

# 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<sub>i</sub>). 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<sub>i</sub>, and whether human PXE patients have low plasma PP<sub>i</sub> 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<sub>i</sub>. The absence of functional *ABCC6* in PXE patients leads to strongly reduced plasma PP<sub>i</sub> concentrations.

### *Conclusions*

Hepatic *ABCC6*-mediated ATP release is the main source of circulating PP<sub>i</sub>, revealing an unanticipated role of the liver in systemic PP<sub>i</sub> homeostasis. PXE patients have a strongly reduced plasma PP<sub>i</sub> level, explaining their mineralization disorder. Our results indicate that systemic PP<sub>i</sub> is relatively stable and that PXE, GACI and other ectopic mineralization disorders could be treated with PP<sub>i</sub> 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<sub>i</sub>, 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<sup>1</sup>. 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<sup>2</sup>.

In 2000, several groups reported that PXE is caused by inactivating mutations in the *ATP-binding cassette sub-family C member 6 (ABCC6)* gene<sup>3-5</sup> and more recently ABCC6 defects were also found to cause some forms of generalized arterial calcification of infancy (GACI)<sup>6</sup>, 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<sup>7</sup>. 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<sup>8</sup>. 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*<sup>9</sup>. Secreted ATP was extracellularly converted into AMP and the mineralization inhibitor inorganic pyrophosphate (PP<sub>i</sub>) by ectonucleotide pyrophosphatase-phosphodiesterase (ENPP)-type ectonucleotidases. The *in vivo* relevance of these findings was demonstrated in *Abcc6*<sup>-/-</sup> mice, which have plasma PP<sub>i</sub> 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<sup>10</sup>. ABCC6 is mainly present in the sinusoidal membrane of the hepatocytes<sup>11</sup>. 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<sup>9</sup>. 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)<sup>12</sup> does not allow PP<sub>i</sub> formation from liver-derived ATP in the periphery. The current study was performed to show that ABCC6 affects plasma PP<sub>i</sub> 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<sup>9</sup>. 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<sup>11</sup>. 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<sub>i</sub> in the culture medium. PP<sub>i</sub> 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<sub>i</sub>. We also detected some PP<sub>i</sub> in medium from *Abcc6*<sup>-/-</sup> 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<sub>i</sub> 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<sub>i</sub> and AMP levels (Figure 1 D). The AMP and PP<sub>i</sub> that we detect in the liver perfusates must be derived from ATP: *Enpp1*<sup>-/-</sup> mice have PP<sub>i</sub> levels that are less than 5% of those found in wild-type mice<sup>13</sup>, implying that also the PP<sub>i</sub> in plasma that depends on ABCC6 must come from ATP. Conversion of released ATP into AMP and PP<sub>i</sub> 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<sub>i</sub> 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<sub>i</sub> 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*<sub>1/2</sub> of PP<sub>i</sub> has been estimated to be 33 min, which requires a hepatic release rate of 6 nmoles PP<sub>i</sub> per hour to achieve the steady-state levels of 2.3 μmol/L (μM) that we have reported for mice<sup>9</sup> (for calculation see the Materials and Methods section). Importantly, the amount of PP<sub>i</sub> detected in liver perfusates of wild-type mice is high enough to explain these steady-state PP<sub>i</sub> 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<sub>i</sub> 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<sub>i</sub> levels we previously reported for *Abcc6*<sup>-/-</sup> mice<sup>9</sup>.

