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ABCC6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation – Brief report

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Running Title
ABCC6 mediates hepatic ATP release

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Abstract

Objective
Mutations in *ABCC6* underlie the ectopic mineralization disorder pseudoxanthoma elasticum (PXE) and some forms of generalized arterial calcification of infancy (GACI), both of which affect the cardiovascular system. Using cultured cells, we recently showed that ABCC6 mediates the cellular release of ATP, which is extracellularly rapidly converted into AMP and the mineralization inhibitor inorganic pyrophosphate (PP_i). The current study was performed to determine which tissues release ATP in an ABCC6-dependent manner *in vivo*, where released ATP is converted into AMP and PP_i, and whether human PXE patients have low plasma PP_i concentrations.

Approach and results
Using cultured primary hepatocytes and *in vivo* liver perfusion experiments we found that ABCC6 mediates the direct, sinusoidal, release of ATP from the liver. Outside hepatocytes, but still within the liver vasculature, released ATP is converted into AMP and PP_i. The absence of functional ABCC6 in PXE patients leads to strongly reduced plasma PP_i concentrations.

Conclusions
Hepatic ABCC6-mediated ATP release is the main source of circulating PP_i, revealing an unanticipated role of the liver in systemic PP_i homeostasis. PXE patients have a strongly reduced plasma PP_i level, explaining their mineralization disorder. Our results indicate that systemic PP_i is relatively stable and that PXE, GACI and other ectopic mineralization disorders could be treated with PP_i supplementation therapy.

Non-standard Abbreviations and Acronyms

ABCC6, *ATP-binding cassette sub-family C member 6;*
ACDC, Arterial Calcification due to Deficiency of CD73;
ANKH, progressive ankylosis protein homolog;
ENPP, ectonucleotide pyrophosphatase-phosphodiesterase;
GACI, General Arterial Calcification of Infancy;
MRP6, Multidrug Resistance-associated Protein 6;
NT5E, ecto-5'-nucleotidase;
PP_i, inorganic pyrophosphate;
PXE, pseudoxanthoma elasticum

Introduction

Pseudoxanthoma elasticum (PXE) is an autosomal recessive disease characterized by progressive ectopic mineralization of the skin, eyes and arteries[1]. Approximately 150,000 PXE patients world-wide suffer from stigmatizing skin lesions, progressive loss of vision and cardiovascular complications, against which no effective therapy exists[2].

In 2000, several groups reported that PXE is caused by inactivating mutations in the *ATP-binding cassette sub-family C member 6* (*ABCC6*) gene[3-5] and more recently ABCC6 defects were also found to cause some forms of generalized arterial calcification of infancy (GACI)[6], a severe form of arterial calcification. ABCC6 (also known as Multidrug Resistance Protein 6, MRP6) is an ATP-dependent orphan efflux transporter that is primarily expressed in the liver[7]. Importantly, PXE is not caused by a lack of ABCC6 in the affected tissues, but by the absence of an unknown factor in the central circulation requiring active ABCC6[8]. Despite extensive research, the identity of this factor has long remained a mystery.

We recently showed that overexpression of ABCC6 in HEK293 cells induces the release of nucleoside triphosphates, predominantly ATP, *in vitro*[9]. Secreted ATP was extracellularly converted into AMP and the mineralization inhibitor inorganic pyrophosphate (PP_i) by ectonucleotide pyrophosphatase-phosphodiesterase (ENPP)-type ectonucleotidases. The *in vivo* relevance of these findings was demonstrated in *Abcc6*$^{-/-}$ mice, which have plasma PP_i levels less than 40% of those found in wild-type control animals. ABCC6 is a member of the ABCC (MRP) family, which contains large proteins transporting a variety of organic anions[10]. ABCC6 is mainly present in the sinusoidal membrane of the hepatocytes[11]. As we could not demonstrate direct ABCC6-mediated ATP transport *in vitro*, we postulated that ABCC6 secretes an organic anion, factor X, into the circulation that induces local ATP release in the periphery[9]. The alternative possibility that the liver directly releases ATP in an ABCC6-dependent manner seemed unlikely. Secretion of ATP over the sinusoidal membrane of hepatocytes has never been described and the extremely short half-life of ATP in the blood circulation (<1 sec)[12] does not allow PP_i formation from liver-derived ATP in the periphery. The current study was performed to show that ABCC6 affects plasma PP_i levels in humans and to assess whether ABCC6 directly affects hepatic ATP release or indirectly induces peripheral ATP release.

