Final report for the ""*In Vivo Studies supporting the Allele-Specific Conformational Therapy In Genaralized Arterial Calcification in Infancy (GACI)"* project

Inactivating mutations in *Enpp1* (ectonucleotidase pyrophosphatase phosphodiesterase 1) result in low circulating levels of the calcification inhibitor pyrophosphate (PPi) and, consequently, ectopic calcification and the development of GACI. Several clinically relevant missense mutations do not affect the enzyme activity of ENPP1, but result in misfolding, destabilization and degradation and/or mistrafficking, misslocalization and retention of the protein in the cytoplasm. This type of ENPP1 mutants can potentially be refolded, stabilized and redirected to the plasmamembrane by chemical chaperones like 4-phenylbutyrate (4-PBA) or its analog molecule, Ravicti. According to the international GACI registry, created by our collaborator Prof. Frank Rutsch (Munster University Children's Hospital, Germany), majority of the GACI patients have missense mutation, thus the therapeutic potential of the pharmacological correction of the missfolded, destabilized and/or misslocalized and intracellularly retained ENPP1 is considerable. This work is aimed to identify disease-causing ENPP1 missense mutations that are amendable to treatment with 4-PBA or Ravicti. Based on this work patients can be selected for enrolment into clinical trial.

The main aim of the current research plan was to identify *in vivo* those missense ENPP1 mutants that:

1. Are misfolded, destabilized and undergo degradation and/or mistrafficked and retained intracellularly.
2. Can be refolded, stabilized and/or redirected to the plasma membrane using 4-PBA or Raviti.
3. Have retained their capacity to attenuate soft tissue calcification after 4-PBA or Ravicti treatment ("functional rescue").

Selection of ENPP1 mutants for in vivo testing

Our collaborator Prof. Rutsch selected 13 putative pathogenic *ENPP1* missense variants (c.583T>C, p.Cys195Arg; c.583C>A, p.Cys195Ser; c.902A>G, p.Tyr301Cys; c.913A>G p.Pro305Thr; c.1412A>G, p.Tyr471Cys; c.1510A>C, p.Ser504Arg; c.1538A>G, p.Tyr513Cys; c.1612G>C, p.Asp538His; c.1756G>A, p.Gly586Arg; c.1976A>G, p.Tyr659Cys; c. 2330A>G, p.His777Arg; c.2462 G>A, p.Arg821His; c.2662C>T, p.Arg888Trp) for *in vitro* studies from their registry (Stella et al. 2016). Selection was based on the fact that the missense variants were novel and/or not functionally characterized before, but also with respect of their frequency in the registry. Additionally, they selected variants of unknown significance that were distributed over diverse regions and domains of *ENPP1*, located in highly conserved but also in nonconserved regions. These missense variants were analysed regarding their functional properties, that is, activity, expression, localization, and PPi generation. They showed that transfection of eight of the 13 variants into HEK293 cells led to complete loss of ENPP1 activity, whereas four mutants (p.Tyr471Cys; p.Ser504Arg; p.Tyr659Cys; p.His777Arg) showed residual activity compared with the wild-type ENPP1. One putative pathologic variant (p.Arg821His) showed normal activity in vitro. These five mutants with normal or residual ENPP1 enzyme activity were still able to generate PPi and localized in the plasma membrane. Based on this in vitro study I choose those 4 mutants for the in vivo studies that showed residual activity (p.Tyr471Cys, p.Ser504Arg, p.Tyr659Cys, and p.His777Arg) therefore would be potential therapeutical targets for conformational-stabilizing agents, like 4-phenylbutyrate or Ravicti. I also included two inactive mutants as negative controls, p.Pro305Thr; p.Tyr301Cys, being expressed in the plasma membrane and the other with intracellular localization, respectively.

