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PXE, a mysterious inborn error clarified..

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Since Garrod deduced the existence of inborn errors in 1901, a vast array of metabolic diseases has been identified and characterized in molecular terms. Anno 2018 it is hard to imagine that there is any uncharted backyard left in the metabolic disease landscape. Nevertheless, it took until 2013 to identify the cause of a relatively frequent inborn error, *Pseudoxanthoma elasticum* (PXE) a disorder resulting in aberrant calcification. The mechanism found was not only biochemically interesting, but also points to possible new treatments for PXE, a disease that has remained untreatable. In this review we sketch the tortuous road that led to the biochemical understanding of PXE and to new ideas for treatment. We also discuss some of the controversies still haunting the field.

PXE, the disease

PXE is caused by a late-onset generalized calcification of the body, most prominently affecting skin, blood vessels and the eye, as illustrated in Fig. 1. The symptoms result from the precipitation of amorphous calcium phosphate, gradually remodeled into hydroxyapatite. Usually the disease develops slowly, but the clinical course is highly variable. Some patients are spared the vision defects and cardiovascular symptoms until they pass 60, but there are also rare cases with fulminant calcification and cardiac infarction in young infants [1,2]. Attempts to slow down the disease process with dietary or pharmacological interventions have been unsuccessful until very recently [2,3].

PXE was long known to be an autosomal, recessive inborn error with a frequency of 1:50,000. A search for the gene defect(s) responsible for the disease, vigorously supported by the active PXE patient association PXE International, led in 2000 to the unexpected discovery that PXE is caused by defects in the gene encoding the ABC-transporter Multidrug Resistance Protein 6 (MRP6), now known as ABCC6 [4–6]. This was a surprise, because it was not obvious how a drug transporter, mainly found in liver and kidney [7],

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could affect calcification of peripheral tissues. This paradox initiated the first *PXE* controversy: some investigators found small amounts of ABCC6 in peripheral tissues [8] and postulated that this peripheral ABCC6 was crucial for explaining the disease, the “*PXE* cell” hypothesis; others focused on the liver, where the bulk of the ABCC6 is, and proposed that *PXE* is a metabolic disease [9]. This would be caused by the absence of an unknown compound secreted by ABCC6 from the liver into the circulation and counteracting peripheral calcification, as depicted in Fig. 2.

***PXE* is a metabolic disease**

As in many inborn errors, *Abcc6* KO mouse models that faithfully reproduce essential features of the human disease have been important for detailed mechanistic studies on the biochemical basis of *PXE* [10,11]. We mention here only a few key experiments:

Jiang et al used an *in vitro* calcification assay of cultured smooth muscle cells to demonstrate that serum from *PXE* patients and mice was unable to prevent calcium-phosphate precipitation, whereas normal serum did [12]. This suggested that normal serum contains an anti-calcification factor diminished in patients. This was confirmed in experiments with parabiotic mice. Linking the circulation of a wild-type mouse with that of an *Abcc6*^{-/-} mouse, counteracted the peripheral calcification in the *PXE* mouse, whereas linking two *Abcc6*^{-/-} mice had no effect on disease symptoms [13]. Jiang et al then used tissue transplantation experiments to test whether local tissue factors contributed to the calcification in *Abcc6*^{-/-} mice. One of the first tissues to be affected by calcification is the connective tissue capsule of the vibrissae (whiskers) in the *Abcc6*^{-/-} mice [11]. This is easily detected already at 12 weeks after birth. When Jiang et al transplanted muzzle skin of *Abcc6*^{-/-} mice onto the skin of wild-type mice, the calcification was strongly diminished. Conversely, transplantation of the muzzle skin of wild-type mice onto *Abcc6*^{-/-} mice resulted in calcification of the normal muzzle transplant [9]. To most investigators these experiments suggested that the absence of circulating serum factors in *PXE* was far more important for the peripheral calcification than local factors in the calcifying tissues. Hence, these experiments strengthened the basis for the “metabolic hypothesis” for *PXE* (Fig. 2) and led to a search for the putative factor X secreted by ABCC6 from the liver into the circulation. The liver contains the bulk of ABCC6 in the body and its importance in *PXE* was also suggested by incidental observations on human liver transplantations resulting in *PXE*. Transplanted livers thought to be devoid of ABCC6 resulted in full-blown *PXE* in the recipients, even though the *ABCC6* genes in the recipients were normal [14]. Unfortunately these intriguing observations were not conclusive, as data showing that the transplanted livers were deficient in ABCC6 were not obtained, nor the genotype of the liver donors, who were surmised to be ABCC6-deficient.

Not everybody was convinced by the evidence for the “metabolic hypothesis”, however, and we return to the arguments of these dissenters in the section on controversies.

Will the real substrate of ABCC6 please stand up?

