ABCC6–Mediated ATP Secretion by the Liver Is the Main Source of the Mineralization Inhibitor Inorganic Pyrophosphate in the Systemic Circulation—Brief Report


Objective—Mutations in ABCC6 underlie the ectopic mineralization disorder pseudoxanthoma elasticum (PXE) and some forms of generalized arterial calcification of infancy, both of which affect the cardiovascular system. Using cultured cells, we recently showed that ATP-binding cassette subfamily C member 6 (ABCC6) mediates the cellular release of ATP, which is extracellularly rapidly converted into AMP and the mineralization inhibitor inorganic pyrophosphate (PPi). 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 PPi, and whether human PXE patients have low plasma PPi 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 PPi. The absence of functional ABCC6 in patients with PXE leads to strongly reduced plasma PPi concentrations.

Conclusions—Hepatic ABCC6-mediated ATP release is the main source of circulating PPi, revealing an unanticipated role of the liver in systemic PPi homeostasis. Patients with PXE have a strongly reduced plasma PPi level, explaining their mineralization disorder. Our results indicate that systemic PPi is relatively stable and that PXE, generalized arterial calcification of infancy, and other ectopic mineralization disorders could be treated with PPi supplementation therapy. (Arterioscler Thromb Vasc Biol. 2014;34:1985-1989.)

Key Words: multidrug resistance-associated proteins ■ nucleotides ■ pathologic calcification ■ pyrophosphatases ■ vascular calcification

Pseudoxanthoma elasticum (PXE) is an autosomal recessive disease characterized by progressive ectopic mineralization of the skin, eyes, and arteries. Approximately 150,000 patients with PXE worldwide experience stigmatizing skin lesions, progressive loss of vision, and cardiovascular complications, against which no effective therapy exists.

In 2000, several groups reported that PXE is caused by inactivating mutations in the ATP-binding cassette subfamily C member 6 (ABCC6) gene. More recently, ABCC6 defects were also found to cause some forms of generalized arterial calcification of infancy (GACI), a severe form of arterial calcification. ABCC6 (also known as multidrug resistance protein 6) is an ATP-dependent orphan efflux transporter that is primarily expressed in the liver. 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. Despite extensive research, the identity of this factor has long remained a mystery.

We recently showed that overexpression of ABCC6 in human embryonic kidney 293 (HEK293) cells induces the release of nucleoside triphosphates, predominantly ATP, in vitro. Secreted ATP was extracellularly converted into AMP and the mineralization inhibitor inorganic pyrophosphate (PPi) by ectonucleotide pyrophosphatase-phosphodiesterase (ENPP)-type ectonucleotidases. The in vivo relevance of these findings was demonstrated in Abcc6−/− mice, which have plasma PPi levels <40% of those found in wild-type control animals. ABCC6 is a member of the multidrug...
resistance protein) family, which contains large proteins transporting a variety of organic anions. ABCC6 is mainly present in the sinusoidal membrane of the hepatocytes. Because 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. 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 seconds) does not allow PPi formation from liver-derived ATP in the periphery. The current study was performed to show that ABCC6 affects plasma PPi levels in humans and to assess whether ABCC6 directly affects hepatic ATP release or indirectly induces peripheral ATP release.

**Materials and Methods**

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

**Results**

**HEK293 and HeLa Cells Release ATP on the Expression of ABCC6**

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. To determine whether ABCC6-dependent ATP release is cell type-dependent, we generated HeLa cells in which the expression of rat ABCC6 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 rat ABCC6, cells released almost no ATP (Figure 1A and 1B). However, on induction of rat ABCC6, 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.

**Appearance of PPi in the Culture Medium of Sandwich-Cultured Hepatocytes Depends on ABCC6**

ABCC6 is predominantly present in the liver. Next, we therefore 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 detect ATP release directly in these experiments, presumably because of the high ectonucleotidase activity of hepatocytes. We, therefore, followed the appearance of the ATP metabolite PPi, in the culture medium. PPi 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 PPi. We also detected some PPi in medium from Abcc6−/− cells, which we attribute to ATP release unrelated to ABCC6, or leakage from damaged cells.