Materials and Methods

Methods and Materials are available in the online-only Data Supplement.

Results

We have previously shown that the introduction of ABCC6 in HEK293 cells results in the release of large amounts of ATP into the culture medium[9]. To determine whether ABCC6-dependent ATP release is cell type-dependent, we generated HeLa cells in which the expression of rat ABCC6 (rABCC6) could be induced by doxycycline. A luciferin/luciferase-based assay was used to follow the appearance of ATP in the cell culture medium in real-time. In the absence of rABCC6 cells released almost no ATP (Figure 1A and B). However, upon induction of rABCC6 both 293 and HeLa cells released substantial amounts of ATP into the cell culture medium (Figure 1). These data show that ATP release is a general feature of ABCC6-containing cells and not specific for HEK293 cells.

ABCC6 is predominantly present in the liver[11]. We therefore next explored in sandwich-cultured hepatocytes the possibility that hepatocytes directly release ATP over their basolateral membrane in an ABCC6-dependent manner. We were unable to directly detect ATP release in these experiments, presumably due to the high ectonucleotidase activity of hepatocytes. We therefore followed the appearance of the ATP metabolite PP_i in the culture medium. PP_i levels clearly increased in culture medium of wild-type hepatocytes over time, with substantially lower levels detected in medium of hepatocytes lacking ABCC6 (Figure 2A). These results indicate that hepatocytes release ATP over their sinusoidal membrane in an ABCC6-dependent manner and are also able to convert it to PP_i. We also detected some PP_i in medium from *Abcc6*$^{-/-}$ cells, which we attribute to ATP release unrelated to ABCC6, or leakage from damaged cells.

To assess whether ABCC6 is an important factor in hepatic ATP release *in vivo*, we performed liver perfusion experiments. PP_i and AMP levels in the liver perfusates strongly depended on the presence of ABCC6 (Figure 2, panels B and C). Interestingly, ATP levels did not differ between the two genotypes and were extremely low, representing less than 1% of the PP_i and AMP levels (Figure 1 D). The AMP and PP_i that we detect in the liver perfusates must be derived from ATP: *Enpp1*$^{-/-}$ mice have PP_i levels that are less than 5% of those found in wild-type mice[13], implying that also the PP_i in plasma that depends on ABCC6 must come from ATP. Conversion of released ATP into AMP and PP_i within the liver is fast. We calculated that during our single-pass perfusion experiments, the buffer is present in the liver for approximately 10 seconds (for the calculation see the Materials and Methods section). During this short period the substantial amounts of ATP released are almost quantitatively converted into PP_i and AMP (Figure 2B, C and D). This rapid and efficient conversion also explains why we were unable to detect ATP release from cultured wild-type hepatocytes: any released ATP is almost instantaneously converted into AMP and PP_i by hepatic NPP1.

From our perfusion experiments we calculate that ABCC6 mediates ~ 90% of the hepatic nucleotide release. Over 24 hours this corresponds to at least 5% of the total hepatic adenine nucleotide pool (Figure 2 B; for the calculation see the Materials and Methods section). The plasma $t_{1/2}$ of PP_i has been estimated to be 33 min, which requires a hepatic release rate of 6 nmoles PP_i per hour to achieve the steady-state levels of 2.3 µmol/L (µM) that we have reported for mice[9] (for calculation see the Materials and Methods section). Importantly, the amount of PP_i detected in liver perfusates of wild-type mice is high enough to explain these steady-state PP_i levels in mouse plasma.

An important question is whether our mouse results translate to human PXE patients. We have therefore studied a group of 12 Dutch PXE patients with known *ABCC6* mutations (Materials and Methods Table 1). The plasma PP_i concentrations were approximately 2.5-fold lower in patients than in healthy individuals (Figure 2 E). This difference did not depend on sex and is in line with the reduced plasma PP_i levels we previously reported for *Abcc6*$^{-/-}$ mice[9].

