shaded area (C). Mean ± SD values are plotted, n=6.

The potassium salt, which had similar bioavailability as $Na_2H_2P_2O_7$ in mice and humans as well, was also tested in $Abcc6^{-/-}$ mice subjected to DCC in order to confirm it has the same anti-mineralization effect as that previously established in the case of the tetrasodium salt of PP$_i$. As Figure 33 shows, $Na_2H_2P_2O_7$ and $K_2H_2P_2O_7$ treatment of mice significantly reduced ectopic calcification induced by cryo-injury, confirming the effectiveness of these alternative salt forms.
Both $K_2H_2P_2O_7$ and $Na_2H_2P_2O_7$ inhibits dystrophic cardiac calcification of Abcc6$^{-/-}$ mice when given orally. Total Ca$^{2+}$-content of the heart tissue was measured from mice subjected to cryoinjury; mean ± SEM are graphed, $p$-values indicated were calculated by Mann-Whitney U test, and animal numbers in each treatment group (n) are displayed on the appropriate bars of the graph.

**Personal contribution in the experiments listed:** long-term oral PP$i$ treatment, histology of arteries and vibrissae, blood sampling by cardiac puncture, plasma preparation for PP$i$ analysis (mouse and healthy human volunteers), PP$i$ determination (mouse and healthy human volunteers), statistical analysis.

II. Plasma PPi levels in calcification diseases.

**A. Plasma PPi in PXE**

PXE patients of the Utrecht cohort had significantly lower plasma PP$i$, levels than healthy controls, as displayed on the inset of Figure 34, the PP$i$, levels in the two groups compared being 0.53±0.15 vs. 1.25±0.25 µM, respectively.
Among PXE patients, PP$_i$ levels showed moderate positive correlation with increasing age (see Figure 34, $r=0.39$, 95%CI:0.27;0.5, $p<0.0001$) and were found to be slightly higher in females than males (0.55±0.17 vs. 0.51±0.13 µM, $p=0.03$).

However, when adjusted for age and sex, no association between plasma PP$_i$ and ABCC6 genotype was found (see Figure 35). In addition, the age and sex adjusted data did not reveal any correlation between vascular or ophthalmological phenotype of PXE; patients having two truncating mutations, and, as a consequence, little to no ABCC6 protein, did not have more severe manifestation of disease than those who harbored one or two missense alleles, potentially retaining some decreased transport activity.

![Graph showing plasma PP$_i$ levels in PXE patients](image)

**Fig. 34. Plasma PP$_i$ in PXE.** Plasma PP$_i$ levels of PXE patients showed slight but significant positive correlation with age ($r=0.39$, 95%CI:0.27;0.5, $p<0.0001$). The inset displays a comparison of plasma PP$_i$ levels of healthy volunteers and PXE patients. Patients had lower plasma PP$_i$ than healthy controls (0.53±0.15 vs. 1.25±0.25 µM, $p<0.0001$, Mann-Whitney test) and female PXE patients appeared to have slightly higher PP$_i$ levels than males (0.55±0.17 vs. 0.51±0.13 µM, $p=0.03$, Student’s t test).
Fig. 35. **Lack of genotype-plasma PP$_i$ level correlation in the Utrecht PXE cohort.** No significant difference was observed between plasma PP$_i$ levels of PXE patients who have 2 truncating mutations, mixed genotype of a truncating and a non-truncating, or 2 non-truncating $ABCC6$ mutations, analyzed by Student's $t$ test.

*Personal contribution in the experiments listed:* determination of PP$_i$ in PXE and control plasma samples.

**B. Plasma PP$_i$ in systemic sclerosis (SSc) patients**

Plasma PP$_i$ levels were lower in systemic sclerosis patients than in age- and sex-matched healthy control subjects in our cohort of Hungarian patients recruited at the University clinics of Szeged and Debrecen (1.4±0.3 vs. 1.7±0.3 μM, see Figure 36A). Similar observations have been made in a US cohort of SSc patients, compared to a control group consisting of patients having non-scleroderma rheumatic disease. The data collection and analysis in the US and Hungarian cohorts were carried out independently, without prior knowledge of each other's projects or results, strengthening the conclusion of PP$_i$ being decreased in SSc patients, which was published in a joint paper in *Rheumatology* [145].
Hungarian patients having either localized or diffuse type of scleroderma had significantly lower plasma PP\textsubscript{i} than controls, but there was no significant difference between PP\textsubscript{i} levels measured in these two groups (see Figure 36C). 42.3% (11/26) patients had calcinosis symptoms in the localized and 52.6% (10/19) in the diffuse cutaneous subgroup of SSc patients. PP\textsubscript{i} levels proved to be decreased in all SSc patients, irrespective of whether they had calcinosis symptoms or not (see Figure 36B).

