In this final report we discuss in detail two of our major achievments, both are preclinical studies for precision medicine and best described as bridges between basic and translational science.

I. Pyrophosphate as a therapeutical metabolite in connective tissue calcification disorders:

Physiological mineralization is essential for the normal development of vertebrates and restricted to specific sites of the body. In mammals, biominerals predominantly consist of calcium and phosphate which form hydroxyapatite. In plasma and other body fluids calcium and phosphate are present at concentrations far exceeding their solubility constant. Vertebrates have evolved mechanisms to stabilize this supersaturated solution permitting precipitation of calcium and phosphate only at specific sites.

Pyrophosphate (PPi) is a central factor in the prevention of precipitation of calcium and phosphate in soft peripheral tissues . The liver is the most important source of circulatory PPi, via a pathway depending on ABCC6-mediated ATP release. Within the liver vasculature, released ATP is rapidly converted into AMP and PPi by the ectoenzyme ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1). Inactivating mutations in the genes encoding enzymes involved in PPi homeostasis result in rare hereditary calcification disorders. Absence of functional ABCC6 results in pseudoxanthoma elasticum (PXE), a late onset ectopic calcification disorder, with lesions found in the skin, eyes and cardiovascular system . Biallelic inactivating mutations in *ENPP1* cause generalized calcification of infancy (GACI), a condition that can become life threatening shortly after birth due to massive calcification of the large and medium-sized arteries. While in PXE plasma PPi concentration is reduced to 40% of normal, GACI patients have virtually no PPi in their blood, which explains the severity of the disease.

Primary hepatocytes cultured between two layers of collagen, so called sandwich-cultured hepatocytes, retain their polarization. We utilized sandwich-cultured *Abcc6*$^{-/-}$ mouse hepatocytes to study the localization and function of introduced wild type and mutant forms of ABCC6. These conditions favour cell-cell contacts in a similar fashion as observed in liver tissue. Cells even form bile canaliculi. Moreover, in a collaborative effort we were involved to show that medium of sandwich-cultured hepatocytes of *Abcc6*$^{-/-}$ mice contains less PP_i than medium of wild-type hepatocytes(Jansen et al 2014). This

result provided one of the ultimate proofs of the essential role of PPi in development of PXE.

Because reduced PPi concentrations in the circulation underlies the ectopic calcification disorders PXE and GACI, a treatment for these disorders would be PPi supplementation. Due to the necessity to treat patients life-long oral administration would be preferred. Phosphatases are abundantly present in the gut therefore it has been always claimed that orally administered PPi does not reach the circulation and therefore it is not effective in inhibiting ectopic calcification. We have tested this assumption in healthy human individuals and in two mouse models of human hereditary calcification disorders, PXE and GACI.

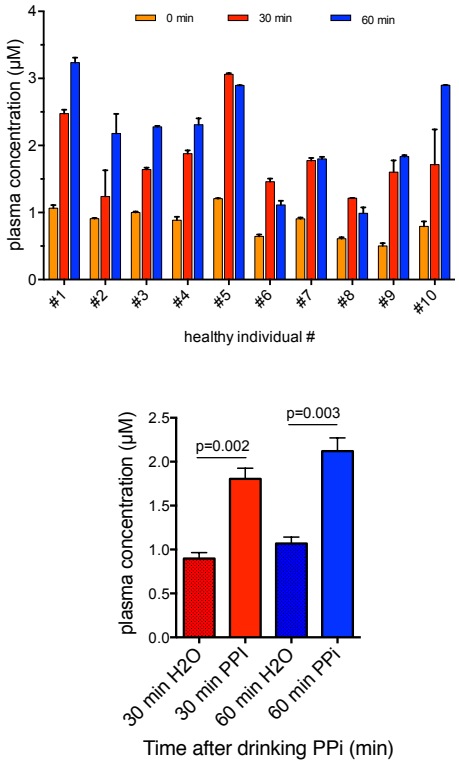
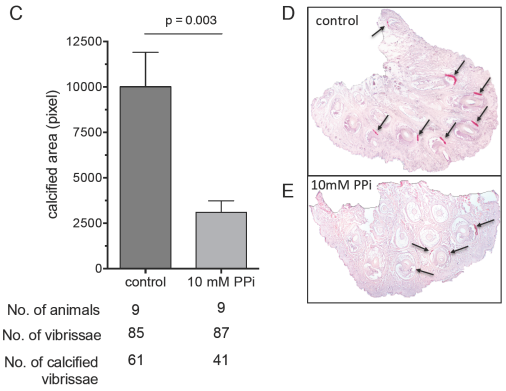


