FINAL REPORT

"*A novel way of intervention in soft tissue calcification: preclinical investigations of pyrophosphate action*", K-177513

The experimental work faced unexpected difficulties due to the Covid pandemic. This slowed down several steps of the project.

1) The PI of the project (A.Váradi) was invited to be a co-author of a review article in highly prestigious Trend of Biochemical Sciences (IF: 15.30). Trend in Biochem. Sci., 2019 44 125-140)

2) The Pi of the project (A.Váradi) was invited to give the key-note opening lecture on the 2020 PXE-Calcification Meeting (Philadelphia, USA; 2020 Oct 15-17).

3) We discovered that modulating dietary PPi can also be an effective approach to reducing calcification in *Abcc6*–/– mice. Our findings were prompted by a change in institutional rodent diet. The new chow was enriched in PPi, which increased plasma PPi, and significantly reduced mineralization in *Abcc6*–/– mice. We also found that dietary PPi is readily absorbed in humans. Our results suggest that the consumption of food naturally or artificially enriched in PPi represents a possible intervention to mitigate calcification progression in pseudoxanthoma elasticum, that dietary preferences of patients may explain pseudoxanthoma elasticum heterogeneous manifestations, and that animal chow has the potential to influence data reproducibility. (Pomozi et al, 2019, J.Invest. Dermatol, IF: 6.450).

4) Based on our previous reports that oral PPi administration have shown to decrease tissue calcification in a murine model of PXE a clinical case study has been executed with the outcome of one patient treated with oral PPi and further operated for critical limb ischemia. During the one-year follow-up the operated area has not re-occluded and there have been no significant side effects. Previous reports have shown early high failure after revascularization by unknown mechanism. Our publication is the first clinical intervention based on our discovery of the effectivity of oral pyrophosphate treatment. The case report has been published (Väärämäki et al, 2019, Surgical Case Reports, IF: 2.130).

5) Trauma-induced calcification is the pathological consequence of complex injuries which often affect the central nervous system and other parts of the body simultaneously. We have established a novel an animal model recapitulating the calcification of the above condition and found the calcification due to trauma follows the general calcification pathway of ectopic calcification. We discovered that adrenaline transmits the stress signal of brain injury to the calcifying tissues. We have also 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. (Tőkési et al, 2020, J Cell Mol Med., IF: 4.670).

6) Craniometaphyseal dysplasia (CMD), a rare genetic bone disorder, is characterized by lifelong progressive thickening of craniofacial bones and metaphyseal flaring of long bones. The

autosomal dominant form of CMD is caused by mutations in the progressive ankylosis gene *ANKH* (mouse ortholog *Ank*), a key factor controlling local PPi level. The experiments described led to the conclusion that lowering but not depleting dietary Pi delays the development of craniofacial hyperostosis in CMD mice without severely compromising serum levels of Pi, Ca, PTH, and 25-OHD, thus point to the crucial role of PPi in the disease. Our group contributed in performing plasma PPi assays (Yasuyuki Fuji et al, 2020, Journal of Bone and Mineral Research, IF: 5.860).

7) We created single and double mutants for the functional paralogs of Abcc6 of zebrafish (Danio rerio) and characterized their calcification defects with a combination of techniques. Zebrafish deficient in abcc6a show defects in their vertebral calcification and also display ectopic calcification foci in their soft tissues. This result puts the mutant into the position of a novel animal model of human diseases due to ABCC6 deficiency and other human calcification diseases (see below). Our results also suggest that the impairment of abcc6b.1 does not affect this biological process and suggest that impairment abcc6b.1 does not affect this biological process. On the other hand, abcc6b.1 loss-of- function results in considerable shorter lifespan. The paper was published in Front Cell Dev Biol (Czimer et al 2021, IF: 6.45). The last author is participant of the project.
We have embarked upon a project to create a novel transgenic line suitable for high- throughput functional testing of different human ABCC6 alleles. This line makes possible the site-specific, precise introduction of ABCC6 sequences into the second exon of the abcc6a gene. As abcc6a is the sole functional zebrafish ortholog of ABCC6, this approach will create “humanized” zebrafish where the functionality of the respective allele can be assessed by testing the calcification of the larvae.To create the line we used an efficient knock-in technique that relies on homologous-recombination and designed a minimal attP site that can be used later for efficient site-specific recombination based on the PhiC31 integrase system. The efficiency of integration was tested using PCR and Sanger- sequencing. Currently we are raising the founder generation of these fish. By this tool we obtain a highly specific disease model of PXE in zebrafish.
Our preliminary results show that Zebrafish deficient in abcc6a is a valuable model to test compound preventing or attenuating calcification. We have demonstrated that presence of 1 mM Na2H2P2O7 in the media reduced substantially the extent of ectopic calcification in developing animals (manuscript in preparation). This finding has translational value in future drug testing.

8) Our earlier work demonstrated that orally administered pyrophosphate inhibits ectopic calcification in the animal models of PXE and GACI, and that orally given Na4P2O7 is absorbed in humans. Our intention was to optimize absorption of PPi in human. We reported that gelatin-encapsulated Na2H2P2O7 has similar absorption properties in healthy volunteers and people affected by PXE. The sodium-free K2H2P2O7 form resulted in similar uptake in healthy volunteers and inhibited calcification in Abcc6-/- mice as effectively as its sodium counterpart. Novel pyrophosphate compounds showing higher bioavailability in mice were also identified. Our results provide an important step towards testing oral PPi in clinical trials in PXE, or potentially any condition accompanied by ectopic calcification including diabetes, chronic kidney disease or ageing (Kozak et al, 2022, Exp Dermatol. IF: 4.297). For the translation/application of these results in the clinic: see below).