**Fig. 36. Plasma PP\textsubscript{i} in systemic sclerosis.** Pyrophosphate levels are significantly decreased in SSc patients compared to healthy controls of similar age and sex distribution in the Hunagrian cohort (A). Both SSc patients who have have calcinosis symptoms (SSc\_calc) and who don’t (SSc patients) had significantly lower PP\textsubscript{i} levels compared to healthy controls (B), but there was no significant difference between the PP\textsubscript{i} levels of scleroderma patients with or without calcinosis. Grouping SSc patients according to disease subtype revealed decreased plasma PP\textsubscript{i} in both localized (SSc\_l) and diffuse (SSc\_d) scleroderma patients, compared to healthy controls (C). Mean ± SEM values are displayed. \(p\)-values were calculated by Mann-Whitney test.
Personal contribution in the experiments listed: Plasma preparation for PP\textsubscript{i} determination in the Hungarian SSc and healthy control cohorts, PP\textsubscript{i} measurements in the Hungarian SSc and healthy control cohorts.

C. Plasma PP\textsubscript{i} in a mouse model of CMD

Ank\textsuperscript{K/K} mice, which carry the CMD-causing Phe377del mutation, had a slight but significant reduction of PP\textsubscript{i} concentration in their plasma compared to Ank\textsuperscript{+/+} C57/Bl6 animals, as shown in Figure 37. Both female and male mice were analyzed, and the figure displays the combined data.

ank/ank mice appeared to have similarly decreased plasma PP\textsubscript{i}, (1.15±0.27), but due to the limited number of available samples, statistical analysis was not possible.

![Graph showing plasma PP\textsubscript{i} levels in Ank\textsuperscript{+/+} and Ank\textsuperscript{K/K} mice]

Fig. 37. **Mouse models of CMD have decreased PP\textsubscript{i} levels in their circulation.** Ank\textsuperscript{K/K} mice have significantly reduced plasma PP\textsubscript{i} levels compared to Ank\textsuperscript{+/+} mice (mean 1.03±0.28 SD vs. mean 1.53±0.24 SD µM). Mean and SEM values are plotted, p-value was calculated by Mann-Whitney test.

Personal contribution: plasma PP\textsubscript{i} measurement.
III. PP<sub>i</sub> as a therapeutic intervention in a non-PXE ectopic calcification disease

Subjecting mice to complex injury of cardiotoxin (CTX) injection in the hamstring muscle following brain trauma resulted in the calcification of the injected muscle which was both detectable by µCT and apparent on Alizarin Red and von Kossa stained tissue sections obtained from the legs of mice (see Figure 38). As opposed to combined injury, CTX injection or head trauma alone was not sufficient to trigger mineralization.

![Fig. 38](image)

**Fig. 38. Calcification induced by complex trauma in our mouse model.** Panel (A) shows that injection of cardiotoxin (CTX) or traumatic brain injury (TBI) alone do not induce calcification detectable by µCT, while their combination does. Alizarin Red (B) and von Kossa (C) staining of a section of muscle tissue obtained from a mouse subjected to combined TBI+CTX treatment demonstrates histologically evident calcification (indicated by arrows) [146].

Oral and injected pyrophosphate treatment showed a powerful anti-calcification effect in mice subjected to complex injury. As shown in Figure 39, PP<sub>i</sub> applied immediately after trauma, or even delayed by 30 minutes (mimicking a potential emergency intervention in a hospital setting) could block the precipitation of calcified deposits in the injured muscle tissue.
Fig. 39. *Calcification following trauma and effect of pyrophosphate treatment.* Panel (A) shows µCT images of a TBI+CTX treated animal’s leg (I), compared to TBI+CTX treated mice injected with pyrophosphate immediately (II) or 30 minutes after (III) combined injury. White arrow indicates mineral deposits (red). Panel (B) displays the quantitative measurement results obtained from µCT images. Volumes of calcified deposits were quantified in (mm$^3$), animal numbers (n) and the exact treatment conditions in each group are displayed below the bars. Mean ± SEM values are shown, $p$-values were calculated by Mann-Whitney test.