**Hepatic ABCC6 Mediates the Sinusoidal Release of ATP, Which Is Converted Into AMP and PPi Within the Liver Vasculature**

To assess whether ABCC6 is an important factor in hepatic ATP release in vivo, we performed liver perfusion experiments. PPi and AMP levels in the liver perfusates strongly depended on the presence of ABCC6 (Figure 2B and 2C). Interestingly, ATP levels did not differ between the 2 genotypes and were extremely low, representing <1% of the PPi and AMP levels (Figure 1D). The AMP and PPi, that we detect in the liver perfusates must be derived from ATP: Enpp1−/− mice have PPi levels that are <5% of those found in wild-type mice, implying that also the PPi in plasma that depends on ABCC6 must come from ATP. Conversion of released ATP into AMP and PPi within the liver is fast. We calculated that

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**Figure 1.** HEK293 and HeLa cells overproducing rat ATP-binding cassette subfamily C member 6 (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.
during our single-pass perfusion experiments the buffer is present in the liver for ≈10 seconds (for the calculation, see the Materials and Methods section in the online-only Data Supplement). During this short period, the substantial amounts of ATP released are almost quantitatively converted into PPi and AMP (Figure 2B–2D). 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 PPi by hepatic NPP1. From our perfusion experiments, we calculate that ABCC6 mediates ≈90% of the hepatic nucleotide release. During 24 hours, this corresponds to ≥5% of the total hepatic adenine nucleotide pool (Figure 2B; for the calculation, see the Materials and Methods section in the online-only Data Supplement). The plasma t½ of PPi has been estimated to be 33 minutes, which requires a hepatic release rate of 6 nmoles PPi per hour to achieve the steady state levels of 2.3 μmol/L that we have reported for mice9 (for calculation, see the Materials and Methods section in the online-only Data Supplement). Importantly, the amount of PPi detected in liver perfusates of wild-type mice is high enough to explain these steady state PPi levels in mouse plasma.

Patients With PXE Have Strongly Reduced PPi Plasma Levels
An important question is whether our mouse results translate to human PXE patients. We have, therefore, studied a group of 12 Dutch patients with PXE with known ABCC6 mutations (Table I in the online-only Data Supplement). The plasma PPi concentrations were ≈2.5-fold lower in patients than in healthy individuals (Figure 2E). This difference did not depend on sex and is in line with the reduced plasma PPi levels we previously reported for Abcc6−/− mice.9

Figure 2. Hepatic ATP-binding cassette subfamily C member 6 (ABCC6) raises inorganic pyrophosphate (PPi) levels via ATP release. Released ATP is rapidly converted into AMP and PPi within the liver vasculature. A, PPi levels in culture medium of sandwich-cultured primary wild-type (WT) and Abcc6−/− hepatocytes (n=3 for WT, n=4 for Abcc6−/−); total amount of (B) PPi, (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, PPi levels in platelet-free plasma samples from healthy subjects (n=14) and patients with PXE (n=12). Patient and control characteristics are given in the online-only Data Supplement. Data are presented as mean±SD. **P<0.01, ***P<0.001. Note that AMP and PPi levels are in nmoles, whereas ATP levels are in pmoles and close to background levels.

Figure 3. Proposed model for hepatic ATP-binding cassette subfamily C member 6 (ABCC6)–mediated pyrophosphate generation and ectopic mineralization. ATP released from the liver by an ABCC6-dependent mechanism is converted into the mineralization inhibitor pyrophosphate (inorganic pyrophosphate [PPi]) by hepatic ectonucleotide pyrophosphatase-phosphodiesterase 1 (ENPP1). In the periphery, PPi 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). Nonfunctional ecto-5′-nucleotidase results in arterial calcification due to deficiency of CD73 (ACDC), and inactive TNAP causes hypophosphatasia (HOPS). Local PPi levels also depend on the transmembrane protein progressive ankylosis protein homolog (ANKH), a protein postulated to be a PPi channel/efflux transporter. Mutations in ANKH can result in chondrodysplasia type 2 (CCAL2) or cranioectodermal dysplasia (CMD).
Discussion

PP is a key regulator of ectopic mineralization acting by inhibiting hydroxyapatite crystal growth. As a result, mutations in genes encoding known PP-regulating enzymes like ENPP1, ecto-5′-nucleotidase, progressive ankylosis protein homolog, and tissue-nonspecific alkaline phosphatase (TNAP) cause various mineralization disorders. The clinical symptoms of the mineralization disorders caused by non-functional ENPP1 (GACI) and ecto-5′-nucleotidase (artificial calcification due to deficiency of CD73) highly overlap those of PXE. 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 ABC6. Our data unexpectedly falsify the factor X-hypothesis and show that ABC6-mediated ATP release from the liver is the principal source of plasma PP. A factor involved in the local release of PP is progressive ankylosis protein homolog, a membrane protein postulated to mediate the direct release of PP, from cells. Progressive ankylosis protein homolog does, however, not substantially contribute to plasma PP levels, which almost exclusively depend on ENPP1 activity and hence ATP release. Based on the currently available data, we propose the model presented in Figure 3.