Discussion

PP_i is a key regulator of ectopic mineralization acting by inhibiting hydroxyapatite crystal growth[14]. As a result, mutations in genes encoding known PP_i-regulating enzymes like ENPP1, ecto-5'-nucleotidase (NT5E), progressive ankylosis protein homolog (ANKH) and tissue-nonspecific alkaline phosphatase (TNAP) cause various mineralization disorders[15-18]. The clinical symptoms of the mineralization disorders caused by non-functional ENPP1 (generalized arterial calcification of infancy; GACI) and NT5E (arterial calcification due to deficiency of CD73; ACDC) highly overlap those of PXE[19]. The similarity between GACI and PXE is underlined by the recent observations that both GACI and PXE can be caused by mutations in *ENPP1* as well as *ABCC6*[6]. Our data unexpectedly falsify the factor X-hypothesis[9] and show that ABCC6-mediated ATP release from the liver is the principal source of plasma PP_i. A factor involved in the local release of PP_i is ANKH, a membrane protein postulated to mediate the direct release of PP_i from cells. ANKH does, however, not substantially contribute to plasma PP_i levels, which almost exclusively depend on ENPP1 activity and hence ATP release[13]. Based on the currently available data we propose the model presented in Figure 3.

Our finding that PP_i generated within the liver is able to act in the periphery shows that increased systemic PP_i levels are sufficient to inhibit local ectopic mineralization. Importantly, Lomashvili et al. very recently showed in *Enpp1*$^{-/-}$ mice that ectopic calcification indeed depends on plasma PP_i levels and not local PP_i production[13]. The crucial role of plasma PP_i in the prevention of ectopic calcification has important therapeutic consequences: Raising PP_i levels in the blood circulation of PXE, GACI and ACDC patients should suffice to halt ectopic mineralization. The short plasma half-life and lack of a suitable dosage form do not make PP_i an attractive candidate for supplementation therapy in humans[20], but it might be possible to generate suitable PP_i precursors. Alternatively, bisphosphonates, a class of metabolically stable, synthetic PP_i analogs that have been used in GACI with reasonable success[21], may represent an attractive treatment strategy for PXE and ACDC.

The AMP metabolite adenosine is known to inhibit the expression of TNAP (Figure 2)[16]. It is therefore tempting to speculate that the increased TNAP activity seen in fibroblasts isolated from PXE patients[22] and *Abcc6*$^{-/-}$ mice[23] is due to a reduction in the amount of released AMP. Low AMP levels might reduce local formation of adenosine and subsequent TNAP inhibition. AMP-derived adenosine might, therefore, be involved in "priming" of the periphery for subsequent PP_i influx. This model would imply that both AMP and PP_i, are necessary to prevent ectopic mineralization: PP_i by directly inhibiting the formation of calcium phosphate crystals and AMP after being metabolized to adenosine by inhibiting premature degradation of circulating PP_i by TNAP.

In vitro, ABCC6 transports glutathione conjugates and the synthetic cyclic peptide BQ-123, suggesting that ABCC6 is a bona-fide transporter[11, 24]. We were unable, however, to demonstrate ABCC6-mediated nucleoside triphosphate transport in vesicular transport experiments[9]. Factors could be missing *in vitro*, however, that allow ABCC6 to transport ATP *in vivo*, or ABCC6 could indirectly stimulate ATP release by regulating vesicular transport or ion-channels[25].

Taken together, we show that ABCC6 mediates the release of ATP directly from the liver into the circulation. Within the liver vasculature, ATP is converted into AMP and PP_i and represents the main source of the mineralization inhibitor PP_i in plasma. This fully explains why absence of ABCC6 results in the ectopic mineralization observed in PXE patients. Our data indicate that correcting PP_i to normal levels could prevent the ectopic mineralization observed in PXE, GACI and ACDC.

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Disclosures
The authors declare that they have no conflict of interest.