*Personal contribution in the experiments listed:* TBI induction and long-term follow-up after trauma induced calcification. Compiling figures, statistical analysis.
Discussion

I. Therapeutic approaches in the treatment of PXE

A. Personalized approach involving ABCC6 mutation-specific conformational therapy

1. ABCC6 specific mAb development

We aimed to generate a mouse monoclonal antibody that can detect ABCC6 and its variants on the intact cell surface, confirming physiological localization, and have created the first mAb capable of detecting ABCC6 on the intact surface. In addition, this is the first mAb with such activity raised against the long-type ABCC proteins (such as ABCC1, ABCC2, ABCC3, ABCC6, ABCC9, ABCC10). The EC epitopes of the proteins in the ABCC family have proved to be notoriously difficult targets for antibodies, and - to our current knowledge - our murine mAb is the only one that has ever been demonstrated to bind its target in vivo. bFcRn transgenic mice have made the generation of this mAb possible, opening future research opportunities into the function of human ABCC6, and possibly the targeting of other ABCC family members including CFTR and ABCC1.

2. Investigating the subcellular localization of PXE-causing ABCC6 mutations in mouse liver

Six PXE-causing missense mutations were expressed in vivo in hepatocytes of Abcc6−/− mice. Liver samples obtained from these animals revealed the variant T1130M to be localized in the basolateral membrane as the wild type protein. The remaining five variants demonstrated various degrees of trafficking problems, with a portion of the expressed ABCC6 retained in intracellular compartments. 4-PBA treatment of mice improved the apparent trafficking problems in 3 of the mislocalized variants: R1314Q, L1335P and E1440K.
These results suggest that patients carrying the missense variants R1314Q, L1335P and E1440K may benefit from treatment with 4-PBA. In the cases of R518Q and G992R, 4-PBA appeared ineffective, but since these were found to be mislocalized, testing the effect of other drugs chemical chaperone properties on these could be warranted.

B. Oral PP\textsubscript{i} supplementation as a potential therapy in PXE

Orally given pyrophosphate was found to be efficient in the prevention of both induced and spontaneous ectopic calcification in mouse models of two rare ectopic calcification diseases, PXE and GACI. Ingesting sodium or potassium salt of pyrophosphate caused significant elevation of plasma PP\textsubscript{i} levels in mice as well as in healthy volunteers, albeit with significant individual differences. Our results regarding oral pyrophosphate treatment of ectopic calcification are in harmony with the findings of Pomozi et al., who have found that increased PP\textsubscript{i} content of chow has a similar anti-calcification effect in \textit{Abcc6}\textsuperscript{-/-} mice as oral treatment given via the drinking water [147]. These results suggest that PP\textsubscript{i} treatment could be a viable alternative to bisphosphonates like etidronate, which is not without risk of serious side effects. Etidronate was found to be effective in reducing arterial calcifications [101], even though it could not prevent CNV events in PXE patients [110].

Since patients would require lifelong treatment to compensate for the lack of the physiological metabolite, oral PP\textsubscript{i} administration is preferable to injectable alternatives. Food grade pyrophosphate is cheap and available as either sodium and potassium salts. The maximum tolerable daily intake of Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7} is 70 mg/kg \textsuperscript{6}. The relatively poor absorption of PP\textsubscript{i} means that oral treatment requires the ingestion of a substantial amount, and the sodium content of a 40 mg/kg Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7} dose required to induce a significant elevation of plasma levels in human volunteers would result in an undesirable amount of sodium intake. This effect can be reduced, or avoided altogether by substituting Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7} with Na\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, or a sodium free PP\textsubscript{i} formulation like the

dipotassium salt of PPI, or one of the amino acid derivatives: monoarginine-H$_2$P$_2$O$_7$ and the monolysine-H$_2$P$_2$O$_7$, which resulted in the highest concentration at the peak of the absorption curve, and would eliminate the issue of high sodium intake altogether.