Our finding that PP, generated within the liver is able to act in the periphery shows that increased systemic PP levels are sufficient to inhibit local ectopic mineralization. Importantly, Lomashvili et al. recently showed in ENPP1−/− mice that ectopic calcification depends on plasma PP levels and not local PP production. The crucial role of plasma PP in the prevention of ectopic calcification has important therapeutic consequences: raising PP levels in the blood circulation of patients with PXE, GACI, and arterial calcification due to deficiency of CD73 should suffice to halt ectopic mineralization. The short plasma half-life and lack of a suitable dosage form do not make PP an attractive candidate for supplementation therapy in humans, but it might be possible to generate suitable PP precursors. Alternatively, bisphosphonates, a class of metabolically stable, synthetic PP, analogs that have been used in GACI with reasonable success, may represent an attractive treatment strategy for PXE and arterial calcification due to deficiency of CD73.

The AMP metabolite adenosine is known to inhibit the expression of TNAP (Figure 3). It is, therefore, tempting to speculate that the increased TNAP activity seen in fibroblasts isolated from patients with PXE and Abcc6−/− mice is because of 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 influx. This model would imply that both AMP and PP are necessary to prevent ectopic mineralization: PP, by directly inhibiting the formation of calcium phosphate crystals and AMP after being metabolized to adenosine by inhibiting premature degradation of circulating PP, by TNAP.

In vitro, ABC6 transports glutathione conjugates and the synthetic cyclic peptide BQ-123, suggesting that ABC6 is a bona fide transporter. We were unable, however, to demonstrate ABC6-mediated nucleoside triphosphate transport in vesicular transport experiments. Factors could be missing in vitro, however, that allows ABC6 to transport ATP in vivo, or ABC6 could indirectly stimulate ATP release by regulating vesicular transport or ion channels.

Taken together, we show that ABC6 mediates the release of ATP directly from the liver into the circulation. Within the liver vasculature, ATP is converted into AMP and PP, and represents the main source of the mineralization inhibitor PP, in plasma. This fully explains why absence of ABC6 results in the ectopic mineralization observed in patients with PXE. Our data indicate that correcting PP to normal levels could prevent the ectopic mineralization observed in PXE, GACI, and arterial calcification due to deficiency of CD73.

Acknowledgments

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Disclosures

None.

References

Pseudoxanthoma elasticum is a hereditary ectopic mineralization disorder caused by the absence of functional ATP-binding cassette subfamily C member 6 that affects ≈150,000 patients worldwide. An effective therapy does not exist because the pathology underlying the disease is not well understood. Here, we show that ATP-binding cassette subfamily C member 6–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 patients with pseudoxanthoma elasticum. Our data indicate that correcting inorganic pyrophosphate to normal levels could prevent the ectopic mineralization observed in pseudoxanthoma elasticum and related mineralization disorders.
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Materials and Methods

**ABCC6 overexpressing cells.**
Flp-In T-REx 293 rABCC6 cells have been described\(^1\). Flp-In T-REx HeLa rABCC6 cells were generated by transfecting Flp-In T-REx HeLa cells with a mixture of 1:9 of pcDNA5-FRT-TO-rABCC6\(^1\) and pOG44 (Invitrogen) using lipofectamine (Invitrogen), according to the manufacturer’s instructions. The presence of rABCC6 was confirmed after induction with 1 µg doxycycline per ml in clones resistant to hygromycin (400 µg/ml) by immunoblot analysis (K14 antibody; provided by Bruno Stieger\(^2\)).

**Real-time ATP efflux assay.**
Flp-In T-REx 293 and HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS and 100 units pen/strep per ml at 37°C and 5% CO\(_2\) under humidifying conditions. Cells were seeded in white clear-bottom 96-well plates (Porvair) at a density of 2.0 x 10\(^4\) cells per well. After 24 hours, doxycycline was added and the cells were allowed to grow to confluence in 2 days. At this point, the medium was removed and replaced by 100 µl of the ATP detection reagent BactiterGlo (Promega) dissolved in DMEM containing 50 mM HEPES (pH 7.4) and 5 mM MgCl\(_2\). Immediately after addition of the ATP detection reagent, bioluminescence was determined in real-time for 2 hours using a microplate reader (Tecan M200 Pro reader) at 37 °C.