The “metabolic hypothesis” depicted in Fig. 2 predicts that ABCC6 transports an unknown anti-calcification compound X. What might X be? The known properties of ABCC6 provided clues. ABCC6 belongs to the large ABCC family of drug transporters [15,16]. All family members are promiscuous organic anion transporters of some sort and there was no reason to assume that ABCC6 would be wildly different from the others. *ABCC6* is directly adjacent to *ABCC1* on chromosome 6p13 and looks like its non-identical twin-brother created in evolution by gene duplication. Both genes have the same exon-intron structure with 31 introns, even though their amino acid sequences are only 54% identical [17]. *In vitro* vesicular transport experiments provided the first list of substrates transported by ABCC6. The principle of the assay is depicted in Fig. 3 and the substrates found were an odd and uninspiring lot: BQ-123, an anionic cyclopentapeptide and endothelin receptor antagonist [18]; leukotriene C4 (LTC4); N-ethylmaleimide S-glutathione (NEM-GS) [19] and S-(2, 4-dinitrophenyl)glutathione [20]. All organic anions of some sort, as expected for an authentic ABCC-type transporter. These transport experiments were useful to prove that *PXE* was connected to the ATP-dependent transport function of ABCC6, as disease-causing mutants inactivating the ATP-binding catalytic site were found to be transport-inactive [19]. However, none of the substrates looked as if it could help prevent calcification. Moreover, none of these compounds was transported at the rates obtained with physiological substrates of other ABCC transporters. Hence, most investigators remained unconvinced that the real physiological substrate of ABCC6 had been found.

This conundrum came to the attention of the entire transporter field at the ABC-transporter meeting in Innsbruck in 2008. In a session dedicated to the mystery of *PXE* Jouni Uitto presented his persuasive evidence that *PXE* is due to the absence of functional ABCC6 from the liver, whereas the other speakers were unable to come up with a plausible candidate for the ABCC6 substrate able to counteract calcification. This enticing discrepancy induced a brain wave in one of us (PB), as an interested outsider sitting in the audience: “The unknown substrate X is a vitamin K derivative!”. Biochemical brain waves are usually wrong and this one was no exception. The hypothesis was plausible enough to get it published [21] and it made sense to investigators in the field, as there are plasma proteins known that bind calcium and that require vitamin K for their post-translational modification. Indeed, several labs started to test the vitamin K hypothesis and proved it conclusively incorrect [22–25]. Nevertheless, vitamin K has remained an interesting player in the background, as vitamin K is an essential cofactor of the posttranslational gamma carboxylation of several Ca-binding proteins [26]. Moreover, the disease known as *PXE-like calcification syndrome* is caused by mutations in the *GGCX* gene (gamma-glutamyl carboxylase) and not in the *ABCC6* gene [27].

Fortunately, incorrect hypotheses can sometimes start a detour on the garden path leading to the correct answer. KvdW, the postdoc involved in the ABCC6 experiments in Amsterdam, had set up a new line of research in the Borst lab, -a search for physiological substrates of transporters using metabolomics-, and this had resulted in a large catalogue of new substrates for ABCC2 and 3 [28,29]. It seemed probable that metabolomics would also be

able to unearth the elusive ABCC6 substrate, factor X. And so it did, but it was no plain sailing.

Finding the physiological substrate of ABCC6

ABCC6 is located in the basolateral membrane of the hepatocyte [7,30], where it secretes its substrate(s) into the circulation [9]. To find these substrates, the obvious experiment was to compare all compounds in plasma from *PXE* patients and their non-affected controls. Experiments with humans are complicated and Jansen and vdW [31] therefore used one of the mouse *PXE* models [10] for a systematic metabolomics analysis of *Abcc6*^{+/+} and *Abcc6*^{-/-} plasma. No differences were found.

After 2 years of negative experiments with increasingly sophisticated metabolomics methods, a change of tactics finally led to positive results. HEK cells were transfected with constructs encoding human or rat ABCC6 and the supernatants of the transfected cells were compared with supernatants of cultured untransfected cells. The first indication that this approach would work came when the supernatants of the transfected cells were shown to inhibit calcification in the *in vitro* calcification assay, whereas the supernatants of control cells did not. Metabolic analysis showed that a range of ABCC6-specific compounds were coming out of the transfected cells, most prominently AMP and GMP [31]. None of the extruded compounds could easily be imagined to inhibit calcification. We quickly realised, however, that cells often contain powerful ectonucleotidases that convert nucleosidetriphosphates (NTPs) into the corresponding nucleosidemonophosphates (NMPs) and pyrophosphate (PPi), a known inhibitor of calcification [32]. PPi does not show up in the metabolomics analysis, but a dedicated PPi assay confirmed that the transfected HEK cells produced large amounts of PPi. If an inhibitor of the ectonucleotidase was added to the transfected cells, the levels of PPi went down and ATP appeared in the cell supernatant [31]. This proved that ABCC6 mediates the extrusion of ATP.