PP$_i$ is a natural metabolite in our bodies, and as a consequence, it can be broken down to phosphates. The hydrolysis of each PP$_i$ molecule will yield 2 phosphates, according to Equation(1).

$$\text{P}_2\text{O}_7^{4-} + \text{H}_2\text{O} \rightarrow 2 \text{PO}_4^{3-} + 2\text{H}^+ \quad \text{(Eq1)}$$

The phosphate formed in the above reaction will then be processed according to the homeostatic phosphate metabolism; it may cause transient elevation of serum phosphate levels, it may be incorporated into nucleotide triphosphates, or indeed any other phosphate containing molecule formed in our bodies, and the excess can be eliminated via renal excretion. It is important to point out however, that just as PP$_i$ can be considered a natural inhibitor of mineralization in our bodies, the inorganic phosphate resulting from its breakdown may exert the opposite effect. High P$_i$ levels enhance ectopic calcification, by serving as building blocks of HAP formation and triggering the osteochondrogenic phenotype change of vascular smooth muscle cells exposed to high P$_i$ concentrations [148]. For this reason, the benefit of PP$_i$ treatment must be weighed against the potential deleterious effects of the P$_i$ load it is expected to produce, and even though high doses of PP$_i$ are considered safe, the smallest dose which can produce an elevation in plasma levels would probably be more beneficial therapeutically than a larger dose. Individual differences in absorption may necessitate calculating the optimal dose for each person. The 50 mg/kg Na$_2$H$_2$P$_2$O$_7$ or K$_2$H$_2$P$_2$O$_7$ dose used in our experiments correspond to ~39 mg/kg PP$_i$ intake, and although this resulted in a measurable increase in plasma P$_i$ concentrations, it never caused an elevation that exceeded the normal range of phosphate in plasma.

In PXE patients, similar absorption rates as those observed in healthy volunteers were confirmed, and the anti-calcification effect of Na$_2$H$_2$P$_2$O$_7$ and K$_2$H$_2$P$_2$O$_7$ were
validated in mouse experiments, suggesting these corroborating the therapeutic potential of orally given PP in PXE.

Our current understanding of pyrophosphate's inhibitory effect on calcification suggests that continuous daily oral PP, treatment may prevent the formation of new calcifications, or the exacerbation of existing lesions. It cannot, however, be expected to decrease the size of mineral deposits already present at the start of treatment. Animal studies with PP, and BPs, which act through a similar mechanism, suggest that with this type of treatment ectopic mineralization cannot be reversed. Pyrophosphate treatment of young ttw mice, if started after weaning, had no mitigating effect on the calcification of vibrissae. In contrast, the same treatment if started early on, even in utero by treating the mother of the ttw offspring, could significantly reduce mineralization. This effect persisted even if treatment was immediately discontinued following birth, and even if the dose was reduced more than tenfold (from 10 mM PP, solution to 0.3 mM) [143].

In spite of these observations, in some cases, etidronate treatment of GACI patients has been documented to decrease the size of calcifications detected before treatment was initiated [56].

As for PXE, a randomized controlled trial of etidronate involving 74 patients also reported a slight but significant decrease of calcium mass detected by CT in the femoral arteries in the intervention group [101], suggesting that resolution of calcification is in fact possible. In addition, a case series testing the therapeutic application of phosphate binders in PXE has also documented improvement of skin symptoms and reduction of histologically apparent dermal calcification following 1-year treatment with aluminium-hydroxide [149]. These results suggest that currently unexplored mechanisms which can clear up ectopic mineralization do exist, and if the causal metabolic imbalance in the PP/Pi homeostasis can be corrected, the reduction of previously formed calcifications is within the realm of possibilities.

Since PP, appears to have therapeutic potential in mouse experiments, and is considered safe, testing its efficacy in pseudoxanthoma elasticum would be warranted. However the variabile symptom severity and disease course exhibited by PXE patients poses further challenges in judging the benefit of any treatment in a clinical setting; if the progression of calcification symptoms is halted, it may be difficult to determine if it
is due to treatment effect, or merely the result of the variable natural progression rate of the disease. Differentiating between the two may require longer trial duration, or larger cohort size, which may prove to be a formidable obstacle, given the rarity of the disease. Currently, a phase 2 clinical trial (NCT04441671) is being organized to test the absorption of oral PP\textsubscript{i} in PXE patients, which is the first step towards determining whether oral pyrophosphate supplementation has a potential to benefit these patients.

II. Plasma PP\textsubscript{i} levels in calcification diseases

A. Plasma PP\textsubscript{i} in PXE

We have measured PP\textsubscript{i} levels in plasma samples of 207 PXE patients from Utrecht, the Netherlands. For 18 of the patients, genotype data was either not available, or no pathogenic ABCC6 mutations could be identified, and as a consequence, they needed to be excluded from genotype-plasma PP\textsubscript{i} correlation analysis. As expected, PXE patients in the Utrecht cohort were found to have significantly reduced plasma PP\textsubscript{i} levels compared to healthy controls (0.53±0.15 and 1.25±0.25 µM, respectively). Female PXE patients had slightly higher plasma PP\textsubscript{i} than males (0.55±0.17 vs. 0.51±0.13 µM), and surprisingly, older age appeared to be associated with higher plasma PP\textsubscript{i} levels in PXE patients.

