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*$^{-/-}$ [3, 5]. In addition, microarray analysis did not uncover significant differences in gene expression profiles between livers of *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_2PO_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 $CaCl_2$ 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/cm^2 Collagen I (BD, 356234) at a density of $0.5x10^6$ 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_i determination.
To quantify PP_i we used ATP sulfurylase to convert PP_i 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 $MgCl_2$ 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, PP_i 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 PP_i 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 PP_i, 40 µmol/L PEP, 50 µmol/L dithiothreitol, 1 mmol/L EDTA, 7.5 mmol/L $MgSO_4$ 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.

$C_{ss} = R_0 / CL$ (equation 1)
$CL = K_e * V_d$ (equation 2)
$K_e = Ln_{(1/2)} / T_{1/2}$ (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

	Male		Female	
	Control	PXE[a]	Control	PXE[a]
Mean age (y)	59.0	54.5	53.7	57.1
SD (y)	11.0	16.8	7.6	10.8
n	7	5	7	7

[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

1. Jansen RS, Kucukosmanoglu A, de Haas M, Sapthu S, Otero JA, Hegman IE, Bergen AA, Gorgels TG, Borst P, van de Wetering K. Abcc6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc. Natl. Acad. Sci. U. S. A.* 2013;110:20206-20211
2. 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
3. Gorgels TG, Hu X, Scheffer GL, van der Wal AC, Toonstra J, de Jong PT, van Kuppevelt TH, Levelt CN, de Wolf A, Loves WJ, Scheper RJ, Peek R, Bergen AA. Disruption of abcc6 in the mouse: Novel insight in the pathogenesis of pseudoxanthoma elasticum. *Hum. Mol. Genet.* 2005;14:1763-1773
4. Gorgels TG, Waarsing JH, Herfs M, Versteeg D, Schoensiegel F, Sato T, Schlingemann RO, Ivandic B, Vermeer C, Schurgers LJ, Bergen AA. Vitamin k supplementation increases vitamin k tissue levels but fails to counteract ectopic calcification in a mouse model for pseudoxanthoma elasticum. *J. Mol. Med. (Berl.)*. 2011;89:1125-1135
5. Klement JF, Matsuzaki Y, Jiang QJ, Terlizzi J, Choi HY, Fujimoto N, Li K, Pulkkinen L, Birk DE, Sundberg JP, Uitto J. Targeted ablation of the abcc6 gene results in ectopic mineralization of connective tissues. *Mol. Cell. Biol.* 2005;25:8299-8310
6. Groen AK, Van Wijland MJ, Frederiks WM, Smit JJ, Schinkel AH, Oude Elferink RP. Regulation of protein secretion into bile: Studies in mice with a disrupted mdr2 p-glycoprotein gene. *Gastroenterology*. 1995;109:1997-2006
7. Helenius M, Jalkanen S, Yegutkin G. Enzyme-coupled assays for simultaneous detection of nanomolar atp, adp, amp, adenosine, inosine and pyrophosphate concentrations in extracellular fluids. *Biochim. Biophys. Acta*. 2012;1823:1967-1975
8. Sakakibara T, Murakami S, Imai K. Enumeration of bacterial cell numbers by amplified firefly bioluminescence without cultivation. *Anal. Biochem.* 2003;312:48-56
9. Greenway CV, Stark RD. Hepatic vascular bed. *Physiol. Rev.* 1971;51:23-65
10. Abadi RH. Analysis of free nucleotide pools of mouse liver tissue by high-pressure liquid chromatography (hplc). *Indian J. Biochem. Biophys.* 2003;40:209-212

11. McLaughlin AC, Takeda H, Chance B. Rapid atp assays in perfused mouse liver by 31p nmr. *Proc. Natl. Acad. Sci. U. S. A.* 1979;76:5445-5449
12. Selzner M, Selzner N, Jochum W, Graf R, Clavien PA. Increased ischemic injury in old mouse liver: An atp-dependent mechanism. *Liver Transpl.* 2007;13:382-390
13. O'Neill WC, Lomashvili KA, Malluche HH, Faugere MC, Riser BL. Treatment with pyrophosphate inhibits uremic vascular calcification. *Kidney Int.* 2011;79:512-517