A) ECTOPIC CALCIFICATION: preclinical models and treatment options

In mammals, biominerals predominantly consist of calcium and various forms of phosphate ions, together forming hydroxyapatite. In plasma and several other body fluids, calcium and phosphate (mostly hypophosphate) can be present at concentrations exceed their solubility constant. Vertebrates have evolved mechanisms to stabilize this supersaturated state and allow the regulated precipitation of calcium and phosphate in specific compartments. An important requirement for physiological mineralization is the presence of a suitable collagen matrix that favours the nucleation of calcium and phosphate and shapes the growing hydroxyapatite crystal. Ectopic mineralization, *i.e.,* deposition of calcium-phosphate complexes in connective tissues at aberrant locations, has been linked to several clinical conditions, such as aging, cancer, diabetes, and autoimmune diseases, represents major causes of morbidity and mortality. Recent studies on Mendelian ectopic mineralization disorders caused by mutations in distinct genes, with defined pathomechanistic consequences, have been extremely helpful toward elucidation of the mechanisms of ectopic mineralization processes in general. Additionally, some of the encoded proteins regulate the level of extracellular pyrophosphate (PPi), a potent anti-calcifying metabolite. Inactivating mutations in these genes are associated with rare soft tissue calcification disorders, which include: **pseudoxanthoma elasticum** (**PXE**, OMIM 264800) and **Generalized Arterial Calcification of Infancy** (**GACI**, OMIM 208000), representing the prototypes of such conditions. Pseudoxanthoma elasticum (PXE), a multi-system genetic disorder due to mutations in the *ABCC6* gene, what affects the skin, eyes, and the cardiovascular system. PXE is a prototype of heritable ectopic mineralization disorders. The spectrum of ectopic mineralization also includes generalized arterial calcification of infancy (GACI) and arterial calcification due to CD73 deficiency (ACDC). While PXE is a late-onset, slowly progressive disease, it carries a high risk of morbidity due to involvement of retina leading to loss of visual acuity and blindness. PXE can also lead to early demise from vascular complications, including gastrointestinal hemorrhage, early myocardial infarct and stroke. The prevalence of PXE is estimated at 1:25,000. PXE severely affects the quality of life: all patients eventually develop eye lesions resulting in loss of vision and a subset of patients suffers from serious cardiovascular problems, sometimes with a fatal outcome. In contrast to PXE, **GACI** presents shortly after birth with extensive calcifications in the walls of major arteries. The majority of GACI cases is caused by mutations in *ENPP1*, the gene encoding the ectonucleotidase pyrophosphatase phosphodiesterase 1 (ENPP1), whereas PXE is caused by mutations in *ABCC6* (ATP-binding cassette subfamily C member 6), which encodes an efflux transporter expressed at high levels in the liver.

Clearly, GACI and PXE represent the two extremes of a clinical spectrum of calcification disorders caused by the absence of functional ABCC6 or ENPP1. Mechanistically, ENPP1 catalyzes the conversion of extracellular ATP into AMP and inorganic pyrophosphate (PP_i). Mice that lack functional Enpp1 have very low circulating levels of PP_i, similar to GACI patients, which is the cause of soft tissue calcification. Recent studies have shown that ABCC6 is involved in the generation of extracellular PP_i and functions in the same pathway as ENPP1. Hepatocytes release nucleoside triphosphates, predominantly ATP, *via* an ABCC6-dependent mechanism. Released ATP is rapidly converted within the vasculature of the liver into AMP and PP_i by ENPP1. Importantly, the absence of ABCC6-mediated ATP release results in PXE, with patients showing highly reduced levels of circulating PP_i (4), similar to *Abcc6*$^{-/-}$ mice.

Pyrophosphate (PPi) is an endogenous metabolite inhibiting connective tissue calcification (CTC).

We have established two preclinical models for intervening of PXE, i.e. progression of calcification.

I. Most mutations in *ABCC6* are missense, and many of these mutations preserve transport activity but cause intracellular retention. We have shown that the chemical chaperone 4-phenylbutyrate (4-PBA) promotes the maturation of ABCC6 mutants to the plasma membrane. In a humanized mouse model of PXE, we investigated whether 4-PBA treatments could rescue the calcification inhibition potential of selected disease-causing ABCC6 mutants. We used the dystrophic cardiac calcification (DCC) phenotype of *Abcc6*$^{-/-}$ mice as an indicator of ABCC6 function to quantify the effect of 4-PBA on human ABCC6 mutants transiently expressed in the liver of these animals. 4-PBA administrations restored the physiological function of ABCC6 mutants resulting in calcification inhibition. This study identifies 4-PBA treatments as a promising strategy for allele-specific therapy of ABCC6-associated calcification disorders.