## Discussion

PP<sub>i</sub> is a key regulator of ectopic mineralization acting by inhibiting hydroxyapatite crystal growth<sup>14</sup>. As a result, mutations in genes encoding known PP<sub>i</sub>-regulating enzymes like ENPP1, ecto-5'-nucleotidase (NT5E), progressive ankylosis protein homolog (ANKH) and tissue-nonspecific alkaline phosphatase (TNAP) cause various mineralization disorders<sup>15-18</sup>. 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<sup>19</sup>. 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*<sup>6</sup>. Our data unexpectedly falsify the factor X-hypothesis<sup>9</sup> and show that ABCC6-mediated ATP release from the liver is the principal source of plasma PP<sub>i</sub>. A factor involved in the local release of PP<sub>i</sub> is ANKH, a membrane protein postulated to mediate the direct release of PP<sub>i</sub> from cells. ANKH does, however, not substantially contribute to plasma PP<sub>i</sub> levels, which almost exclusively depend on ENPP1 activity and hence ATP release<sup>13</sup>. Based on the currently available data we propose the model presented in Figure 3.

Our finding that PP<sub>i</sub> generated within the liver is able to act in the periphery shows that increased systemic PP<sub>i</sub> levels are sufficient to inhibit local ectopic mineralization. Importantly, Lomashvili et al. very recently showed in *Enpp1*<sup>-/-</sup> mice that ectopic calcification indeed depends on plasma PP<sub>i</sub> levels and not local PP<sub>i</sub> production<sup>13</sup>. The crucial role of plasma PP<sub>i</sub> in the prevention of ectopic calcification has important therapeutic consequences: Raising PP<sub>i</sub> 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<sub>i</sub> an attractive candidate for supplementation therapy in humans<sup>20</sup>, but it might be possible to generate suitable PP<sub>i</sub> precursors. Alternatively, bisphosphonates, a class of metabolically stable, synthetic PP<sub>i</sub> analogs that have been used in GACI with reasonable success<sup>21</sup>, may represent an attractive treatment strategy for PXE and ACDC.

The AMP metabolite adenosine is known to inhibit the expression of TNAP (Figure 2)<sup>16</sup>. It is therefore tempting to speculate that the increased TNAP activity seen in fibroblasts isolated from PXE patients<sup>22</sup> and *Abcc6*<sup>-/-</sup> mice<sup>23</sup> 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<sub>i</sub> influx. This model would imply that both AMP and PP<sub>i</sub> are necessary to prevent ectopic mineralization: PP<sub>i</sub> by directly inhibiting the formation of calcium phosphate crystals and AMP after being metabolized to adenosine by inhibiting premature degradation of circulating PP<sub>i</sub> by TNAP.

*In vitro*, ABCC6 transports glutathione conjugates and the synthetic cyclic peptide BQ-123, suggesting that ABCC6 is a bona-fide transporter<sup>11, 24</sup>. We were unable, however, to demonstrate ABCC6-mediated nucleoside triphosphate transport in vesicular transport experiments<sup>9</sup>. 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<sup>25</sup>.

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<sub>i</sub> and represents the main source of the mineralization inhibitor PP<sub>i</sub> in plasma. This fully explains why absence of ABCC6 results in the ectopic mineralization observed in PXE patients. Our data indicate that correcting PP<sub>i</sub> 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.