The crucial importance of PPi in *PXE*

The Amsterdam group backed up their initial findings by showing that plasma PPi was only about 40% of normal in the *Abcc6*^{-/-} mice [31], as well as in *PXE* patients [33]. With liver perfusion experiments Jansen *et al.* showed that mouse liver secretes substantial amounts of PPi into the circulation, but not in the *Abcc6*^{-/-} mice (Fig. 4). Virtually no ATP was detected in the perfusate of the wild-type livers, but Jansen *et al.* attribute this to a rapid conversion of ATP into AMP and PPi once it enters the circulation and meets the ecto-nucleotidase in the endothelial lining [33]. The results of Jansen *et al.* in the mouse have fully been duplicated in the recently produced *Abcc6* KO rat [34]. In addition, Li *et al.* showed that the rat liver produced the bulk of the plasma PPi. The kidney contains only 5% of the *Abcc6* RNA of the liver and a corresponding small amount of PPi came out of the kidney in perfusion experiments. A crucial role for PPi is plausible, as plasma PPi is normally kept in a narrow range, whereas elevated or abnormally low PPi can result in serious disease, as shown by inborn errors leading either to elevated or lowered plasma PPi [35]. The near complete absence of PPi in the plasma of *GACI* patients [36], usually caused by homozygous loss of the gene for ENPP1, leads to devastating calcification already early in life [37]. This disease

also shows that ATP is by far the most important source of PPi in plasma both in humans and in mice [36,38]. Other sources exist, as discussed below, but the near-complete loss of PPi in ENPP1 deficiency shows that hydrolysis of NTPs by ENPP1 is essential for generating plasma PPi. The crucial role of PPi in *PXE* is also supported by animal experiments showing that supplementation of PPi can delay the appearance of calcification in *Abcc6* mice (see below) [39,40].

Although the available evidence shows that PPi is crucial in calcium-phosphate homeostasis, PPi is clearly not the only player. The hydrolysis of ATP also yields AMP, which contributes to maintaining plasma PPi as well. We return to an overview of all players below.

Doubts and controversies

From the start the ABCC6 field has been plagued by errors of experiment and interpretation. The *ABCC6* gene entered the lime-light as the *anthracycline resistance-associated gene* (*ARA*), a factor associated with multidrug resistance of cancer cells [41]. Because the cDNAs found to be associated with resistance did not encode a complete ABC-transporter, the association looked suspicious from the start. Experiments in Amsterdam then showed that the *ABCC6* gene is directly adjacent to the *ABCC1* gene and that its 3'-end was often coamplified with *ABCC1* in cell lines made resistant to doxorubicin [17]. Since the complete *ABCC1* gene was co-amplified with the truncated *ABCC6* gene in the amplicons and since *ABCC1* was known to confer resistance to doxorubicin, it was obvious that the MDR connection of *ABCC6* was a spurious one, a conclusion confirmed in later work.

A second controversy emerged when the results of Jansen *et al.* [31,33] on diminished PPi in *PXE* patients and mice, were initially not reproduced in other labs. This became clear in heated discussions during a *PXE* meeting in 2014. In the end this controversy boiled down to a technical problem. PPi determinations are complicated and there are many pitfalls. For instance, minor damage to platelets will swamp the plasma with PPi [42,43]. Indeed, published plasma values for PPi can be off by an order of magnitude [44,45]. Once KvdW had gone round the world to teach the proper way to determine PPi, full agreement was reached.

Some investigators questioned whether a relatively minor decrease in plasma PPi, to about 40% of normal both in *PXE* mice and *PXE* patients [31,33], was sufficient to explain calcification of peripheral tissues. The plasma PPi concentration is micromolar, whereas Ca^{2+} and phosphate are in the millimolar range. How can a little more or less PPi make a difference? The explanation of this paradox was found long ago: PPi coats hydroxyapatite crystals and prevents their further growth [35]. Apparently, micromolar PPi is sufficient to accomplish this feat, 40% of normal levels are not. This delicate balance may also explain why the symptoms of *PXE* are so variable. There are several sources of plasma PPi besides *ABCC6* (see below), and the rate of PPi degradation could also vary (normal $t_{1/2}$ about 0.5 hour), depending on the genetic background.

A long-standing controversy in the *PXE* field concerns the potential contribution of peripheral *ABCC6* to the prevention of symptoms [9,46]. Extensive data support the activity

of some ABCC6 in cultured fibroblasts [47,48], but it has not been shown conclusively that fibroblasts in tissues also contain ABCC6. Culture can induce artifactual gene expression and most experiments with tissue RNA or protein have given negative results. The issue is complicated by a commercial antibody against ABCC6, used by some labs as the only antibody to detect the protein [49]. Detailed analysis of this antibody by one of us (AV) has shown that it does not react with ABCC6 at all (Report available on request). On the basis of this work the company has withdrawn the antibody.

Another controversy came with the publication of a high-profile paper in Science Translational Medicine [50], challenging the central role of PPI in the circulation and of ABCC6 in the liver in *PXE*. The authors find that knocking out *Abcc6* in the *Enpp1* KO mice has no effect on the (severe) calcification or on plasma PPI and they conclude that ABCC6 must therefore be DOWNSTREAM of ENPP1. We find this conclusion incomprehensible. Plasma PPI virtually disappears in the *Enpp1* KO mouse [38], as this enzyme is the only significant contributor to plasma PPI. If ATP cannot be converted into PPI, providing more ATP through ABCC6, whether upstream (where it is), or downstream of ENPP1, cannot have any influence on plasma PPI.