Generation of expression plasmids

Coding sequence of human wtENPP1 was cloned from pcDNA-wtENPP1 (gift of Prof. Rutsch) into pLIVE (Mirus Bio) via digestions with specific restrictions enzymes (BamHI-XhoI). Correct insertion was verified

via sequencing. This pLIVE-wtENPP1 plasmid served as template for the construction of the mutant plasmids. Mutations were introduced via site-directed mutagenesis using the Quick Change Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). As the DNA polymerase tends to make mistakes after certain DNA length (600-1000bp), I minimalized the chance of these mistakes by moving only ~1000bp long DNA cassettes. With the help of the program SnapGene, I identified restriction sites within the sequence of *ENNP1* and choose optimal cassettes surround the required mutation points. These cassettes were between the following restriction sites: BamHI-StuI to generate p.Tyr301Cys and p.Pro305Thr mutations, StuI-XcmI in case of p.Tyr471Cys, p.Ser504Arg and p.Tyr659Cys, XcmI-BsttZ17I for p.His777Arg. To move a cassette it is necessary to have unique restriction sites. To achieve this aim first I had to mutate one of the two XcmI restriction sites within the sequence of *ENPP1* (silent mutation). In this way I managed to have a template plasmid that contains only one from each restriction sites that I wished to use. This plasmid was denoted as pLIVE-wtENPP1 1xXcmI and was used to introduce all the mutations afterwards. The correct sequence and insertion were verified via sequencing.

Determination of intracellular localization of ENPP1 mutants in *Enpp1*$^{-/-}$ mice

To determine the intracellular localization of wtENPP1 and its mutants we have used our previously established complex experimental strategy tested on *Abcc6* $^{-/-}$ mice with ABCC6 mutants as a model of another soft tissue calcification disease: pseudoxanthoma elasticum (PXE) (Pomozi et al., 2016). Mutants were expressed *in vivo*, in mouse liver via hydrodynamic tail vein injection (HTVI) of the plasmid constructs. The mechanism of how the rapid injection of nucleic acid in a large volume leads to increased liver expression is under investigation by a number of laboratories. The increased volume and increased pressure for direct injection into liver vessels was initially proposed as a means to increase the size of the sinusoid fenestrae, thereby increasing extravasation and delivery of pDNA to liver cells. The injected volume is delivered to the liver because of a transient heart failure that is accompanied by decreased heart rate, increased cardiac ventricular size and an increase in the inferior vena cava pressure. As a result, the pDNA solution back fills the hepatic vein into the liver (Sebestyen et al., 2006). Four days after the injection, the liver of the mice were isolated and frozen. To determine the intracellular localization of the *wtENPP1* and its mutants immunohistochemistry on these liver sections was carried out after cryo-sectioning, using human ENPP1 specific antibody. Investigation of the immunostained liver sections by confocal microscopy showed that the wild type human ENPP1 is localized physiologically. It was visible within the plasma membrane of the liver cells (Fig1).

