One of the major goals of this research project has been “In Vivo Preclinical Studies supporting a Clinical Trial of Allele-Specific Conformational Therapy in Pseudoxanthoma Elasticum”

Inactivating mutations in ABCC6 result in low circulating levels of the calcification inhibitor PPi and, consequently, ectopic calcification. Several clinically relevant missense mutations do not affect the transport activity of ABCC6, but result in misfolding, mistrafficking and retention of the protein in the endoplasmic reticulum. This type of ABCC6 mutants can potentially be redirected to the plasma membrane by chemical chaperones like 4-phenylbutyrate (4-PBA). Using 4-PBA in our experiments has a great advantage, because it is also approved by U.S. Food and Drug Administration for clinical use in urea cycle disorders and thalassaemia.

During this study, we have identified missense mutations with preserved transport function but with abnormal intracellular processing (no or only partial plasma membrane localization) in two PXE patients’ cohorts (Angers/France and Ghent/Belgium). The PXE expertise centres in Angers and Ghent have cohorts of fully characterized PXE patients (also with known genotypes). We set up criteria to select ABCC6 mutants for preclinical testing in vivo: 1) ABCC6 missense mutations that are linked to PXE. 2) The mutation should be present in at least one of the patients seen at the PXE expertise centres at the University of Angers in France or at the University of Ghent in Belgium. 3) Preferably, the mutation is present in several patients of the Angers/Ghent cohort. 4) Alleles encoding ABCC6 variants with mutations in the catalytic ATP-binding sites will be excluded, as these will lack intrinsic transport activity. Based on the above criteria PXE patients present in both cohorts having at least one allele were chosen and their mutations were analyzed for their eligibility for 4-PBA treatment in the first year of this study (a total of 29 patients fell into this category). The selected mutations are the followings:

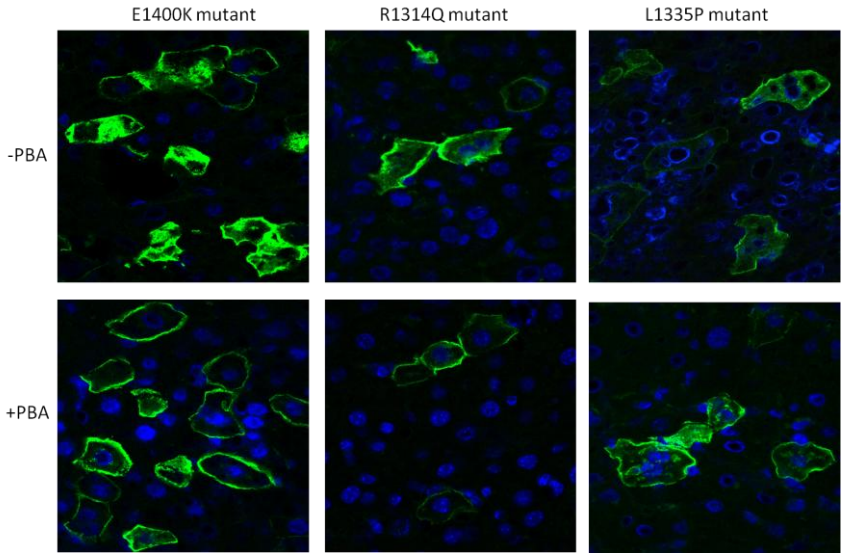
-R518Q mutant (9 patients)
-G992R (4 patients)
-T1130M (4 patients)
-R1314Q mutant (3 patients)
-L1335P (3 patients)
-E1400K (5 patients)

As a second step, we had to identify the localization of the mutants to study the effect of 4-PBA on the selected mutations in vivo. For the localization identification, the mutated cDNAs (encoding these specific human ABCC6-mutants) were cloned into a liver-specific pLIVE vector carrying the mouse albumin promoter and a fetoprotein enhancer that ensured a liver-specific expression. Then we utilized hydrodynamic tail vein injection (TVI) of pLIVE vectors into 12-15 weeks old Abcc6-/- mice. Mice were injected with the optimal dosage of 70 to 90 μg of plasmid. Previously we have demonstrated many times that we could achieve a high level of liver expression in mice with TVI. Each mutant was injected into at least 6 mice and the livers were collected four days after the TVI. The subcellular localization defects of ABCC6 mutants were determined by immunohistochemistry on cryo-preserved liver sections using confocal microscopy. Based on the confocal images we found that four of the six mutants were mislocalized and two of the six mutants were partly in the plasma membrane in mouse liver:

-R518Q mutant showed mainly intracellular (IC) localization
-G992R mutant located in the IC
-T1130M mutant was targeted to the plasma membrane (PM)

-R1314Q mutant located mostly IC
-L1335P mutant located IC
-E1400K located mostly in the PM

One of the main aims of this study was to evaluate the effect of 4-PBA on the different ABCC6 mutants in Abcc6-/- mice. In the first year, we examined three ABCC6 mutants (R1314Q, R518Q, E1400K). No 4-PBA induced plasma membrane rescue was observed for R518Q in Abcc6-/- mouse liver. The 4-PBA treatment resulted in plasma membrane targeting in the case of E1400K and R1314Q mutants. In the second year we continued to examine the effect of 4-PBA on the other three mutants (G992R, T1130M, L1335P). Mice received intraperitoneal injections of 4-PBA (1000 mg/kg/day) right after the hydrodynamic tail vein injections. Injection were continued daily thereafter until euthanasia. In addition, drinking water contained 12mg/ml 4-PBA to ensure continuous exposure to the drug. No 4-PBA induced plasma membrane rescue was observed for G992R in Abcc6-/- mouse liver. The T1130M mutant was located in the plasma membrane without 4-PBA treatment. The 4-PBA treatment resulted in plasma membrane targeting in the case of L1335P mutant.