Our results did not reveal any correlation between plasma PP\textsubscript{i} levels and patient genotype, in accordance with the observations in previous works investigating the relationship of genotype and phenotypic manifestations in PXE [35], [122],[125]. Patients with missense/missense genotype were underrepresented in our cohort, since the most common mutations were ones resulting in a frameshift or a premature termination codon. It is possible - though not too likely - that the inclusion of more patients with missense/missense genotype could have revealed some moderate correlation between genotypes and the observed plasma PP\textsubscript{i} levels in our analysis.

However, as discussed in the relevant part of the Aims section, variants classified as ‘missense’ are in fact a diverse group. Some of these result in transporters that have only slight impairment, while others could cause severely diminished function. A subset of these may result in a transporter that is entirely non-functional - either because of
very severe folding defects, or a complete loss of transport activity - and this may be another confounding variable that our current analysis does not address.

On the other hand, it is entirely possible that no correlation between genotype and plasma PP$_i$ exists. For instance, the mutation R1141* is present in homozygous form in 21 (10.4%) of the 201 PXE patients of whom we have both complete genotype and plasma PP$_i$ data, making R1141*/R1141* the most common ABCC6 genotype in the cohort examined. This high prevalence is not unexpected, since this mutation is quite common in European PXE patients [150],[122]. It results in a C-terminally truncated ABCC6 protein lacking a part TMD2 and one of the ATP-binding cassette domains, rendering it nonfunctional, and decreasing its expression to undetectable levels [151]. Figure 40 displays patients homozygous for R1141* highlighted in orange color on the Age vs. Plasma PP$_i$ plot, revealing that plasma PP$_i$ concentrations observed in these patients vary widely between 0.35 and 0.85 µM, and that the ABCC6 genotype of these patients apparently does not account for these differences.

![Fig. 40. Plasma PP$_i$ concentrations in PXE patients of different age](image)

Fig. 40. Plasma PP$_i$ concentrations in PXE patients of different age, with individuals homozygous for the R1141* mutation highlighted on the plot in orange color. The graph reveals significant differences in plasma PP$_i$ of patients who share the same genotype. Orange triangles indicate three R1141* homozygous patients from the same family.
In addition, four of the missense mutations from the Angers cohort which were analyzed in the \textit{in vivo} localization studies were also found in the Utrecht cohort of PXE patients. Subcellular localization data obtained in mice for these mutations could be paired with the corresponding plasma PPi levels measured in patients (see Figure 41).

The mutation R1314Q, which gave rise to a mislocalized ABCC6, was found in a PXE patient with 0.35±0.03 µM, and another who in contrast had 0.7±0.04 µM. The difference is striking, even though the patients' genotypes are very similar, with R1314Q on one allele, and one resulting in frameshift on the other.

In the case of the mutations T1330M and E1400K, only a single patient carrying the mutation in a homozygous form could be identified. R518Q was found in several patients, but it was paired with a different missense variant, present in a homozygous form in one case, or with a deletion on the other allele, making it difficult to draw a comparison between these three patients.
Fig. 41. **Subcellular localization of ABCC6 variants expressed in mouse hepatocytes and the corresponding plasma PP, levels measured in PXE patients.** As (C) illustrates, plasma PP, levels were found to be different even in patients who have the same R1314Q missense mutation on one allele, and a frameshift mutation on the other.

As previous results suggest, the relationship between ABCC6 mutations and PXE is far from being straightforward. Identical ABCC6 mutations have been described to result in a different clinical course [35], and in some cases, ABCC6 mutations have been documented to cause GACI phenotype characterized by severe calcification symptoms, which present much earlier than expected in PXE [152]. Our results suggest that ABCC6 mutations cause a significant decrease in plasma PP, but the decrease observed is no greater in those harboring truncating mutations on both alleles than in those who have
one or two missense *ABCC6* variants. Furthermore, plasma PP$_i$ levels are not the sole factors determining disease severity in PXE, which may be influenced by modifier genes or even lifestyle and environmental factors. In conclusion, it appears plasma PP$_i$ levels cannot be used to predict disease course or severity of PXE symptoms of patients.