Figure 1. Uptake of PPi in human

Uptake of PPi in human and mice:

We tested whether orally consumed PPi is absorbed in human. Healthy human volunteers (five females and five males) ingested tetrasodium pyrophosphate solution (110 mM, pH 8.0) resulting in a dose of 98mg tetrasodium pyrophosphate/kg body weight or water (three females and three males). The ingested amount of tetrasodium pyrophosphate corresponds to 33% of the maximal tolerable daily intake published by the World Health Organization, WHO. We detected elevated plasma PPi in each individual 30 and 60 minutes after ingestion of the PPi solution (see Figure 1 upper panel). Statistical analysis showed that the difference in the plasma PPi-level between the water-drinking and PPi-drinking group was significant, both at 30 and 60 min after ingestion (see Figure 1 lower panel). The ~1 μM elevation we detected would apparently restore plasma PPi to the level of normal in GACI and in PXE patients. Next we tested the effect of direct delivery of PPi into the oral cavity, stomach or small intestine of mice and found that PPI was remarkably well absorbed from all sites tested (not shown).

Oral PPi inhibits calcification in Abcc6$^{-/-}$ mice:



We tested whether the spontaneous ectopic calcification in *Abcc6*$^{-/-}$ mice could also be attenuated by PPi administration *via* the drinking water. A hallmark phenotype seen in *Abcc6*$^{-/-}$ mice is the gradual calcification of the connective tissue surrounding the vibrissae. We therefore determined the effect of oral PPi on calcification of the dermal sheet surrounding the vibrissae by quantitation of calcified areas on the Alizarin Red-stained microscopic slices. Just after weaning *Abcc6*$^{-/-}$ mice received PPi-containing drinking water (10mM) for 19 weeks when the effect was determined by quantitation of calcified areas on the Alizarin Red-stained microscopic slices. Orally available PPi potently inhibited the spontaneous calcification seen in *Abcc6*$^{-/-}$ mice. (Figure 2 and D).

Figure 2. ***Oral PPi inhibits calcification in Abcc6$^{-/-}$ mice***

Oral PPi attenuates calcification in Enpp1$^{-/-}$ (ttw) mice: Tiptoe walking (*ttw*) mice have an inactivating mutation in *Enpp1*. These mice have extremely low plasma PPi levels and like GACI patients, develop extensive calcifications in blood vessels and joints shortly after birth. Just like *Abcc6*$^{-/-}$ mice, *the Enpp1*$^{-/-}$ mice show extensive calcification of the dermal sheet surrounding the vibrissae and in these animals the phenotype shows up much earlier than in the PXE mice. We have studied the *Enpp1*$^{-/-}$ offsprings of heteroyzgous pairs.

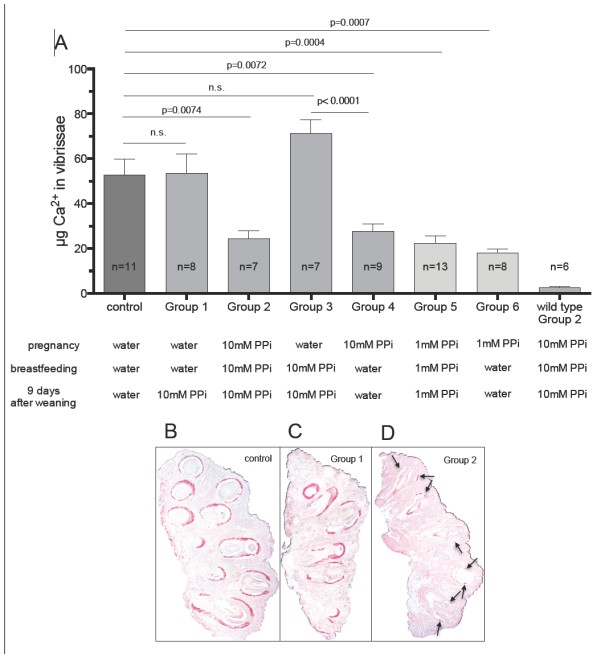


Figure 3. ***Oral PPi inhibits calcification in Enpp1$^{-/-}$ mice***

Orally administered PPi during pregnancy and lactation followed by oral PPi treatment of the pups, resulted in reduced calcification of the dermal sheet surrounding the vibrissae in the *ttw* mice (see Figure 3A, B, C and D). To determine when PPi treatment was most effective, we analysed the effect of PPi separately during pregnancy, lactation and after weaning. When *ttw* pups were treated only after weaning or PPi was provided only during lactation, the amount of calcification was not different from the control-treated group. Calcification was inhibited in *ttw* mice only when their heterozygous mothers had received PPi during pregnancy. It is worth to note that as low as 1mM PPi triggered even larger inhibitory effect in the "*in utero* PPi" group than 10 mM PPi ($p=0.022$; compare Group 4 and 6 on Figure 3A).