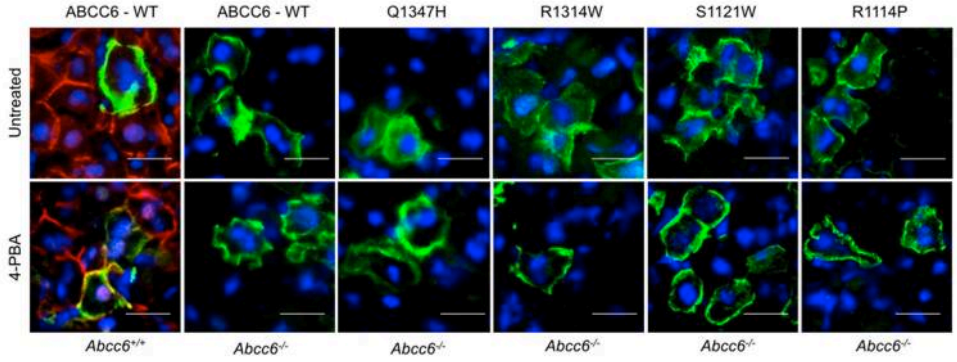

Figure 1: Intracellular localization of human ABCC6 variants expressed in *Abcc6−/−* mice
Hydrodynamic tail vein injections led to the successful expression of human wild type and mutant ABCC6. ABCC6 was detected on liver frozen sections by immunofluorescence using the M6II-31 monoclonal antibody (green) to verify the plasma membrane localization with or without 4-PBA treatments. Co-labeling with the S-20 polyclonal antibody (red) was used to control for the absence of the endogenous mouse Abcc6 and wild type mice (*Abcc6*+/+) were used as positive controls. DAPI was used as counter staining (blue). With 4-PBA treatments, ABCC6 mutant proteins were predominantly located in the plasma membrane. Scale bar = 50µm. (Pomozi et al, 2017)

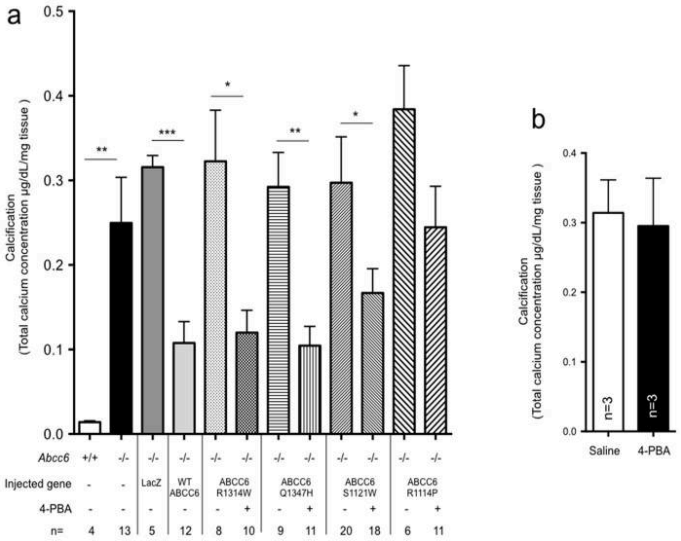

Figure 2: 4-PBA treatments rescue ABCC6 function
A: The wild type ABCC6 protein, four PXE/GACI mutants and LacZ were transiently expressed in *Abcc6−/−* liver prior to inducing dystrophic cardiac calcification (DCC). *Abcc6−/−* mice were exposed to 4-PBA for 7 days (controls received saline injections) with a cumulative dose of 1,000 mg/kg/day. The level of calcification (DCC phenotype) was measured as total heart calcium content and normalized to tissue weight. **B:** The p.Q1347H variant was transiently expressed in *Abcc6−/−* liver prior to inducing dystrophic cardiac calcification (DCC). *Abcc6−/−* mice were administered 4-PBA 36 hrs after DCC induction (controls received saline injections) until the harvesting of hearts. The level of calcification (DCC phenotype) was measured as total heart calcium content and normalized to tissue weight. Results are +/− SEM. * $p<0.05$, ** $p<0.01$. (Pomozi et al, 2017)

II. It was always assumed that the bioavailability of orally administered PPi is zero. In contrast to this assumption we detected increased PPi concentrations in the circulation of humans that ingesting pyrophosphate. In mouse models of PXE and GACI, PPi provided *via* the drinking water attenuated the ectopic calcification phenotype (skin, kidney and arteries). Strikingly, providing drinking water with 0.3 mM PPi to mice heterozygous for inactivating mutations in *Enpp1* exclusively during pregnancy, robustly inhibited ectopic calcification in their *Enpp1*$^{-/-}$ offsprings.