References

1. Neldner KH. Pseudoxanthoma elasticum. *Clin. Dermatol.* 1988;6:1-159
2. Uitto J, Varadi A, Bercovitch L, Terry PF, Terry SF. Pseudoxanthoma elasticum: Progress in research toward treatment: Summary of the 2012 pxe international research meeting. *J. Invest. Dermatol.* 2013;133:1444-1449
3. Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards A, Terry S, Bercovitch L, de Paepe A, Boyd CD. Mutations in a gene encoding an abc transporter cause pseudoxanthoma elasticum. *Nat. Genet.* 2000;25:223-227
4. Bergen AA, Plomp AS, Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, van Soest S, Baas F, ten Brink JB, de Jong PT. Mutations in abcc6 cause pseudoxanthoma elasticum. *Nat. Genet.* 2000;25:228-231
5. Ringpfeil F, Lebwohl MG, Christiano AM, Uitto J. Pseudoxanthoma elasticum: Mutations in the mrp6 gene encoding a transmembrane atp-binding cassette (abc) transporter. *Proc. Natl. Acad. Sci. U. S. A.* 2000;97:6001-6006
6. Nitschke Y, Rutsch F. Generalized arterial calcification of infancy and pseudoxanthoma elasticum: Two sides of the same coin. *Front. Genet.* 2012;3:302
7. Kool M, van der Linden M, de Haas M, Baas F, Borst P. Expression of human mrp6, a homologue of the multidrug resistance protein gene mrp1, in tissues and cancer cells. *Cancer Res.* 1999;59:175-182
8. Jiang Q, Oldenburg R, Otsuru S, Grand-Pierre AE, Horwitz EM, Uitto J. Parabiotic heterogenetic pairing of abcc6-/-/rag1-/- mice and their wild-type counterparts halts ectopic mineralization in a murine model of pseudoxanthoma elasticum. *Am. J. Pathol.* 2010;176:1855-1862
9. Jansen R, Küçükosmanoğlu A, de Haas M, Sapthu S, Otero J, Hegman I, Bergen A, Gorgels T, Borst P, van de Wetering K. Abcc6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc. Natl. Acad. Sci.* 2013
10. Borst P, Oude Elferink RP. Mammalian abc transporters in health and disease. *Annu. Rev. Biochem.* 2002;71:537-592
11. Madon J, Hagenbuch B, Landmann L, Meier PJ, Stieger B. Transport function and hepatocellular localization of mrp6 in rat liver. *Mol Pharmacol*. 2000;57:634-641
12. Mortensen SP, Thaning P, Nyberg M, Saltin B, Hellsten Y. Local release of atp into the arterial inflow and venous drainage of human skeletal muscle: Insight from atp determination with the intravascular microdialysis technique. *J. Physiol.* 2011;589:1847-1857
13. Lomashvili KA, Narisawa S, Millan JL, O'Neill WC. Vascular calcification is dependent on plasma levels of pyrophosphate. *Kidney Int.* 2014
14. Fleisch H, Russell RG, Straumann F. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. *Nature*. 1966;212:901-903
15. Rutsch F, Ruf N, Vaingankar S, Toliat MR, Suk A, Hohne W, Schauer G, Lehmann M, Roscioli T, Schnabel D, Epplen JT, Knisely A, Superti-Furga A, McGill J, Filippone M, Sinaiko AR, Vallance H, Hinrichs B, Smith W, Ferre M, Terkeltaub R, Nurnberg P. Mutations in enpp1 are associated with 'idiopathic' infantile arterial calcification. *Nat. Genet.* 2003;34:379-381
16. St Hilaire C, Ziegler SG, Markello TC, Brusco A, Groden C, Gill F, Carlson-Donohoe H, Lederman RJ, Chen MY, Yang D, Siegenthaler MP, Arduino C, Mancini C, Freudenthal B, Stanescu HC, Zdebik AA, Chaganti RK, Nussbaum RL, Kleta R, Gahl WA, Boehm M. Nt5e mutations and arterial calcifications. *N. Engl. J. Med.* 2011;364:432-442

17. Henthorn PS, Raducha M, Fedde KN, Lafferty MA, Whyte MP. Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia. *Proc. Natl. Acad. Sci. U. S. A.* 1992;89:9924-9928
18. Pendleton A, Johnson MD, Hughes A, Gurley KA, Ho AM, Doherty M, Dixey J, Gillet P, Loeuille D, McGrath R, Reginato A, Shiang R, Wright G, Netter P, Williams C, Kingsley DM. Mutations in ankh cause chondrocalcinosis. *Am. J. Hum. Genet.* 2002;71:933-940
19. Rutsch F, Nitschke Y, Terkeltaub R. Genetics in arterial calcification: Pieces of a puzzle and cogs in a wheel. *Circ. Res.* 2011;109:578-592
20. O'Neill WC, Lomashvili KA, Malluche HH, Faugere MC, Riser BL. Treatment with pyrophosphate inhibits uremic vascular calcification. *Kidney Int.* 2011;79:512-517
21. Rutsch F, Boyer P, Nitschke Y, Ruf N, Lorenz-Depierieux B, Wittkampf T, Weissen-Plenz G, Fischer RJ, Mughal Z, Gregory JW, Davies JH, Loirat C, Strom TM, Schnabel D, Nurnberg P, Terkeltaub R. Hypophosphatemia, hyperphosphaturia, and bisphosphonate treatment are associated with survival beyond infancy in generalized arterial calcification of infancy. *Circ. Cardiovasc. Genet.* 2008;1:133-140
22. Boraldi F, Annovi G, Bartolomeo A, Quaglino D. Fibroblasts from patients affected by pseudoxanthoma elasticum exhibit an altered ppi metabolism and are more responsive to pro-calcifying stimuli. *J. Dermatol. Sci.* 2014
23. Boraldi F, Bartolomeo A, Li Q, Uitto J, Quaglino D. Changes in dermal fibroblasts from abcc6-/- mice are present before and after the onset of ectopic tissue mineralization. *J. Invest. Dermatol.* 2014
24. Ilias A, Urban Z, Seidl TL, Le Saux O, Sinko E, Boyd CD, Sarkadi B, Varadi A. Loss of atp-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human abcc6 (mrp6). *J. Biol. Chem.* 2002;277:16860-16867
25. Lazarowski ER. Vesicular and conductive mechanisms of nucleotide release. *Purinergic Signal*. 2012;8:359-373