The second argument presented by Ziegler *et al.* [50] rests on experiments in which functional ABCC6 is eliminated by disruption of the gene by the Cre recombinase. When the *Cre* construct is expressed throughout the body, the mice develop full-blown *PXE*. If a liver-specific albumin gene promoter is used, the mice are initially fine and only develop more calcification than WT mice after one year. The authors conclude that this experiment “excluded the prevailing pathogenic hypothesis that singularly invokes failure of hepatic secretion of an endocrine inhibitor of calcification” (i.e. PPI). It is notoriously difficult, however, to get complete gene disruption in all cells of a tissue with promotor-driven *Cre* and the CMV promotor, used for body-wide gene disruption, is far more active than the albumin promotor [51]. We therefore expect that the putative liver-specific KO of *Abcc6* produced by Ziegler *et al.* [50] is incomplete. It is known that the *PXE* symptoms in the *Abcc6*^{-/-} mouse can be mitigated by a rather inefficient transfection of *Abcc6* cDNA into the liver, resulting in a very limited restoration of liver *Abcc6* expression [52,53]. Obviously, results of a liver-specific KO of *Abcc6* can only be interpreted if the KO is shown to be near-complete. The issue will eventually be settled by liver transplantation experiments. These are hard to do in mice, but should be doable in the recently produced *Abcc6* rat KO [34].

Another curious controversy erupted with the publication of a high-profile paper in Circulation Research [54]. This paper reported an unusual localisation of ABCC6 in heart in an ill-defined para-mitochondrial structure and the authors presented imaginative speculations about the possible function of ABCC6 in this new location. In the early days of ABCC6 research there was some controversy about its cellular location [18], but with the generation of mono-specific, high-affinity MABs the transporter was conclusively localised in the apical membrane of liver and kidney cells [7]. The intracellular location of ABCC6 reported by Martin *et al.* [54] in heart was therefore completely unexpected. A detailed reinvestigation of the issue with multiple MABs, confirmed, however, that ABCC6 is only in the plasma membrane [30]. Martin *et al.* were not convinced [55], but they are probably the only investigators in the field who still believe in para-mitochondrial ABCC6.

Finally, there is the ongoing controversy, already mentioned, about the possible contribution of peripheral ABCC6 to the prevention of *PXE*. There is no doubt that cultured *PXE* fibroblasts behave differently from their normal cultured counterparts but there is also no unambiguous evidence for the presence of ABCC6 or *ABCC6*RNA in connective tissue. The question remains therefore whether CULTURED *PXE* fibroblasts differ from fibroblasts *in situ*, for instance, because the culture conditions induce ABCC6 expression; or alternatively, whether available methods fail to detect *ABCC6* (RNA) *in situ*. The issue was re-investigated recently by Li *et al.* [34] in their rat *Abcc6* KO, using a sensitive RT-PCR method. *Abcc6* RNA was abundant in liver, much less in kidney (5% of liver) and undetectable in peripheral tissues, including tissues affected by calcification in *PXE* patients, rats and mice [34]. Unfortunately, the authors did not titrate in decreasing amounts of liver RNA in the tissue samples to determine how low undetectable was, 1%, 0.1%, or even lower than liver RNA. Rats are no humans, but we think that the results of Li *et al.* further detract from the concept that peripheral ABCC6 exists and is significant in preventing *PXE*.

Known players in calcium phosphate homeostasis that might affect the severity of symptoms in *PXE* patients.

PPI homeostasis

ABCC6 is part of a network keeping plasma PPI levels within the physiological range of 1.5–3 μ M in healthy individuals. Fig. 5 shows the major participants in this network. The crucial role of ENPP1 in PPI homeostasis was already stressed. *Enpp1*^{-/-} mice virtually lack PPI in their blood circulation [38]. All PPI present in the circulation must therefore be produced by ENPP1 from NTPs, predominantly ATP, released into the extracellular milieu and converted into their respective monophosphate and PPI. In contrast, PPI plasma levels in *PXE* patients are about 40% of those found in healthy individuals [33]. This remaining plasma PPI must therefore come from sources of extracellular NTPs that are independent of ABCC6 activity. Other pathways used by cells to get ATP out of the cell include a range of non-specific ion channels like pannexins, connexin hemichannels and maxi-anion channels, in addition to exocytosis of ATP-loaded vesicles [56]. The relative contributions of each of the alternative ATP conduits to plasma PPI concentrations is unknown and warrants further study, as ABCC6-independent ATP release pathways might represent potential pharmacological targets to increase ATP release and PPI formation in *PXE* patients. Differential activity of these alternative ATP conduits might also contribute to the variation in plasma PPI concentrations seen in *PXE* patients [33].