9) The pathogenesis of calcinosis cutis, a disabling complication of systemic sclerosis (SSc), is poorly understood and effective treatments are lacking. We sought to test the hypothesis that SSc may be associated with reduced circulating PPi, which might play a pathogenic role in calcinosis cutis.

Subjects with SSc and age-matched controls without SSc were recruited from the outpatient rheumatology clinics at Rutgers and Northwestern Universities (US cohort), and from the Universities of Szeged and Debrecen (Hungarian cohort). Calcinosis cutis was confirmed by direct palpation, by imaging or both. Plasma PPi levels were determined in platelet-free plasma using ATP sulfurylase to convert PPi into ATP in the presence of excess adenosine 5' phosphosulfate.
Eighty-one patients with SSc (40 diffuse cutaneous, and 41 limited cutaneous SSc) in the US cohort and 45 patients with SSc (19 diffuse cutaneous and 26 limited cutaneous SSc) in the Hungarian cohort were enrolled. Calcinosis was frequently detected (40% of US and 46% of the Hungarian cohort). Plasma PPi levels were significantly reduced in both SSc cohorts with and without calcinosis (US: $P = 0.003$; Hungarian: $P < 0.001$).
Summarizing: **c**irculating PPi are significantly reduced in SSc patients with or without calcinosis. Reduced PPi may be important in the pathophysiology of calcinosis and contribute to tissue damage with chronic SSc. Administering PPi may be a therapeutic strategy and larger clinical studies are planned to confirm our findings. Hsu VM, Kozák E, et al, 2022, Rheumatology (Oxford), IF: 7.58). For the translation/application of these results in the clinic: see below).

10) The “Utrech PXE cohort” is the largest of this kind including more than 250 patients. We had a unique opportunity to investigate the association between plasma PPi level and ABCC6 genotype (mutations affecting the ABCC6 protein). PPi was measured in 193 PXE patients and ABCC6 genotyping of each patients was performed in Utrecht as part of the diagnosis of the disease. We found that PPi levels are on average 60% lower in PXE patients compared to controls. PPi was correlated with increasing age but we found no clear association between PPi and the type of mutations (non-sense/truncating and missense). We have studied a few individual mutations in details: i.e. the R1141X mutation was present in homozygous form in 20 out of the 193 patients. Plasma PPi concentrations observed in these patients vary widely (between 0.35 and 0.85 μM) and the ABCC6 genotype of these patients apparently does not account for these differences. We also studied the cellular localization of two missense mutants by overexpressing the human ABCC6 protein variants in the liver of $Abcc6^{-/-}$ mice. A strength of this experimental approach is that the expression happens in vivo in the native environment of the liver, in the tissue and cell type where ABCC6 protein is physiologically present. In addition to confirming protein expression, this method can also establish the subcellular localization of a given mutant in its physiological conditions. Missense mutant R1138Q was found in five
patients of the mixed group (non-sense/truncating and missense), i.e., each patient carried this mutation on one allele and a truncated one on the other allele. The cellular localization of this mutant showed partial intracellular retention. The plasma $PP_i$ level varied in these five patients between 0.40 μM to 0.96 μM, lacking any apparent correlation between this specific missense mutation and the plasma $PP_i$ measured. The mutation R1314Q, which also gave rise to a partially mislocalized ABCC6 protein, was found in a PXE patient with 0.35 ± 0.03 μM plasma $PP_i$ concentration, and one who, in contrast, had 0.7 ± 0.04 μM of plasma $PP_i$ The difference is striking, even though these patients’ ABCC6 genotypes are similar, with R1314Q on one allele, and a truncating mutation on the other. The major outcome of this analysis argues for factors (genetic and environmental, i.e. nutrition) have an impact on the level of this important anti-calcification inhibitor. We have established a model showing the factors and interactions perturbing the correlation. The paper is published (Kozak et al, J. Clin. Medicine, 2023 *12*(3), 1047; IF: 5.583), both the first and last authors are participants/PI of the project.

Translational value and ongoing clinical applications:
Our group contributed in a decisive manner to interventional inhibition of ectopic calcification by discovering that orally given PPi is absorbed both in human and mice and counteracts calcification in mouse models of PXE and GACI [patent US16/333,856 and EP17781568.5. *"Oral Pyrophosphate for Use in Reducing Tissue Calcification"*] ongoing clinical trials are based on our discovery (NCT04868578 and NCT04966416). Both clinical trials utilizes ouroptimized salt form and dose discovered during the present project (see: Kozak et al, 2022, Exp Dermatol. Detailed under point 7). NCT04966416 aims to treat Ssc patinets with oral pyrophsophate. This trial is baswed on our discovery (publised toigether with a USA group) that plasma pyrophsophate is low in systemic sclerosis, the work is part of the present project (Hsu VM, Kozák E, et al, 2022, Rheumatology (Oxford) as described in details under point 9).