Significance

PXE is a hereditary ectopic mineralization disorder caused by the absence of functional ABCC6 that affects approximately 150,000 patients world-wide. An effective therapy does not exist as the pathology underlying the disease is not well understood. Here we show that ABCC6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation, explaining the ectopic calcification observed in PXE patients. Our data indicate that correcting PP_i to normal levels could prevent the ectopic mineralization observed in PXE and related mineralization disorders.

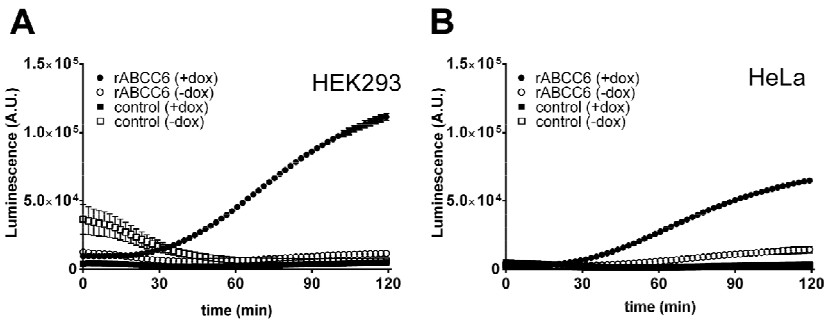

Figure 1. HEK293 and HeLa cells overproducing rABCC6 release ATP. (**A**) Flp-In T-REx 293 control (squares) or Flp-In T-REx 293 rABCC6 (circles) cells were grown in the presence (filled symbols) or absence (open symbols) of 1 µg/ml doxycycline to induce rABCC6 expression. Two days later, ATP efflux was followed in real-time for 2 hours using the ATP-detection reagent BactiterGlo. (**B**) ATP efflux from Flp-In T-REx HeLa control (squares) or Flp-In T-REx HeLa rABCC6 (circles) cells grown in the presence (filled symbols) or absence (open symbols) of 1 µg/ml doxycycline was followed for 2 hours in real-time. Data (*n*=12) represent mean +/- SEM.