PPI is predominantly degraded by Tissue Non-specific Alkaline Phosphatase (TNAP). This enzyme is absent in hypophosphatasia patients and consequently these have high levels of PPI in their plasma and suffer from life-threatening hypomineralization of their bones [57,58]. People with only one functional allele of the gene encoding TNAP do not have clinical manifestations of hypophosphatasia. Inhibition of TNAP represents therefore a potential way to increase plasma PPI in *PXE* patients.

A third layer of regulation of plasma PPI is formed by CD73, an ecto-enzyme that converts adenosine monophosphate (AMP) into adenosine and Pi [59]. Adenosine is an endogenous

TNAP inhibitor and absence of CD73 results in reduced local adenosine formation, higher plasma TNAP activity, lower PPI plasma levels and, consequently, mineralization of arteria, especially in the legs, a disease known as *Arterial Calcification Due to the Absence of CD73 (ACDC)*.

Fetuin-A

Fetuin-A is a major liver-derived plasma protein with an important function in mineral homeostasis. Monomeric fetuin-A binds to amorphous calcium phosphate micro-precipitates, thereby forming colloidal protein-mineral clusters that are also known as “calciprotein particles (CPPs)”. These CPPs prevent microcrystals from further growth and keep them in solution till they are filtered out of the circulation by macrophages [60]. *PXE* patients have reduced serum Fetuin-A concentrations [61]. This might be caused by increased consumption of Fetuin-A to deal with the increased formation of calcium phosphate micro-precipitates due to reduced PPI. In line with this hypothesis the overproduction of fetuin-A in livers of *Abcc6*^{-/-} mice substantially reduced the ectopic mineralization phenotype [62]. The severity of the calcification phenotype seen in *fetuin-A*^{-/-} mice depends to a large degree on the genetic background: On a C57C6 background there is no substantial calcification, whereas in DBA/2 mice, which harbor an inactivating mutation in both *Abcc6* alleles, absence of Fetuin-A results in massive calcification [60]. This further demonstrates the interaction between the different systems that are in place to prevent soft tissue mineralization.

Matrix-Gla protein (MGP) and Gamma-glutamyl-carboxylase (GGCX)

Vascular smooth muscle cells (VSMCs) produce and secrete MGP. Patients lacking MGP suffer from the Keutel syndrome, a mineralization disorder characterized by calcification of arteries and cartilage [63]. This phenotype is reproduced in *Mgp*^{-/-} mice [64]. How MGP exactly prevents calcification is unknown. It needs to undergo two posttranslational modifications, phosphorylation and glutamate carboxylation to become fully active. Interaction via these negatively charged ligands with micro-crystals might prevent hydroxyapatite formation, but inactivation of the osteogenic growth factor bone-morphogenic protein 2 (BMP2) has also been proposed to explain MGP action [65]. The carboxylation of MGP is catalyzed by gamma-glutamyl-carboxylase (GGCX). Patients with defects in the gene encoding this GCCX present with mild connective tissue calcification, in addition to clotting defects [27]. As GCCX needs vitamin K as a cofactor, insufficient intake of vitamin K results in undercarboxylation of MGP and increased vascular calcification [26]. Inhibition of vitamin K dependent carboxylation with warfarin similarly induces vascular calcification [66], but the partial inhibition achieved in patients on coumarin-type anti-coagulants does not seem to contribute to arterial calcification.

New (and old) attempts to treat *PXE*

With the discovery that low plasma PPI plays a central role in *PXE*, new therapeutic interventions came in focus. Fig. 6 summarizes the main players now known to affect plasma PPI and possible ways to alter these. The most obvious one is supplementation of PPI

itself. Indeed, intraperitoneal administration of PPI to *Abcc6*^{-/-} mice fully inhibited acute dystrophic cardiac calcification [39], proving that PPI supplementation therapy could work. Intraperitoneal administration is not practical in human *PXE* patients that need lifelong treatment. The alternative, oral administration of PPI, did not seem to make any sense, as the oral availability of PPI was thought to be zero [35]. The highly charged PPI cannot be expected to pass through membranes and is susceptible to prompt hydrolysis by intestinal phosphatases. Nevertheless, large doses of oral PPI in drinking water were found to raise plasma PPI and to result in significant attenuation of calcification in both the *PXE*- and in the *GAC1* KO mice. Elevation of plasma PPI was also achieved in humans, even though only 0.3 % of the ingested dose reached the circulation [40]. This tiny fraction substantially increased plasma PPI concentrations, however, to levels that are expected to inhibit the soft tissue mineralization seen in *PXE* patients and in several other hereditary ectopic calcification disorders. This encouraging result points the way to an effective, simple, and low-cost medication for these patients. In fact, clinical trials to test oral PPI are already planned in different countries.