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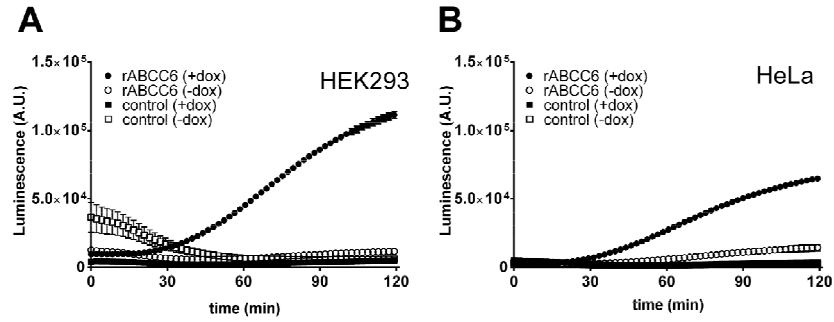
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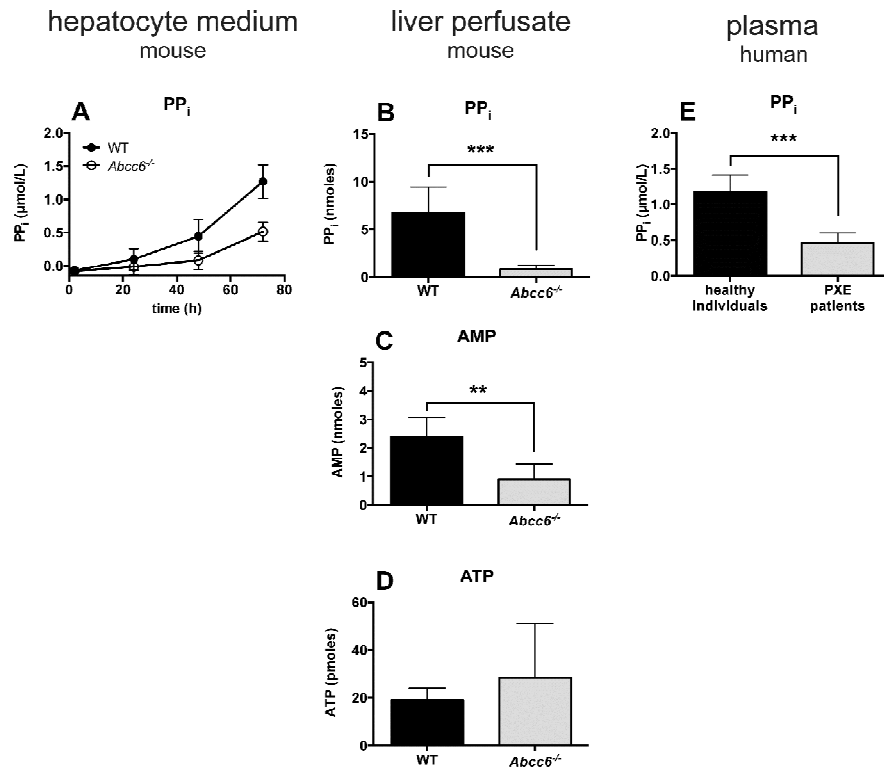
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## **Significance**

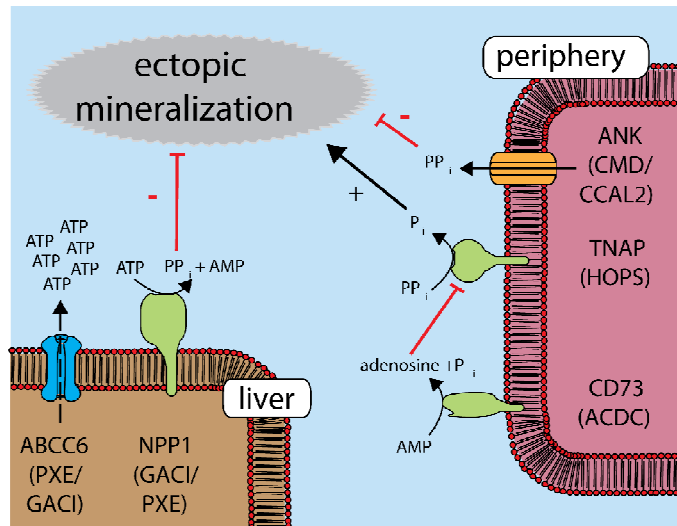
PXE is a hereditary ectopic mineralization disorder caused by the absence of functional ABCC6 that affects approximately 150,000 patients world-wide. An effective therapy does not exist as the pathology underlying the disease is not well understood. Here we show that ABCC6-mediated ATP secretion by the 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  $\mu$ g/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  $\mu$ g/ml doxycycline was followed for 2 hours in real-time. Data ( $n=12$ ) represent mean  $\pm$  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  $\pm$  standard deviation. \*\*:  $P<0.01$ , \*\*\*:  $P<0.001$ . Note that AMP and  $PP_i$  levels are in nmol, whereas ATP levels are in pmol 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).