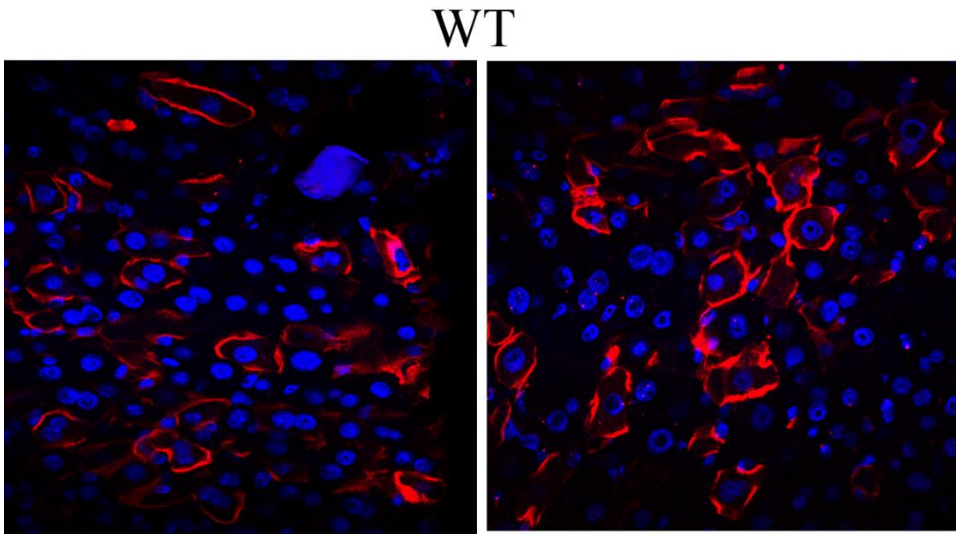

Figure 1

Localization of wtENPP1 (red) within mouse liver (blue: nucleus)

In contrast both the two inactive mutants (Y301C, P305T) and the other four mutants that had residual enzyme activity *in vitro* (Y471C, S504R, Y659C, and H777R) showed homogeneous cytoplasmic distribution (Fig 2). In case of P305T, that was localized in the plasma membrane *in vitro* its intracellular

localization *in vivo* is novel and emphasize the relevance and importance of the *in vivo* investigation besides the *in vivo* studies as we observed similar phenomenon in case of other mutants earlier (Pomozi et al, 2014).