In summary, oral administration may represent a simple route to achieve therapeutic levels of the physiological, non-toxic metabolite PPi in the blood circulation. Our data indicate that oral PPi has potential in treatment of two currently incurable diseases, PXE and GACI. Importantly, oral PPi might have broader applicability and be useful in other conditions involving ectopic calcification, such as hypercholesterolemia, diabetes, chronic renal insufficiency, β-thalassemia and heterotopic ossification of traumatized muscle. Clinical studies in PXE are planned in the US. The risk of orally administrated PPi to patients is probably negligible. The WHO considers PPi a nontoxic physiological metabolite with a high maximal tolerable daily intake value (MTDI). Moreover, it is used generally in the food industry as an additive, and the Code for Federal Regulation by the FDA states: "*This substance is generally recognized as safe when used in accordance with good manufacturing practice*". It may also worth to note that PPi would represent an unusually low cost treatment of connective tissue calcification disorders.

A manuscript describing the above results are under revision at EMBO Molecular Medicine.

A patent "*Oral pyrophosphate for use in reducing tissue calcification*" has been filed in The Netherlands with an 85% intellectual property of scientists of our research group (see Publications).

II. **Pharmacological correction of mislocalized ABCC6 proteins *in vivo:* preclinical basis of an allelel-specific therapy in PXE**

We have established in the present grant period that ABCC6 is a basolateral plasma membrane protein expressed in the sinusoid side of hepatocytes (Pomozi et al, 2013). This result paves the road toward a novel therapeutical solution: to correct the the intracellular traficking of disease-causing missense mutant ABCC6 proteins. As approx. 75% of PXE patients are with at least one missense allele, this approach has a potential to provide therapy for a large portion of PXE patients. (Note that PXE is a monogenic recessive disorder.)

Indeed, we have shown earlier that most of the ABCC6 missense mutations does not affect the transport activity of the protein, but instead results in protein folding problems preventing correct routing to the plasma membrane. Restoration of plasma membrane localization has the potential to restore ATP secretion and PP_i formation in the liver vasculature. We have also demonstrated earlier that several ABCC6 missense mutants can be redirected to the plasma membrane by the FDA-approved chemical chaperone 4-phenylbutyrate (4-PBA) *in vivo* in the liver of living mice. In these experiments we used hydrodynamic tail-vein injections to deliver human ABCC6 cDNAs into the liver. This approach resulted in organ-specific expression in 5-10% of the hepatocytes.

In the present grant period, we addressed whether calcification inhibition could be restored in *Abcc6-/-* mice expressing human ABCC6 mutants in their liver and treated with 4-PBA.

First, we have shown that development of an induced soft tissue calcification, the dystrophic cardiac calcification, DCC is solely depends on the function of ABCC6 (Brampton et al., 2014). DCC is based on applying cryo-injury to the heart muscle and determining the calcium content in the injured tissue afterwards. DCC can be induced in Abcc6-deficient mice, but not in wild-type animals. Therefore, to determine if 4-PBA could restore the calcification inhibition potential of ABCC6 mutants, we quantified DCC as a measure of ABCC6 physiological function.

Furthermore, we found that transient expression of human ABCC6 in livers of Abcc6-deficient mice dramatically reduced (by 62%) the level of DCC (20) see also figure 4C.

Importantly, our recently published data show that overexpression of disease-associated mutants did not rescue the DCC phenotype, but that co-administration of 4-PBA resulted in attenuation of *in vivo* calcification by 50-64% ($p<0.05$ for each of the mutants, Figure 3C) thus providing a **proof of principle** of the clinical potential of chemical chaperones in the treatment of PXE and ABCC6-dependent GACI (Pomozi et al, 2016).

There is now a growing interest in developing and testing a pharmacological correction for mutated transmembrane proteins. Developing new drugs for humans is a long and costly process, and generally unattractive for orphan diseases. One current and very promising trend to circumvent this problem is to re-**purpose existing drugs** and using them towards pathologies not initially identified for these drugs. 4-PBA has been used for many years for the clinical treatment of urea cycle disorders and thalassemia.