Our work shows that orally administered PPi is readily absorbed in human and inhibits connective tissue calcification in mouse models of PXE and GACI. PPi, which is recognized as safe by FDA, therefore not only has great potential as an effective and low cost treatment for these currently intractable genetic disorders, but also in other conditions involving connective tissue calcification.

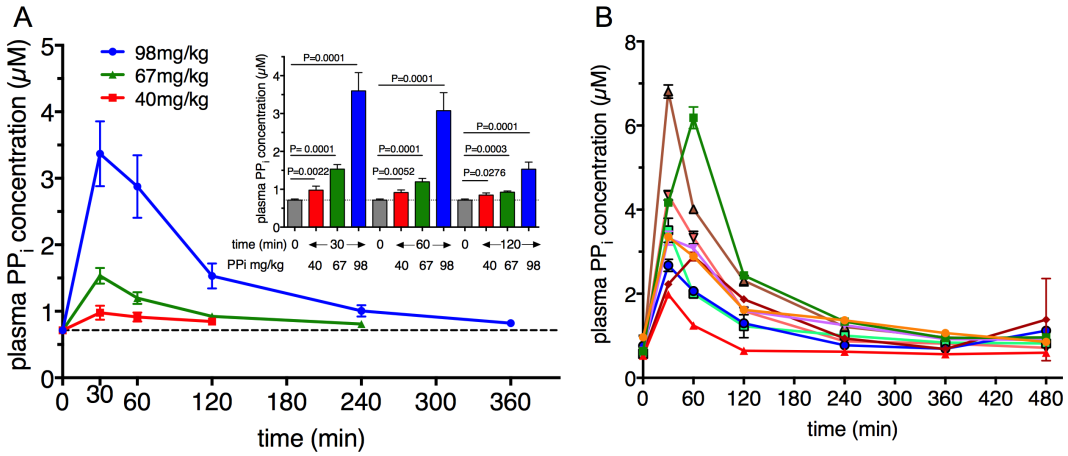

Figure 3: Uptake of PPi from drinking water in human
A: Oral uptake of tetrasodium pyrophosphate in humans. Volunteers ingested tetrasodium pyrophosphate solutions of 43, 72, 110 mM, pH 8.0, resulting in a dose of 40 mg/kg (n = 10) or 67 mg/kg (n = 10) or 98 mg/kg (n = 9), respectively. Plasma PPi levels were determined before (0 min), and 30, 60, 120, 240, and 480 min after ingestion. The insert shows the differences between the basal plasma PPi level (0 min) and that 30, 60, and 120 min after ingestion. **B:** Oral uptake of 98 mg/kg tetrasodium pyrophosphate (n = 9) in human indicating individual differences. (Dedinszki et al, 2017)