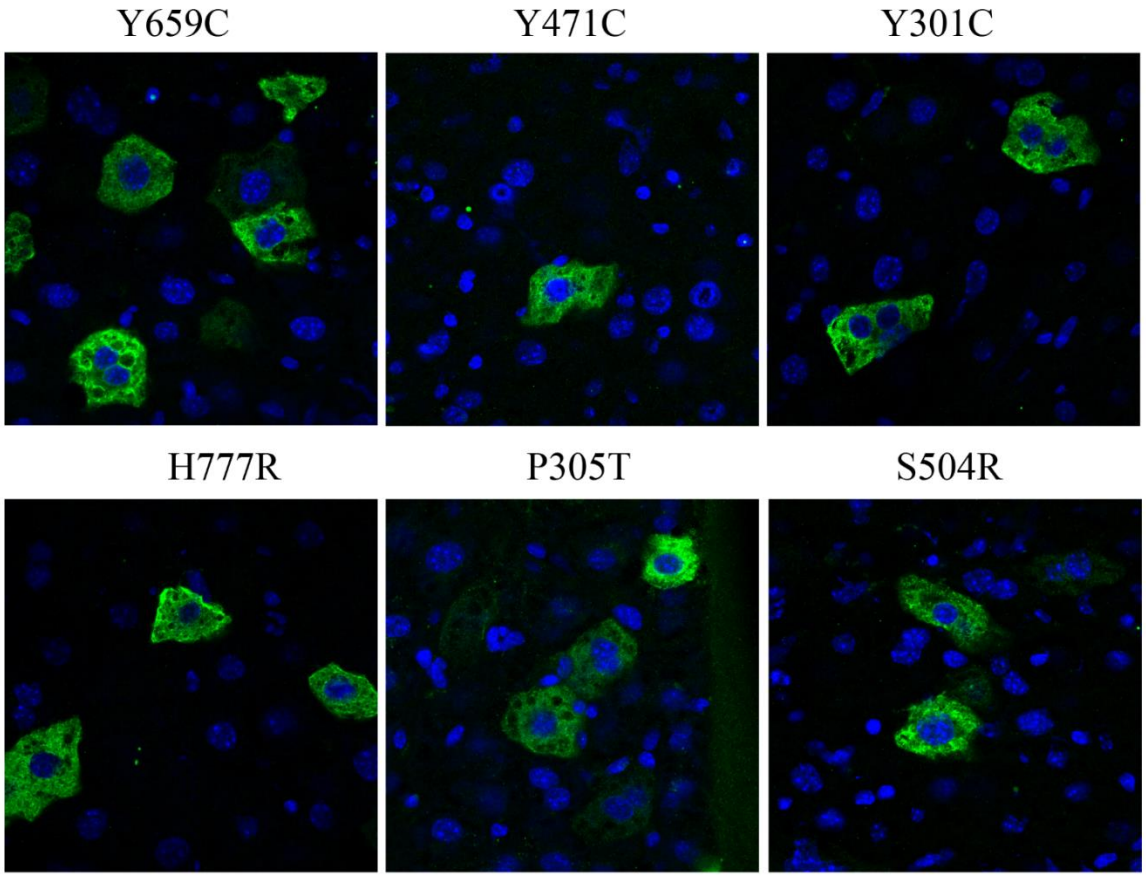

Figure 2

Localization of different ENPP1 mutants (green) within mouse liver (blue: nucleus)

Determination of the effect of 4-PBA on the intracellular localization of ENPP1 mutants

The 4-PBA is a histone deacetylase inhibitor, most probably influencing transcription of endoplasmatic reticulum (ER) chaperones as well as components of the ER-associated degradation system. Three recent reports describe that the liver function of patients suffering from progressive familiar intrahepatic cholestasis type 2 (PFIC2) spectacularly improved by 4-PBA treatment. PFIC2 is caused by mutations in the hepatic efflux transporter ABCB11. In all patients missense mutations resulted in retention of ABCB11 in the ER. 4-PBA treatment enhanced trafficking of these ABCB11 mutants to the canalicular membrane of the hepatocytes and greatly improved liver function (Hayashi et al., 2007; 2009, Lam et al., 2007). Our research group also demonstrated in case of the PXE mouse model system that several of the intracellularly retained ABCC6 mutants can be redirected to the plasma membrane by 4-PBA treatment (Pomozi et al., 2016). Therefore we treated our mice after HTVI with 4-PBA according to the following protocol. Mice typically received intraperitoneal injections of 4-PBA (100 mg/kg/day) right after HTVI and this injection were repeated daily thereafter until euthanasia. In addition, drinking water contained 12,5 mg/ml 4-PBA to ensure continuous exposure to the drug. Four days after the HTVI by the use of immunohistochemistry we could also clearly monitor the changes of the intracellular localization of the mutant ENPP1 due to 4-PBA treatment by confocal microscopy. Our results showed that all the six mutants showed no response to 4-PBA treatment, their cytoplasmic localization did not change after 4-PBA treatment (Fig 3).