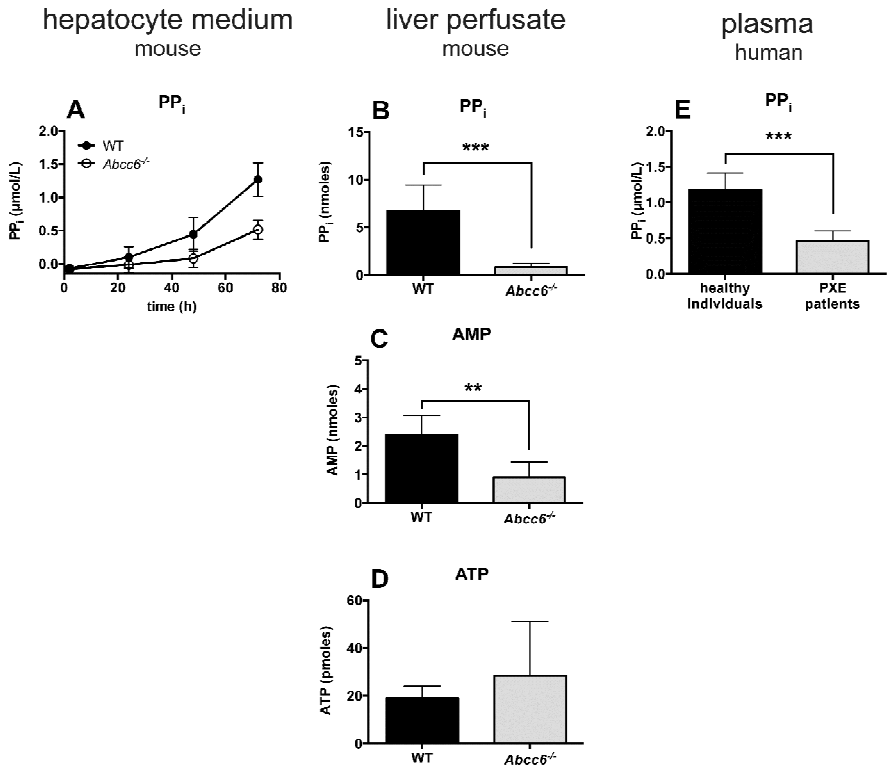

Figure 2. Hepatic ABCC6 raises PP_i levels via ATP release. Released ATP is rapidly converted into AMP and PP_i within the liver vasculature. (**A**) PP_i levels in culture medium of sandwich-cultured primary wildtype (WT) and *Abcc6*$^{-/-}$ hepatocytes (n=3 for WT, n=4 for *Abcc6*$^{-/-}$); Total amount of (**B**) PP_i, (**C**) AMP and (**D**) ATP in mouse liver perfusates collected from WT and *Abcc6*$^{-/-}$ livers during 30 minutes (n=5 for WT, n=6 for *Abcc6*$^{-/-}$);(**E**) PP_i levels in platelet-free plasma samples from healthy subjects (n=14) and PXE patients (n=12). Patient and control characteristics are given in the online-only Data Supplement. Data are presented as mean ± standard deviation. **: P<0.01, ***:P<0.001. Note that AMP and PP_i levels are in nmoles, whereas ATP levels are in pmoles and close to background levels.

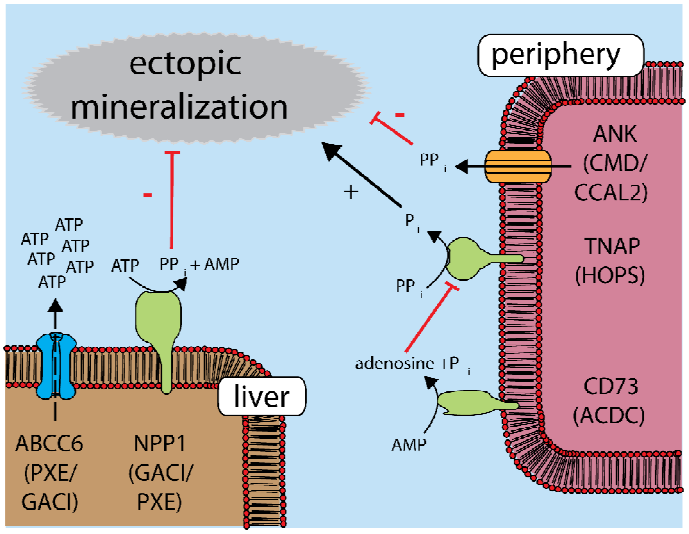

Figure 3. Proposed model for hepatic ABCC6-mediated pyrophosphate generation and ectopic mineralization. ATP released from the liver by an ABCC6-dependent mechanism is converted into the mineralization inhibitor pyrophosphate (PP_i) by hepatic ectonucleotide pyrophosphatase-phosphodiesterase 1 (ENPP1). In the periphery, PP_i is hydrolyzed by tissue-nonspecific alkaline phosphatase (TNAP). Inactive ABCC6 classically causes pseudoxanthoma elasticum (PXE), whereas inactive ENPP1 causes generalized arterial calcification of infancy (GACI). Non-functional ecto-5'-nucleotidase (NT5E) results in arterial calcification due to deficiency of CD73 (ACDC) and inactive TNAP causes hypophosphatasia (HOPS). Local PP_i levels also depend on the transmembrane protein ANKH, a protein postulated to be a PP_i channel/efflux transporter. Mutations in ANKH can result in chondrocalcinosis type 2 (CCAL2) or craniometaphyseal dysplasia (CMD).