**B. Plasma PP$_i$ in systemic sclerosis (SSc) patients**

SSc patients were found to have slightly, but significantly reduced plasma pyrophosphate levels compared to healthy age-matched controls, or age-matched non-scleroderma patients [145]. Surprisingly, this was true irrespective of whether they had cutaneous calcification symptoms. Our findings in Hungarian SSc patients are confirmed by results from an independent cohort based in the US. The data from the Hungarian and US cohorts had been collected and analyzed independently, which strengthens these observations and the conclusion drawn from them.

Calcinosis typically affects at least 18-49% of SSc patients [116]; in the cohorts analyzed, the prevalence of calcinosis was 40.7% and 46.7% in the US and the Hungarian cohort, respectively. The decrease we have observed in plasma PP$_i$ may be the factor which predisposes these patients to developing calcifications. It is worth noting that in PXE, decreased circulating pyrophosphate levels are presumably present from birth, yet typically they do not result in symptomatic mineralization until the early teenage years, suggesting PP$_i$ may be low for many years before these symptoms would occur. This could be the case in SSc as well, in accordance with the observation that calcinosis is associated with longer disease duration in these patients [116].

Since oral PP$_i$ treatment appears safe and well tolerated both in healthy volunteers, as well as two PXE patients [153], its effectiveness in the prevention of SSc-related calcinosis might be worth investigating. To this end, a clinical trial is being organized, with the participation of three university clinics in Hungary (Szeged, Debrecen and Pécs), aiming to test the absorption of Na$_2$H$_2$P$_2$O$_7$ in SSc patients, and assess whether oral PP$_i$ treatment can influence SSc-related calcinosis.
C. Plasma PP\(_i\) in a mouse model of CMD

Low plasma PP\(_i\) levels in Ank\(^{K/J\text{KI}}\) (Phe377del) mice is an interesting but not entirely unexpected finding. Tissue expression of ANKH differs markedly from that of ABCC6 and ENPP1. ANKH was thought not to contribute to the inhibition of calcification in the circulation, because its postulated PP\(_i\) transport activity apparently could not compensate for the lack of Enpp1 in ttw mice: these animals have no PP\(_i\) in their plasma regardless of having functional Ank transporters [60], confirming that nucleoside triphosphate hydrolysis by ENPP1 is the only source of extracellular PP\(_i\) in blood. Therefore it was hypothesized that Abcc6 and Enpp1 regulate PP\(_i\) levels in the circulation, while Ank performs a similar function at a different anatomical site: the synovium and bone tissue.

*ank/ank* mice have a phenotype of severe joint calcification which becomes apparent at 4 weeks of age, and this resembles closely the deleterious effect of Enpp1 deficiency in *ttw* animals. However, while in *ttw* mice PP\(_i\) in plasma is virtually absent, in Ank\(^{K/J\text{KI}}\) animals, we observed only a slight reduction. ANKH was originally thought to be a pyrophosphate transporter, which releases PP\(_i\) from cells to the extracellular space in the joint synovium [154]. This has been recently disproved by Szeri et al. [71], who discovered that ANKH instead releases citrate and nucleoside triphosphates (ATP) from cells. The latter can be converted to PP\(_i\) in the extracellular environment by ubiquitously expressed ENPP1. Modulators of ANK activity which enhance or decrease the amount of ANK-dependent PP\(_i\)-release have been shown to have strikingly similar effects on extracellular ATP release, in accordance with these being generated through a shared pathway [155].

In Ank\(^{K/J\text{KI}}\) mice, the lack of Ank function results in decreased, but not absent plasma PP\(_i\), because it is not the only transporter contributing to extracellular ATP levels: roughly 60% of which comes from Abcc6-mediated release [55], while the rest originates from a different source - possibly Ank. In comparison, *ttw* mice lacking functional Enpp1 suffer a more dramatic reduction, since extracellular ATP cannot be converted to PP\(_i\) in the absence of this enzyme (see Figure 42). Plasma PP\(_i\) levels in humans having ANKH deficiency (CMD patients) may also be reduced, but this remains to be confirmed experimentally.
Fig. 42. **Plasma PP, in Ank, Abcc6 and Enpp1 deficient mice.** Plasma PP$_i$ is significantly decreased in each strain, compared to wt animals (in the order of appearance, 1.53±0.24, 1.03±0.28, 0.64±0.3 and 0.05±0.01 µM). Reduced PP$_i$ levels observed in Ank$^{KI/KI}$ mice suggest Ank may be the source of non-Abcc6 mediated ATP release, which is the source of EC PP$_i$ produced in Abcc6$^{-/-}$ mice. Mean ± SEM values are shown. \( p \)-values were calculated by Mann-Whitney test.