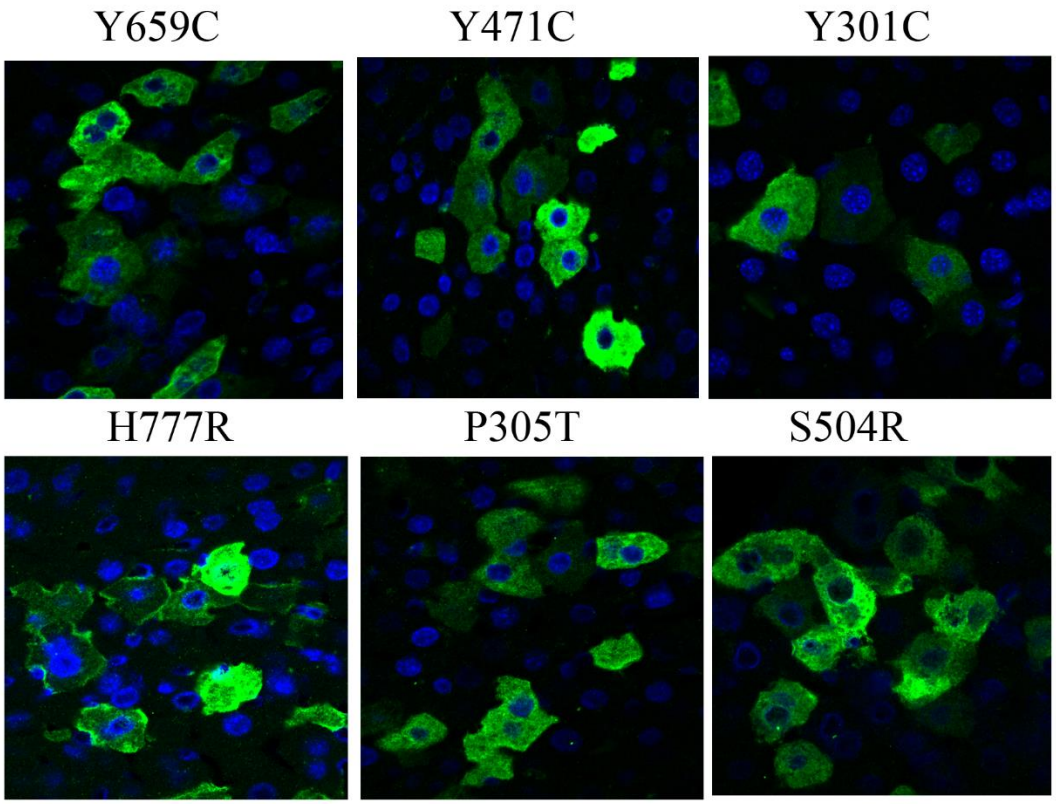

Figure 3

Localization of different ENPP1 mutants (green) within mouse liver (blue: nucleus)

Determination of the effect of 4-PBA on DCC in *Enpp1*$^{-/-}$ mice

A problem with our animal model is that, like human GACI, the calcification phenotype is late onset, so we can not use in our above detailed experimental setup for the detection of calcification inhibition. Therefore we used a short-term ectopic calcification model for the functional analysis, that is called: dystrophic cardiac calcification (DCC) injury (Doehring et al., 2006). It proved to be effective in the PXE mouse model earlier (Pomozi et al., 2016), as a valuable additional phenotype to the spontaneous calcification model and is especially useful to rapidly screen for experimental therapies. The protocol of DCC is based on a myocardial freeze-thaw injury carried out through the abdomen of anaesthetized animal. In brief, one day after HTVI abdomen was opened in midline. The left lobe of the liver was gently flipped over to allow visualization of the beating heart through the translucid diaphragmal base. A blunt steel pin, precooled in liquid nitrogen, was pressed gently for 10 seconds onto the diaphragm aiming at the heart. Then, the abdomen was closed using suture material. Four days after HTVI mice were sacrificed by cervical dislocation and the hearts were quickly excised and Ca content was analyzed by calcification measurements via a colorimetric assay using the Calcium Liquicolor kit (Stanbio), that measures directly the amount of excess calcium as previously described (Brampton et al., 2014). Prior to the measurement hearts were chopped up and decalcified for 48 hours in 0.15N HCl and the total calcium content of the supernatant was assessed after centrifugation. Our results showed massive calcification of the hearts 3 days after the cryo-injury in the *Enpp1* $^{-/-}$ mice, as compared to the wild type *Enpp1*$^{+/+}$ mice (3 μg vs. 35 μg, respectively), suggesting that calcification is ENPP1 dependent. However the expression of the human wild type ENPP1 in the liver, did not rescued this phenotype although it localized nicely at the plasma membrane of the liver cells (34 μg) (Fig 4).