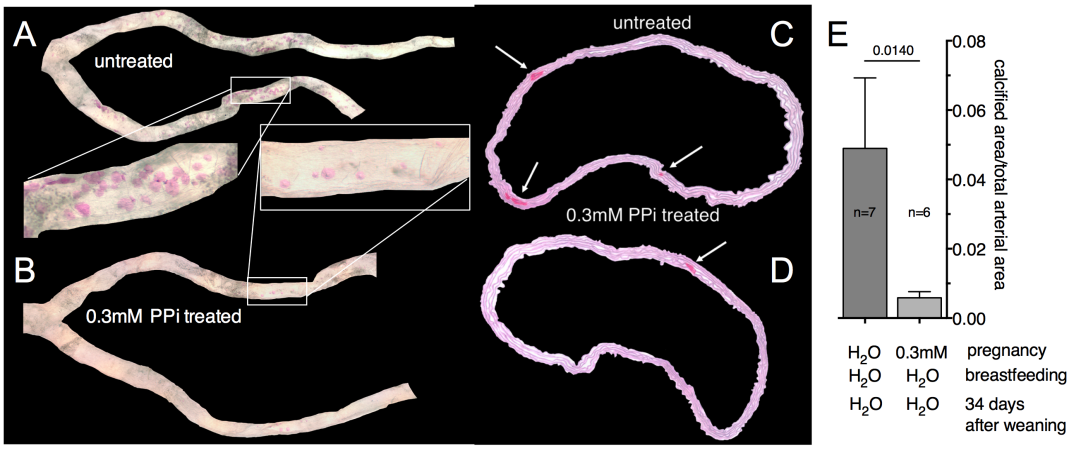

Figure 4: Prenatal PPi treatment of the Enpp1$^{-/-}$ mice attenuates calcification of the arteries of the hind limbs.
A, B: Typical Alizarin Red-stained hind limb arteries of a 55-day-old animal of the control group and those of a 55-day-old animal from the group of 0.3 mM treatment only during pregnancy. **C, D:** Cross sections of the Alizarin Red-stained hind limb arteries in the control group and in the group of 0.3 mM treatment only during pregnancy. **E:** Calcium content of the hind limb arteries. The heterozygous mothers were kept on tap water (n = 7) or 0.3 mM PPi (n = 6) during pregnancy. Offspring was kept on tap water for 55 days. (Dedinszki et al, 2017)

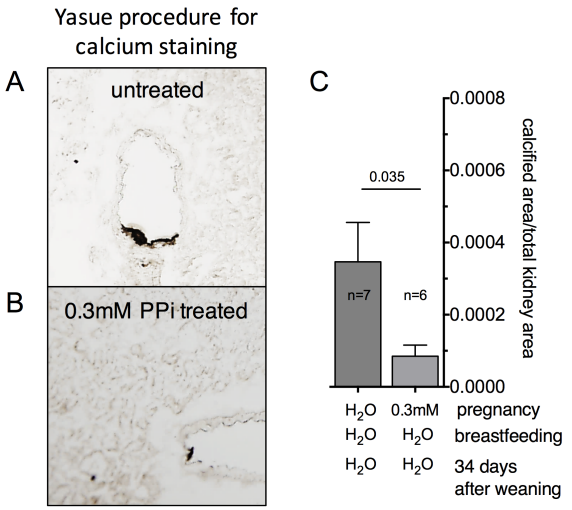

Figure 5: Prenatal PPi treatment of the Enpp1$^{-/-}$ mice attenuates calcification of the kidneys.
A, B: Typical kidney sections of a 55-day-old animal of the control group and of a 55-day-old animal from the group of 0.3 mM treatment only during pregnancy. Sections were stained by the Yasue procedure.

C: Calcification of renal arteries. The Enpp1$^{+/-}$ mothers were kept on tap water (n = 7) or on 0.3 mM PPi (n = 6) during pregnancy. Offspring was kept on tap water for 55 days (Dedinszki et al, 2017)

B) MECHANISM OF ABCC-TYPE TRANSPORTERS

Drosophila multidrug resistance-associated protein (DMRP) is the functional ortholog of human long ABCC transporters, with similar substrate and inhibitor specificity, but higher activity. Exploiting its high activity, we kinetically dissected the catalytic mechanism of DMRP by using E_2-d-glucuronide (E_2G), the physiologic substrate of human ABCC. we show that the second E_2G binding-similar to human ABCC2-allosterically stimulates transport activity of DMRP. Our data suggest that this is achieved by a significant increase in the coupling of ATP hydrolysis to transport.

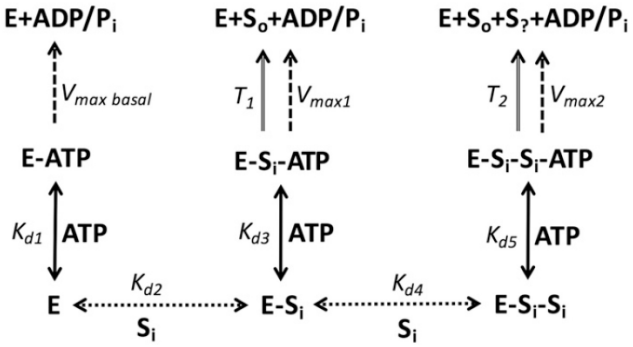

Figure 4. Kinetic parameters are depicted in italics and refer to dissociation constants for ATP (K_{d1}, K_{d3}, K_{d5}), E_2G (K_{d2}, K_{d4}), and initial rate of ATP hydrolysis in the absence of E_2G ($V_{max\ basal}$) or in the presence of 1 or 2 molecules of E_2G (V_{max1}, V_{max2}), respectively, as well as initial rate of E_2G transport in the presence of 1 or 2 molecules of E_2G (T_1, T_2), respectively. (Karasik et al, 2018.)