The acute risk of oral administration PPI to patients is probably negligible as the compound is abundant in our food and considered safe by the FDA. However, there is no experience yet with hefty oral doses of PPI taken for years. Abnormally elevated plasma PPI levels are known to induce defects in bone calcification and very high doses of PPI also yield lots of Pi, which promotes calcification. The therapeutic window for PPI is therefore not unlimited. It is not known either during which fraction of the day the PPI should be normalized to get an optimal therapeutic effect. Is once in 24 hours sufficient to prevent calcification (as animal experiments suggest)? Moreover, more attractive ways of administering PPI should be found than the large volumes of an unpleasantly tasting solution used thus far, if kids need to be seduced to take their daily PPI. We are enthusiastic about this new therapy, but it is early days and oral PPI still needs to prove its long-term usefulness.

An obvious alternative to PPI are the bisphosphonates, non-hydrolysable PPI analogues developed long ago for the treatment of osteoporosis and bone metastases (for a review, see [67]). The potential of these drugs to prevent *PXE*-related calcification was addressed by feeding *Abcc6*^{-/-} mice with chow containing two different bisphosphonates, etidronate disodium (ETD) or alendronate sodium trihydrate (AST) [68]. High doses of ETD significantly reduced mineralization, while AST had no effect. These encouraging results have led to a prospective clinical trial of etidronate treatment in adult *PXE* patients [3]. After one year the treated patients had moderately less calcification symptoms than the controls. This is encouraging in the absence of other treatment options, but it remains to be seen whether the improvement can be maintained over longer periods and whether the (modest) side-effects [69,70] of the drug remain acceptable.

It is obvious from Fig. 6 that another way to increase plasma PPI is the inhibition of PPI degradation by TNAP. Indeed, recent papers reported that an orally bioavailable TNAP inhibitor attenuated both the development and progression of calcification in *Abcc6*^{-/-} mice *in vivo* [50,71]. Ziegler *et al.* [50] found that plasma PPI level did not increase upon TNAP inhibition treatment, although plasma TNAP activity was decreased. This is surprising in the light of previous results demonstrating profound effects of TNAP activity on plasma PPI.

The absence of NT5E results in increased TNAP activity and in *Nt5e*^{-/-} mice plasma PPI concentrations were indeed reduced [45]. In contrast, the absence of TNAP in the inborn error hypophosphatasia leads to high plasma PPI concentrations and a (lethal) block in bone calcification [57,72]. One would therefore expect that inhibiting the PPI hydrolytic activity of TNAP should increase plasma PPI concentrations. This was not found in the mice treated with a TNAP inhibitor. To substantially increase plasma PPI concentrations TNAP most likely needs to be inhibited more than the 50–60% achieved by Li et al. [71]. In *Enpp1*^{-/-} mice subcutaneous administration of ENPP1 dramatically increases plasma PPI concentrations [73]. As there is competition between various ectonucleotidases for available ATP [74], increasing ENPP1 activity in plasma could result in a larger fraction of released ATP being converted into PPI. Studies are now ongoing to find out if ENPP1 enzyme substitution in *Abcc6*^{-/-} mice prevents disease progression. Indications that this approach might actually work came from a recent study showing that ectopic mineralization was greatly reduced in *Abcc6*^{-/-} mice expressing high levels of human ENPP1 [75].

Several attempts have been made to modify the clinical progression of *PXE* by changing the mineral content of the diet. Variations in dietary calcium levels do not affect calcification in *Abcc6*^{-/-} mice [76], possibly because plasma calcium levels are very tightly regulated. Plasma phosphate concentrations are more variable and increasing phosphate in the diet of *Abcc6*^{-/-} mice accelerated ectopic calcification [77]. Despite this clear effect in mice, phosphate binders do not prevent disease progression in mice or humans [78,79]. Why, is unclear. In contrast, increasing the magnesium content of the diet does have an effect, at least in mice. It has long been known that Mg²⁺ counteracts soft tissue mineralization (for a recent review, see Braake et al, 2017 [80]), and increasing Mg²⁺ in the diet completely prevented ectopic mineralization in *Abcc6*^{-/-} mice [76,81]. A prospective Mg-supplementation clinical trial in *PXE* patients (<https://clinicaltrials.gov/ct2/show/NCT01525875>) was completed in March 2015. The results have still not been published and it seems therefore unlikely that spectacular improvements can be obtained in *PXE* patient with dietary magnesium supplementation.

A different route to elevate plasma PPI concentration, indicated in Fig. 6, is to increase ABCC6 activity in the liver. Most mutations in *PXE* are missense, and many of the mutant proteins preserve transport activity, but cause intracellular retention [52]. It has been shown for other large transport proteins that the chemical chaperone 4-phenylbutyrate (4-PBA) promotes the routing of missense mutant proteins to the plasma membrane [82]. Pomozi *et al.* [83] found that this is also the case for ABCC6 mutant proteins. In a humanized mouse model of *PXE*, transiently expressing these human ABCC6 trafficking mutants, 4-PBA administration was able to inhibit calcification [53]. Therapeutic use of 4-PBA therefore looks like a promising strategy for allele-specific therapy of ABCC6-associated calcification disorders [53]. The studies with other defective transporters have already shown that long-term therapy with 4-PBA is well tolerated, even though huge doses of 4-PBA were needed for correction [82,84]. Nevertheless, other pharmacological correctors are needed because 4-PBA was found to be active only in certain missense mutants (in 3 out of randomly selected 7). Correctors with different selectivities would be required, since there are a large array of disease-causing mutations, resulting in intra-cellular retention.