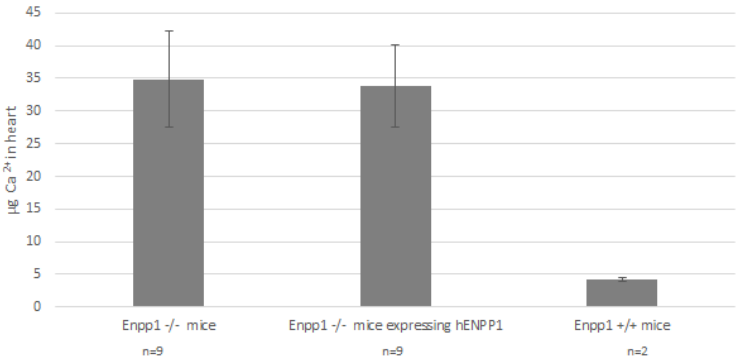

Figure 4

Calcium contents of the heart after DCC

It suggests that the exogeneously expressed ENPP1, at least in this amount and form, is not enough to restore the physiological level of PPi in the circulation that could prevent calcification. Although ENPP1 is expressed in wide range of tissues, and we restore its expression only in the liver with this method, as 60% percent of PPi is produced in the liver (Favre et al., 2017), our general approach to restore ENPP1 function within the liver was reasonable. However our results suggest that we could not achieve the minimum level of PPi in circulation that can inhibit calcification with this method. Taking into account these results, I did not investigated the DCC in case of the 6 ENPP1 mutants as these experiments become irrelevant.

Determination of the effect of Ravicti on the intracellular localization of ENPP1 mutants

Ravicti (glycerol phenylbutyrate), is an oral liquid that is used as a nitrogen-binding agent for chronic management of patients with urea cycle disorders (UCDs). As it is the 4th most expensive drug of the world first we applied it on three of the mutants only. In order to find the optimal dose first we treated our mice with 12.4 g/m2/day via gavage (recommended dose: 5 to 12.4 g/m2/day). The first gavage was performed one hour after HTVI, than repeated once a day for four days. Unfortunately this type of drug administration proved to be a very great expense to the mice as it can be detected by their significantly reduced activity. Therefore we changed this protocol and add 6 g/m2/day except the day of the HTVI, but the general condition of our mice did not turned to better significantly. In order to investigate the intracellular localization of the mutant ENPP1, four days after the HTVI we performed immunohistochemistry and investigated our samples by confocal microscopy. Our results showed that all the three mutants showed no response to Ravicti treatment, their cytoplasmic localization did not change (Fig 5).

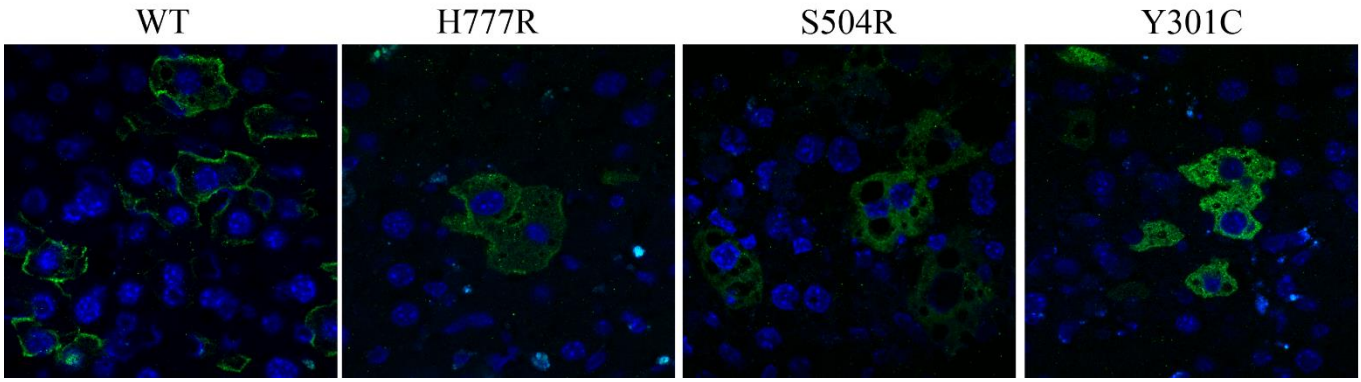

Figure 5

Localization of different ENPP1 mutants (green) within mouse liver (blue: nucleus)

Considering that Ravicti is very expensive and even the second "mildest" protocol also proved to be very straining to our mice, we decided not to repeat the experiment with the other three mutats.