**Mice.**
Abcc6\(^{-/-}\) mice were generated as described\(^3\), and back-crossed into a C57Bl/6 background for 15 generations\(^4\). Wild-type and Abcc6\(^{-/-}\) mice were housed in constant temperature rooms with a 12-hour light/12-hour dark cycle and received food and water ad libitum. All animal experiments were performed according to the guidelines for the care and use of laboratory animals and were approved by the institutional ethical review committee for experiments on laboratory animals of the Netherlands Cancer Institute. Standard clinical chemistry of serum and liver function tests yielded results within the normal range for Abcc6\(^{-/-}\) and wild-type mice (data not shown).

**Plasma collection.**
Blood sampling of human subjects was approved by the Medical Ethical Committee of the Academic Hospital (AMC) Amsterdam and performed after informed consent. Mutations in ABCC6 were confirmed for all PXE patients (Material and Methods Table I). Whole blood was collected in 4.5 mL CTAD Vacutainer tubes (BD) and stored on ice after addition of 50 µL 15% trisodium EDTA (Sigma). After centrifugation (10 min, 1,000 g, 4 °C), plasma was depleted of platelets by filtration (20 min, 2,200 g, 4 °C) through a Centrisart I 300,000 kD mass cutoff filter (Sartorius) and stored at -20 °C until further processing. All collected samples were included in the analysis.

**Sandwich-cultured hepatocytes.**
16 weeks old male C57BL/6 mice were anaesthetized (20 mg/kg tiletamine, 20 mg/kg zolazepam, 12.5 mg/kg xylazine and 3 mg/kg butorphanol) and livers were perfused with 75 ml oxygenized perfusion buffer (120 mmol/L NaCl, 5.4 mmol/L KCl, 0.9 mmol/L NaH\(_2\)PO\(_4\), 26 mmol/L NaHCO\(_3\), 5.6 mmol/L glucose, pH 7.4) supplemented with EGTA (0.5 mmol/L), followed by a second perfusion with 75 ml perfusion buffer without EGTA. Collagenase digestion was performed by 75 ml perfusion buffer supplemented with 2.5
mmol/L CaCl₂ and 0.2 mg/ml collagenase (C5138, Sigma). The digested liver was taken out of the abdominal cavity and minced using tweezers, thereby releasing the hepatocytes. Cells were washed in ice-cold sterile suspension buffer (10 mmol/L HEPES, 142 mmol/L NaCl, 7 mmol/L KCl, pH 7.4), filtered through a 100 µm mesh membrane and centrifuged for 4 min at 4 °C at 80 g. After one additional washing step, dead cells were removed by Percoll (Sigma) centrifugation. After checking their viability by Trypan Blue exclusion staining, cells were seeded on 6-well plates pre-coated with 5 µg/cm² Collagen I (BD, 356234) at a density of 0.5x10⁶ cells/well in Williams E medium (Gibco) supplemented with hepatocyte thawing/plating supplement (Gibco) and 10% FBS. Cells that did not attach were removed by refreshing the culture medium after 2 h. 24 h post seeding medium was collected and replaced by Williams E medium supplemented with hepatocyte maintenance supplement (Gibco) without FBS, but with 6 µl Matrigel Matrix (BD)/ml to generate the upper layer of the sandwich configuration. Williams E medium with hepatocyte maintenance supplement was collected and replaced by fresh medium after 48 and 72 h. Collected medium samples were centrifuged for 4 min. at 4 °C at 120 g and the supernatant was stored at -20 °C until analysis.

Liver perfusion experiments.
Livers of anesthetized male mice were cannulated and perfused (~2 ml/min) with Krebs-bicarbonate buffer in the orthograde direction as previously described 6. Perfusate was collected on ice and stored at -20 °C.

ATP determination.
ATP was determined using the ATP-monitoring reagent BactiterGlo (Promega). To 20 µl sample or standard, 10 µl of BactiterGlo reagent added. Bioluminescence was subsequently determined in a microplate reader (Tecan M200 Pro reader). To exclude matrix effects, ATP calibration curves were prepared in the matrix of the unknowns.