Another allele-specific approach is to “read through” premature termination codons (PTCs) present in some *ABCC6* gene mutants. The R1141X (a PTC-type) mutation is the most frequent disease-causing variant in *PXE* [85] and *in vitro* studies demonstrated low level production of full-length protein upon treatment with PTC124, a nonsense codon read-through-inducing drug [86]. More recent studies have utilized another drug, amlexanox, which also counteracts nonsense-mediated mRNA decay, in addition to promoting PTC read-through, suggesting its potential usefulness for a subset of patients with *PXE* caused by PTC mutations [87].

It should be clear from this brief overview that the number of potential treatment options for *PXE* has considerably increased in the past years through the basic science that has elucidated the molecular defects underlying *PXE*. The treatment problem has not been solved yet, but the future for patients has certainly brightened.

Open questions and future directions

How *ABCC6* mediates ATP secretion is the most pressing question that remains to be answered, biochemically speaking. Is ATP a substrate of *ABCC6*, or is *ABCC6* an activator of another transporter/channel? This looks like a simple question, easily answered by vesicular transport experiments, but it is not. Enzymes using ATP as substrate generally have a low affinity for ATP, as this substrate is present in millimolar concentrations in normal cells [88]. ABC-transporters are no exception to this elementary principle [15]. This makes it hard to find the minute amounts of ATP that might be transported by *ABCC6* in the presence of the vast amounts of ATP required to drive transport. As the members of the *ABCC* family of drug transporters prefer organic anions [15], ATP is not an implausible substrate for *ABCC6*. There are also *ABCC* family members, however, that regulate channels [89]. Since there are several channels mediating export of ATP from cells, such an indirect effect of *ABCC6* is not *a priori* improbable, although a channel specific for large molecules, such as NTPs, would be a novelty. Experiments are in progress to find out how *ABCC6* actually works.

Unsolved is also how plasma PPi is controlled. The study of inborn errors has shown that production of PPi cannot be drastically decreased if degradation is severely diminished, nor can degradation be slowed down if supply fails. Nevertheless, we find it hard to believe that there is not some sort of feed-back control to fine-tune the plasma level in the narrow range found (and required to avoid disease). Both experiments on humans and mice would greatly benefit from a robust micro-determination for PPi, as the presently available assays are cumbersome and need relatively large volumes of plasma.

A further open question concerns the variability of symptoms in *PXE* patients [90]. This is not attributable to the degree of remaining *ABCC6* activity, as zero activity may be associated with widely variable symptoms. Genetic background must play a role given the large range of players involved in PPi metabolism (see Fig. 5). There is also experimental proof for this: several mouse strains are available with exactly the same *Abcc6* inactivating mutation. Disease severity in these animals very much depends on the genetic background of the mouse strain [91]. The intriguing recent finding that variations in dietary PPi can affect

the severity of symptoms in KO mice [40] suggests that dietary PPI could also have this effect in humans. As pointed out in the Therapy Section, PPI is used for preserving food and is even present in tooth paste. Intake could therefore vary between patients depending on food composition. Plasma PPI levels are probably also not the only factor affecting disease in *PXE* patients. Although PPI is clearly essential, the correlation between PPI and symptoms is not a simple one and other factors could therefore affect the severity of the disease, such as Mg^{2+} and phosphate intake.

A major task for the near future is the translation of recent basic insights in *PXE* pathogenesis into better treatments of patients. We expect much from the optimization of oral PPI treatment, but several other options look promising as well. We hope that in the end the *PXE* patients' association *PXE* International may see the practical fruits of its persistent support for research into the molecular basis of *PXE*.

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Abbreviations(box 1)

ABCC1	ABC-transporter C1, also known as Multidrug Resistance Protein 1 (MRP1) ABCC6, idem C6 (MRP6)
Abcc6^{-/-}	KO mice (or rats) with disrupted Abcc6 genes
ACDC	Arterial Calcification Due to the absence of CD73
ARA	the Antracycline Resistance-Associated gene
CD73	ecto-5'-nucleotidase
CMV	Cytomegalo Virus
ENPP1	ecto-nucleotide pyrophosphatase phosphodiesterase 1
GACI	Generalised Arterial Calcification of Infancy
GGCX	gamma-glutamyl carboxylase
HEK	Human Embryonic Kidney cells
Hepatocyte	main (epithelial) cell of the liver, in which ABCC6 is located in the baso-lateral membrane
LC-MS	Liquid Chromatography-Mass Spectrometry
MAB	mono-clonal antibody
MGP	Matrix-Gla Protein