In summary, I have managed to perform all the experiments that were planned in the three years of my post-doc grant. Unfortunately, none of the promising pharmacological chaperons were able to redirect any of the mutants (that I had chosen to investigate) from the cytoplasm to the plasma membrane.

However, meanwhile in relation to soft tissue calcification I developed a mouse model in with the trauma induced calcification can be investigated, and pointed out that PPi therapy inhibits this calcification process:

> **Tőkési N**, Kozák E, Fülöp K, Dedinszki D, Hegedűs N, Király B, Szigeti K, Ajtay K, Jakus Z, Zaworski J, Letavernier E, Pomozi V, Váradi A.: Pyrophosphate therapy prevents trauma-induced calcification in the mouse model of neurogenic heterotopic ossification. *J CELL MOL MED*. 2020

Besides that I was involved in different research project that investigated PPi supplementation as a potential therapy in other diseases:

> Dedinszki D, Szeri F, Kozak E, Pomozi V, **Tokesi N**, Mezei TR, Merczel K, Letavernier E, Tang E, Le Saux O, Aranyi T, van de Wetering K, Varadi A: Oral administration of pyrophosphate inhibits connective tissue calcification., *EMBO MOL MED* 9: (11) 1463-1470, 2017
>
> Pomozi V, Julian CB, Zoll J, Pham K, Kuo S, **Tőkési N**, Martin L, Váradi A, Le Saux O.: Dietary Pyrophosphate Modulates Calcification in a Mouse Model of Pseudoxanthoma Elasticum: Implication for Treatment of Patients., *JOURNAL OF INVESTIGATIVE DERMATOLOGY*, 2019
>
> Väärämäk Suvi, Uusitalo Hannu, **Tőkési Natália**, Pelttari Saku, Váradi András, Nevalainen Pasi I: Pyrophosphate Treatment in Pseudoxanthoma Elasticum (PXE)-Preventing ReOcclusion After Surgery for Critical Limb Ischaemia, *SURGICAL CASE REPORTS*, 2019
>
> Kozák E, Fülöp K, **Tőkési N,** Rao N, Li Q, Terry SF, Uitto J, Zhang X, Becker C, Váradi A, Pomozi V.: Oral supplementation of inorganic pyrophosphate in pseudoxanthoma elasticum and other pyrophosphate deficiency syndromes. *EXP DERMATOLOGY* 2021
>
> Bartstra JW, Kozák E, de Jong PA, Mali W, Fülöp K, **Tőkési N**, Pomozi V, Risseeuw S, Ossewaarde-van Norel J, van Leeuwen R, Váradi A, Spiering W.: Inorganic pyrophosphate is not associated with disease severity in pseudoxanthoma elasticum. *J INVEST DERMATOLOGY (under revision)*

Moreover, I also contributed to the development of an antibody that also can support PXE treatment:

> Kozák E, Szikora B, Iliás A, Jani PK, Hegyi Z, Matula Z, Dedinszki D, **Tőkési N**, Fülöp K, Pomozi V, Várady G, Bakos É, Tusnády GE, Kacskovics I, Váradi A.: Creation of the first monoclonal antibody recognizing an extracellular epitope of hABCC6. *FEBS LETT* 2021

Finally, based on our promising results of PPi treatment using PXE and trauma-induced mouse models, two clinical trial have been initiated for pyrophosphate therapy: one for PXE patients and the other for Scleroderma (another calcification disorder) patients.

- PyROphosPHate Supplementation to Fight ECtopIc Calcification in PseudoXanthoma Elasticum (PROPHECI) https://clinicaltrials.gov/ct2/show/NCT04868578?term=pyrophosphate&cond=PXE&draw=2&rank=2
- Calcinosis Reduction by Pyrophosphate in SSC https://clinicaltrials.gov/ct2/show/NCT04966416?term=pyrophosphate&cond=Scleroderma&draw=2&rank=1

Tőkési Natália

Budapest, 2021.11.24