PP, determination.
To quantify PP, we used ATP sulfurylase to convert PPᵢ into ATP in the presence of excess adenosine 5’ phosphosulfate (APS)⁷. To 30 µl of sample, 10 µl of a mixture containing 32 mU ATP sulfurylase (Sigma), 64 µmol/L APS (Santa Cruz Biotechnology), 80 µmol/L MgCl₂ and 40 mmol/L HEPES (pH 7.4) was added. The mixture was incubated for 30 min at 37 °C, after which ATP sulfurylase was inactivated by incubation at 90 °C for 10 min. Generated ATP was subsequently quantified as described under “ATP determination”. To exclude matrix effects, PPᵢ calibration curves were prepared in the matrix of the unknowns.

AMP determination.
To quantify AMP we used pyruvate orthophosphate dikinase (PPDK) from Microbispora rosea subsp. aerate (Kikkoman Biochemifa, Tokyo, Japan) to convert AMP into ATP in the presence of excess PPᵢ and phosphoenol pyruvate (PEP)⁸. To 10 µl of sample or standard, 10 µl of a solution containing 2.34 U/ml PPDK, 125 µmol/L PPᵢ, 40 µmol/L PEP, 50 µmol/L dithiothreitol, 1 mmol/L EDTA, 7.5 mmol/L MgSO₄ and 30 mmol/L BES (pH 8.0) was added. Conversion of AMP into ATP was allowed to proceed for 20 min at 30 °C, after which PPDK was inactivated by incubation at 90 °C for 10 min. Generated ATP was subsequently quantified as described under “ATP determination”. To exclude matrix effects, AMP calibration curves were prepared in the matrix of the unknowns.
Calculation of the time that the perfusion buffer resides within the liver.
Mouse livers were perfused at a rate of 2 ml/min. The volume of the liver vasculature of an adult mouse of 28 g is reported to be approximately 0.35 ml\(^9\). This means that during single-pass perfusion experiments a given fraction of buffer is present for approximately 10 seconds within the liver (vasculature).

Calculation of hepatic ATP content and release.
Reported ATP levels in mouse liver are variable and range from approximately 1-4 µmole/g wet liver tissue\(^{10-12}\). Based on a liver wet-weight of ~1.5 g for our mice, the total amount of ATP in the liver ranges from approximately 1.5-6 µmoles. In our liver perfusion experiments, wild-type liver excreted 6.8 nmoles ATP in 30 minutes. Extrapolated to 24 hours, this represents 5-22% of the total hepatic ATP pool.

Calculation of PP\(_i\) required to reach observed plasma levels.
At steady state, the concentration found in plasma is determined by the ratio between the PP\(_i\) infusion rate and PP\(_i\) clearance (equation 1). The clearance was calculated using equation 2, in which \(K_e\) was calculated using equation 3. The dose of PP\(_i\) required to reach the steady state PP\(_i\) levels previously observed in mice was calculated to be 5.8 nmol/hour, assuming a volume of distribution that equals the approximate blood volume. This calculated dose is likely an underestimation of the actual dose required because some PP\(_i\) is expected to diffuse into peripheral tissue compartments, increasing the apparent volume of distribution.

\[
C_{ss} = \frac{R_0}{CL} \quad \text{(equation 1)}
\]
\[
CL = K_e \cdot V_d \quad \text{(equation 2)}
\]
\[
K_e = \frac{\ln(1/2)}{T_{1/2}} \quad \text{(equation 3)}
\]

- \(R_0\): Infusion rate (µmol/h)
- \(C_{ss}\): Concentration at steady state (2.3 µmol/L\(^1\))
- \(CL\): Plasma clearance (L/h)
- \(K_e\): Elimination constant
- \(V_d\): Volume of distribution (set at 2 ml)
- \(T_{1/2}\): Plasma elimination half-life (33 min, or 0.55 h\(^{13}\))

Statistical analyses.
P-values of group comparisons were calculated using unpaired, two-tailed students t-tests with equal variance.

Tables
Table I. Patients and controls characteristics

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\(^a\) 10 patients were homozygous for the c.3775delT mutation, one was compound heterozygote for c.3775delT and c.3421C>T(Arg1141Stop) and one for c.4182delG;
c.1937T>C (Leu646Pro). Healthy controls did not have pathogenic ABCC6 variants. The age of males ($P=0.60$), females ($P=0.51$) or both ($P=0.94$) did not significantly differ between controls and PXE patients.

References


