FINAL REPORT (127933)

ABCC6, the key component connective tissue calcification (PI: András Váradi)

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

1). In order to study structure-function relations of ABCC6 we created a monoclonal Ab recognising an extracellular epitope of hABCC6. We have generated a homology model of ABCC6 suggesting very short extracellular segments, thus challenging to generate antibodies recognising extracellular epitopes. We immunized mice overexpressing the bovine neonatal Fc receptor and have augmented humoral immune response allowing generation of mAbs against weakly immunogenic antigens. We obtained a mAb specific to an extracellular epitope of hABCC6. The un vivo test of the mAb detected human ABCC6 expressed in the liver of living mice, verifying both specificity and extracellular binding to the intact cells in the liver. Our mAb did not react with the mouse and with the rat Abcc6, two rodent orthologues. No cross reaction with the human homologues ABCC1, ABCC2 and ABCC3 was demonstrated. We have mapped the epitope to one of the two extracellular loops of the TMD1 domain of the protein. The work was published in FEBS Letters (Kozák et, 2020). The first and last authors are participant of the project.

2). Categorising the members of ABC superfamily.
In recent years, it has become increasingly evident that the distinct TMD folds are best suited to categorise the multitude of ABC transporters. We were involved into a large international consortium formed to propose a new ABC transporter classification that currently comprises seven different types based on structural homology in the TMDs. The work was published in FEBS Letters (Thomas et al, 2020). It generated high interest indicating more than 60 independent citations (more than 100 according to Google Scholar).

3) Zebrafish as a model organism of human calcification diseases.
We developed a new zebrafish (Danio rerio) model for PXE. By resolving some ambiguous assemblies in the zebrafish genome, we showed that there are two functional and one non-functional paralogs for ABCC6 in zebrafish (abcc6a, abcc6b.1, and abcc6b.2, respectively). We created single and double mutants for the functional paralogs 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. 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). 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.

4) 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). The last author of the review is member of the USA laboratory, the NN-OTKA is partly associated to this collaboration.

5) The PI of the project (A.Váradi) was invited to be a co-athor of a review article in highly prestigous Trend of Biochemical Sciences (IF: 15.30). The last author of the review is member of the USA laboratory, the NN-OTKA is partly associated to this collaboration Borst, A.Váradi and vd Wetering, TIBS, 2019 44 125-140)

6) 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. The paper is published (Kozak et al, J. Clin. Medicine, 2023*12*(3), 1047; https://doi.org/10.3390/jcm12031047), both the first and last authors are participants/PI of the project.

Our finding that plasma level of pyrophosphate does not correlate with *ABCC6* genotype is utilized in the clinical trial PPI Supplementation to Fight EctopIc Calcification in PXE started in 2022 in France (NCT04868578) to improve diagnosis. This trial is based on our earlier results that orally given PPi can attenuate ectopic calcification.