III. PP$_i$ as a therapeutic intervention in a non-PXE ectopic calcification disease

Neither plasma PP$_i$ levels, nor the hepatic expression of Abcc6 or Enpp1 were found to be altered in the murine model of neurogenic HO used in our experiments. In spite of this, immediate or slightly delayed (after 30 minutes) PP$_i$ treatment was effective in the prevention of trauma-induced soft tissue calcification, which is an established precursor event of HO formation [119].

These results suggest that even though the combined injury in this murine model would not cause an observable decrease in plasma PP$_i$ levels, the homeostatic plasma or local concentrations of this inhibitor may not be sufficient to prevent mineralization triggered by such major trauma. Externally supplied PP$_i$ however can transiently raise this concentration well above homeostatic levels, effectively preventing calcification in these cases too. Short-term elevation of plasma PP$_i$ appears to be well tolerated without noticeable side-effects in mice and human volunteers alike.
Both physiological and ectopic mineralization are complex processes regulated through many intertwined pathways that act to facilitate or decrease the precipitation of calcium in living tissue. A common denominator between several of these pathways is inorganic pyrophosphate (PP\(_i\)), a natural inhibitor of hydroxyapatite growth.

Pyrophosphate levels in plasma were shown to be decreased in several heritable ectopic calcification diseases including pseudoxanthoma elasticum (PXE), GACI and in animal models of CMD and HGPS. PXE develops due to mutations in the \(ABCC6\) gene, encoding a transporter expressed in the basolateral membrane of hepatocytes. Many patients harbor missense variants that result in transport-competent \(ABCC6\) affected by folding or trafficking problems, which could be corrected by pharmacological chaperones as a personalized therapeutic approach. To allow easier identification of these functional but mislocalized mutants, we have developed mEChC6, a monoclonal antibody which can detect \(ABCC6\) on the cell surface but does not react with the protein in the intracellular compartments.

Our results regarding plasma PP\(_i\) concentrations in human disease confirm previous reports of decreased plasma PP\(_i\) levels in PXE patients in the largest patient cohort investigated as of 2021, but do not indicate any association between genotype and plasma PP\(_i\) levels, or plasma PP\(_i\) levels and PXE phenotype. Since we could not detect any association between lower levels of pyrophosphate and more severe manifestation of disease in a large cohort of PXE patients, this suggests that a more complex relationship exists between patient genotypes, PP\(_i\) levels, and the observed disease phenotype.

Investigation of plasma pyrophosphate levels in scleroderma patients revealed a less pronounced, but significant decrease in the case of systemic sclerosis (irrespective of the presence of calcinosis symptoms), and whether these patients could benefit from supplementing PP\(_i\) is worth exploring.

Data obtained in a mouse model suggest a similar decrease in PP\(_i\) may be demonstrated in the rare disease CMD, but the human relevance of this observation remains to be confirmed.
Externally supplied PP\textsubscript{i} could counteract both induced and spontaneously occurring ectopic mineralization in mouse models of PXE and GACI, as well as a mouse model of trauma-induced calcification, which is recognized as a precursor event of permanent heterotopic ossification, a frequent debilitating complication of complex injuries in trauma patients.

Our results indicate that oral treatment is effective in animal models, furthermore, it appears safe and feasible in humans too; gelatin-encapsulated disodium salt of PP\textsubscript{i} was absorbed in healthy volunteers as well as PXE patients without producing any apparent deleterious effect. Amino acid derivatives of pyrophosphate exhibited equally favorable absorption profiles, but their bioavailability remains to be tested in humans. Based on these results, testing of PP\textsubscript{i} as a therapeutic intervention holds a lot of promise for patients affected by rare ectopic mineralization diseases: clinical trials to assess PP\textsubscript{i} treatment in PXE [156] and systemic sclerosis-related cutaneous calcinosis [157] have already been registered.
Összefoglalás

Az élő szervezetben a mineralizáció fiziológiai és patológiai formái komplex folyamatok, amelyeket a kalcium élő szövetben való kicsapódását serkentő vagy gátló útvonalak összefonódása szabályoz. Ezekben közös nevezőt jelent a pirofoszfát (PP), egy egyszerű szervetlen molekula, ami a hidroxiapatit kristály növekedés természetes inhibitora.