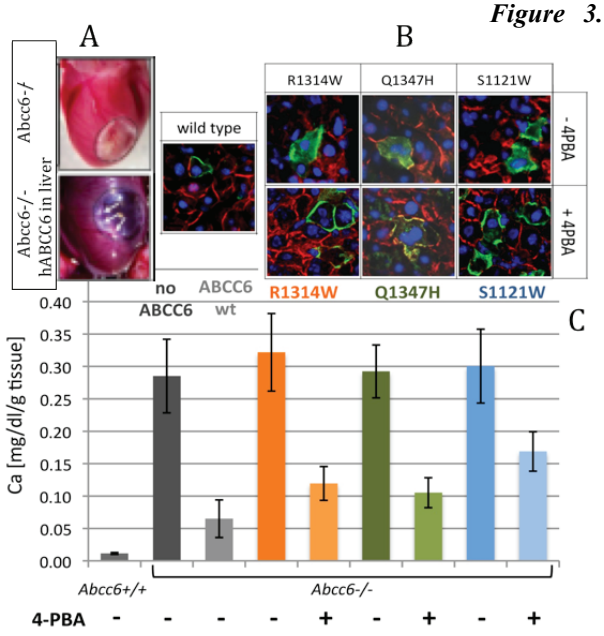


Figure 3. In vivo pharmacological correction of plasma membrane localization of mutant ABCC6 variants by administration of 4-PBA results in inhibition of cardiac calcification. Human ABCC6 variants were overexpressed *in vivo* in the liver of *Abcc6*$^{-/-}$ mice by tail-vein injections of plasmids containing the indicated cDNAs. **A:** Images of the lesions in *Abcc6*$^{-/-}$ mice with and without human ABCC6 expression in the liver; **B:** confocal image of frozen liver sections; green: human ABCC6, red: plasma membrane marker, blue: DAPI to visualize the nuclei; **C:** extent of cryo-injury induced dystrophic cardiac calcification.

In conclusion, our study demonstrated the viability of using 4-PBA to rescue the functional defects of certain ABCC6 missense mutations and restore their calcification inhibition potential. Based on our results, we propose that 4-PBA treatments could serve as an allele-specific therapy for PXE/GACI. Approximately 75% of PXE patients harbor at least one missense allele and many of these patients carrying missense mutants with residual transport activity could be eligible for 4-PBA treatment. As PXE and GACI are

recessive diseases, the rescue of single missense allele should be sufficient. Therefore, if 4-PBA treatment is eventually used to treat PXE patients, one will have to define which clinical criteria to use to assess treatment efficacy. As approximately 75% of the PXE patients have at least one missense allele, the therapeutic potential of pharmacological correction of intracellularly retained ABCC6 is considerable. In a French cohort of PXE patients, together with Dr. L. Martin (Angers Medical School, France) we identified several ABCC6 mutants that are likely to be amendable for 4-PBA treatment. We will test *in vitro* whether these mutants support ATP efflux from cells and prevent ectopic calcification in *Abcc6*$^{-/-}$ mice after 4-PBA treatment *in vivo*. This collaboration is a good example of "Bridging the gap to the clinic" efforts.

The most important **PUBLICATIONS** and a filed patent

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Brampton C, Aherrahrou Z, Chen LH, Martin L, Bergen AA, Gorgels TG, Erdmann J, Schunkert H, **Szabó Z, Váradi A,** Le Saux O. (2014) The level of hepatic ABCC6 expression determines the severity of calcification after cardiac injury.
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Functional Rescue of ABCC6 Deficiency by 4-Phenylbutyrate Therapy Reduces Dystrophic Calcification in Abcc6$^{-/-}$ Mice.
J Invest Dermatol. 2016 Nov 5. doi: 10.1016/j.jid.2016.10.035. [Epub ahead of print]

Váradi A, Dedinszki D, Szeri F, van de Wetering K and Borts, P. (2016) Patent application *Oral pyrophosphate for use in reducing tissue calcification* filed 15/09/20.

Overzicht geregistreerde gegevens bij indiening

Aanvraagnummer	: 2017471	**Ingediend**:	15/09/2016
VNO soort	: Internationaal Onderzoek	**Ingediend:**	15/09/2016
Korte aanduiding	: Oral pyrophosphate for use in reducing tissue calcification		
Tenaamstelling	: Stichting Het Nederlands Kanker Instituut-Antoni van Leeuwenhoek Ziekenhuis te AMSTERDAM, Nederland		
Aanvrager(s)	: Stichting Het Nederlands Kanker Instituut-Antoni van Leeuwenhoek Ziekenhuis Plesmanlaan 121 1066 CX AMSTERDAM Nederland		
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