NT5E	Ecto-5'Nucleotidase, also known as CD73
NTP	nucleoside triphosphate
Parabiotic mice	mice joined together and sharing one blood circulation
4-PBA	4-phenylbutyrate
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
PTC	premature termination codon
PXE	Pseudoxanthoma elasticum, an inborn error of calcium-phosphate metabolism
TNAP	Tissue Non-specific Alkaline Phosphatase
Vibrissae	mouse/rat whiskers

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Highlights (box 2)

- The hereditary mineralization disorder Pseudoxanthoma elasticum (PXE) is due to inactivating mutations in the gene encoding the hepatic efflux transporter ABCC6.
- Recent work has shown that ABCC6 in the liver mediates the secretion of ATP into the circulation, where it is rapidly converted into AMP and the calcification inhibitor pyrophosphate (PPi).
- In PXE patients, absence of functional ABCC6 leads to a substantial decrease of plasma PPi, providing a plausible explanation for their ectopic calcification.
- The discovery of the lowered plasma PPi in PXE patients has led to new initiatives to treat the disease, such as oral PPi supplementation and first generation bisphosphonates, ultra-cheap and relatively simple treatment options.
- The new treatment initiatives for PXE also offer new perspectives for the many other disturbances in calcium-phosphate metabolism for which no treatment exists.

Open questions (box 3)

- How does ABCC6 mediate ATP secretion? Is it an ATP transporter, or does it activate another transporter/channel?
- How is the fine-control of plasma PPi concentrations regulated?
- What are the main reasons for the variability in symptoms and progression in PXE patients?
- What is the function of ABCC6 in the kidney?
- Can oral PPi supplementation in PXE patients be turned into an effective and patient-friendly treatment?
- Can oral PPi also benefit patients with other inborn errors of calcium phosphate metabolism?
- Can an effective, safe genetic modification protocol be developed to repair the defective ABCC6 gene in the livers of PXE patients or to replace it with a functional copy?

Figure 1.

Clinical features and histopathology of ectopic mineralization in pseudoxanthoma elasticum (PXE). **(A)** Characteristic early cutaneous signs of PXE consist of discrete yellowish papules at predilection sites, such as sides of the neck. **(B)** These early lesions coalesce into plaques of inelastic, leathery skin. **(C)** Staining of a parallel section with von Kossa stain reveals mineralization of the elastotic structures. **(D)** Characteristic ocular findings consist of angioid streaks, breaks in Bruch's membrane behind the retina (arrows), which allow neovascularization (arrowheads) of the retina leading to loss of visual acuity and blindness. **(E)** Histopathology of the left renal artery in a GACI patient with *ABCC6* mutations reveals extensive mineralization (Hematoxylin-Eosin stain). Adapted from Li et al (2018 Am. J. Pathol accepted for publication)

Figure 2.

The “metabolic” hypothesis for PXE. ABCC6 is located in the baso-lateral membrane of the hepatocyte where it secretes a compound X into the blood circulation. X is distributed throughout the body via the circulation and hypothesised to counteract the ectopic peripheral calcification that occurs in PXE patients with non-functioning ABCC6.

Figure 3.

How membrane vesicles can be used to study transport of substrates by ABC-transporters. When cells are disrupted, the plasma membrane forms two types of vesicles, inside-in vesicles in which the transporter has its normal orientation pumping substrates out; and inside-out vesicles in which the orientation is inverted, the transporter pumping substrates into the vesicle, if ATP is provided to energise the pump. Following the incubation of the vesicles with radioactive substrate and ATP, the vesicles are washed on filters and counted. The inside-in vesicles do not contribute to the reaction, as under these conditions no ATP is present inside the vesicles to support ATP hydrolysis at the ABCC6 nucleotide-binding domains.

Figure 4.

Liver perfusion experiments showing that mouse liver produces large amounts of AMP and PP_i, but not in mice without functional ABCC6. The very low levels of ATP in the perfusates are contributed to the rapid conversion of any ATP released into the perfusion buffer into AMP and PP_i. In PXE patients reduced ABCC6-mediated ATP release results in low plasma PP_i concentrations.

Figure 5.

The known major mechanisms regulating plasma pyrophosphate level. Quantitatively the most important source PP_i is the ABCC6-mediated ATP-release from hepatocytes. This ATP is rapidly converted into AMP and to PP_i in the liver vasculature by ENPP1. The most important enzyme degrading PP_i is TNAP. The activity of this enzyme is regulated by NT5E, which cleaves AMP to adenosine and phosphate (P_i); the AMP generated inhibits TNAP, suppressing PP_i hydrolysis. Mutations in the ABCC6, the ENPP1 and the NT5E genes cause various calcification diseases. (For more details see text).

Figure 6. Current therapeutic attempts to counteract the ectopic calcification in PXE patients. Interventions targeting different steps in the regulation of ectopic mineralization are indicated by arrows. Yellow block arrows indicate preclinical interventions, blue arrows ongoing or recently completed clinical trials. (For more details see text).