A vérplazmában mérhető pirofoszfát koncentrációjának csökkenése kimutatható több ektopikus meszesedéssel járó genetikai betegség esetében: ezek közé tartozik a pseudoxanthoma elasticum (PXE), a csecsemőkori általános artériás meszesedéssel járó GACI, illetve hasonló eltérés mutatható ki a cranio-metaphysealis dysplasia (CMD) és a Hutchinson-Gilford-progéra szindróma (HGPS) állatmodelljeiben is. A PXE az ABCC6, egy májsejtek basolateralis membránjában kifejeződő transzporter génjét érintő mutációk miatt alakul ki. Számos beteg hordoz olyan missense mutációkat, amelyek transzportképes fehérjét kódolnak ugyan, de azok folding illetve trafficking problémák miatt mégsem jelennek meg a plazmamembránban. Ezekben az esetekben a funkció helyreállítható farmakológiai chaperonokra épülő személyre szabott terápia révén, ami képes helyreállítani a normális lokalizációt. A hibás szubcelluláris lokalizációt mutató missense variánsok könnyebb azonosítására létrehozottak az mEChC6 elnevezésű egér monoklonális antitestet, ami sejten kívüli epitóppal reagál, ezért kizárólag a sejtfelszínen kifejeződő ABCC6-ot detektálja.

A plazma pirofoszfát meghatározására irányuló vizsgálataink megerősítették a PXE betegekben a csökkent PP, szintet leíró irodalmi adatokat az eddigi legnagyobb vizsgált beteg kohort esetében, mindazonáltal nem találtunk összefüggést sem a betegek genotípusa és plazma PP, szintje, sem pedig a PP, szint és a kialakuló PXE fenotípus súlyossága között. Mivel nem sikerült összefüggést igazolnunk a pirofoszfát szint és a PXE klinikai maniszfesztációi között ebben a nagy létszámú kohortban, ez arra utal, hogy a genotípus, a plazma pirofoszfát és a megfigyelt fenotípus közötti kapcsolat komplexebb, mint feltételeztük.

A plazma pirofoszfát szint vizsgálata a szisztémás sclerosis betegek esetében kisebb mértékű, de szignifikáns csökkenést mutatott ki a sclerodermás kohortot hasonló kor- és nem eloszlású egészséges önkéntesekhez viszonyítva. A megfigyelt különbség a kután calcinosist mutató és nem mutató sclerodermás betegcsoport esetében egyaránt
fennállt, ezért a pirofoszfát pótlásának terápiás hatását ebben a betegcsoportban is érdemes tovább vizsgálni.

A betegség egér modelljének segítségével gyűjtött adatok alapján hasonló eltérés állhat fenn a CMD esetében is, de ezt a megfigyelést egyelőre nem tudjuk humán adatokkal alátámasztani.

A pirofoszfát kezelés képes volt az ektopikus meszesedést csökkenteni mind a PXE, mind a GACI egér modelljeiben, legyen szó akár indukált, akár spontán kialakuló kalcifikációs fenotípusról. Hasonlóan pozitív eredményeket értünk el a permanens heterotopikus csontképződés előfutárának tekinthető trauma-indukált meszesedés esetében, ami a komplex sérülések gyakori súlyos szövődménye.

Kísérleteinkben a szájon át adott pirofoszfát kezelés több betegség állat modelljében is hatásosnak bizonyult, továbbá emberek esetében is biztonságos és egyszerűen megvalósítható; a zselatin kapszulákba töltött PPı dinátrium só a PXE betegekben hasonlóan jó felszívódást mutatott, negatív hatások nélkül, mint az egészséges önkéntesek esetében. A pirofoszfát aminosav-származékai kedvező felszívódási profil mutattak egerekben: ezeket az eredményeket további humán vizsgálatokkal tervezzük megerősíteni. Eredményeink alapján a PPı terápiás alkalmazása igéretes lehetőség ektopikus kalcifikációval járó ritka betegségek gyógyításában: ennek megfelelően már két klinikai kísérletet is regisztráltak, amelyek PXE [156], illetve calcinosisos scleroderma [157] betegek esetében vizsgálják pirofoszfát kezelést.
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7 Asterisk (*) indicates shared first authorship, and # indicates shared last authorship


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