*Picture 1: The 4-PBA treatment resulted in plasma membrane targeting in the case of E1400, R1314Q and L1335P mutants*

By the end of the second year, we identified the localization of all mutants in vivo and the effect of 4-PBA treatment. Our next goal is to investigate whether 4-PBA treatments could rescue the calcification inhibition potential of selected ABCC6 mutants. For this kind of examinations we use 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. Firstly, we needed to set up control measurements, we injected wild type ABCC6 (wt ABCC6) into 12-15 weeks old Abcc6-/- mice to show out that wt ABCC6 could enhance the calcification inhibition. I could not start the experiments with the mutants, which show plasmamembrane localization after the 4-PBA treatment, because i was on **MATERNITY LEAVE from August 1., 2018**.

Publications:
1. I took part in a study where we applied similar methods to examine the effect of 4-PBA on different mutant in vitro and in vivo. We have published an article entitled: Pomozie et al., 2017, Functional Rescue of ABCC6 Deficiency by 4-Phenylbutyrate Therapy Reduces

Dystrophic Calcification in Abcc6-/- Mice. (J Invest Dermatol. 2017 Mar;137(3):595-602. doi: 10.1016/j.jid.2016.10.035.) We have previously 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 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. We showed that 4-PBA administrations restored the physiological function of ABCC6 mutants resulting in enhanced calcification inhibition. This study identifies 4-PBA treatments as a romising strategy for allele-specific therapy of ABCC6-associated calcification disorders.

2. To gain a better insight of the mechanism of the action of PXE disease we also investigated the possible inhibitory effect of the oral administration of pyrophosphate (PPi) in mice and humans. We have published an article entitled: Dedinszki et al., 2017, Oral administration of pyrophosphate inhibits connective tissue calcification. (EMBO Mol Med. 2017 Jul 12. pii: e201707532. doi: 10.15252/emmm.201707532.)
Various disorders including pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI), which are caused by inactivating mutations in ABCC6 and ENPP1, respectively, present with extensive tissue calcification due to reduced plasma pyrophosphate (PPi). However, it has always been assumed that the bioavailability of orally administered PPi is negligible. Here, we demonstrate increased PPi concentration in the circulation of humans after oral PPi administration. Furthermore, in mouse models of PXE and GACI, oral PPi provided via drinking water attenuated their ectopic calcification phenotype. Noticeably, provision of drinking water with 0.3mM PPi to mice heterozygous for inactivating mutations in Enpp1 during pregnancy robustly inhibited ectopic calcification in their Enpp1-/- offspring. Our work shows that orally administered PPi is readily absorbed in humans and mice and inhibits connective tissue calcification in mouse models of PXE and GACI PPi, which is recognized as safe by the FDA, therefore not only has great potential as an effective and extremely low-cost treatment for these currently.

3. I took part in a study where we examined the trauma-induced calcification (that is the pathological consequence of complex injuries which often affect the central nervous system and other parts of the body simultaneously) in mouse model. We have published an article entitled: Tokesi et al., 2020, Pyrophosphate therapy prevents trauma-induced calcification in the mouse model of neurogenic heterotopic ossification (J Cell Mol Med. 2020 Oct;24(20):11791-11799. doi: 10.1111/jcmm.15793. Epub 2020 Sep 4.) We found that although the level of plasma pyrophosphate, the endogenous inhibitor of calcification, was normal in calcifying animals, it could not counteract the acute calcification. However, externally added pyrophosphate inhibited calcification even when it was administered after the complex injuries. Our finding suggests a potentially powerful clinical intervention of calcification triggered by polytrauma injuries which has no effective treatment.

4.I took part in a study where we obtained a monoclonal antibody. We have published an article entitled: Kozak et al., 2020 Creation of the first monoclonal antibody recognizing an extracellular epitope of hABCC6 (FEBS Lett. 2021 Mar;595(6):789-798. doi: 10.1002/1873-3468.13991. Epub 2020 Nov 28.) We immunized bovine FcRn transgenic mice exhibiting an augmented humoral immune response with Human Embryonic Kidney 293 cells cells expressing human ABCC6 (hABCC6). We obtained a monoclonal antibody recognizing an EC epitope of hABCC6 that we named mEChC6. Limited proteolysis revealed that the

epitope is within a loop in the N-terminal half of ABCC6 and probably spans amino acids 338-347. mEChC6 recognizes hABCC6 in the liver of hABCC6 transgenic mice, verifying both specificity and EC binding to intact hepatocytes.