Preface:

The PI established the Ectopic Calcification Laboratory in 2020, when returned from her postdoc in the US. Substantial part of the grant's resources had to be used for the salary of the PI as – by that time – it was not covered by the host institution. Therefore, there was no possibility to hire a senior researcher, e.g., a postdoctoral fellow. The laboratory operated with the PI and students supervised by her (3 PhD students starting in the laboratory in 2021, 2022 and 2024), and 6BSc and 6MSc students over the years. Training/mentoring demanded a considerable amount of the PI's time/effort. To obtain the here presented results the PI also used her already existing and newly established wide national/ international collaborations.

Grants obtained in the FK OTKA grant period:

Based on the results of the FK OTKA grant the PI obtained the following grants as PI, co-PI or Senior Participant:

- Senior Participant, OTKA K-146732 "Aortic valve calcification from molecular mechanisms to clinical implications "2024-2028
- Senior Participant, Coordination Office Member, WP leader, INTEC - International Network on Ectopic Calcification; Ghent University PI: Olivier Vanakker; 300.000 EUR, 2022-2027
- Principal Investigator, ELKH-POC-2022-023 Proof-of-Concept grant "High-precision quantification of the mineralization inhibitor pyrophosphate, applicable to clinical samples" 25.000 EUR 2023-2024
- Co-PI, FWO Junior Fundamental Research Project G061521N, FWO Research Foundation Flanders, Belgium; “Identifying mechanisms of clinical variability in soft tissue mineralization, using pseudoxanthoma elasticum as a model” 538.000 EUR, 2021-2024

Fellowships obtained in the FK OTKA grant period:

- The PI obtained the Bolyai János Fellowship of Excellence (BO/00730/19/8), from Hungarian Academy of Sciences, and was awarded the Bolyai Commemorative Certificate from the Bolyai Board for the "outstanding" evaluation of her Final Research Report.
- Annually, the PI obtained the UNKP-Bolyai+ fellowship from New National Excellence Program, Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund, altogether 3 times.

Societal activity during FK OTKA grant period:

The PI became an Executive Board member in International Scientific Society for Ectopic Mineralization (ISSEC, https://issec.org) and Senior Participant, Coordination Office member, Work Package Leader in the INTEC - International Network on Ectopic Calcification (https://www.itnintec.com/network/). The PI organizes and chairs the monthly joint webinar series of INTEC and ISSEC.

Publications and Patentable Intellectual Property obtained in the FK OTKA grant period:

In this final report the relevant publications are briefly summarized. Three independent patentable intellectual property (IP) was generated in the grant period, patent filing is currently ongoing. To prevent premature disclosure that compromise novelty, related publications have been delayed. Consequently, these data are not yet published but included in the report as unpublished data. The here reported work revolves around ectopic calcification, the pathologic calcification of soft tissues (e.g. arteries), a critical public health concern for which currently no therapy exists. Of note, a well-established inhibitor of ectopic mineralization is plasma inorganic pyrophosphate (PPi).

Results of FK OTKA grant:
1. Methodological aspects

1.1. Based on our previous patent on the therapeutic use of PPi supplementation (NL20117471, "Oral Pyrophosphate for Use in Reducing Tissue Calcification"; US 16/333,856; EP3512530), a clinical trial is currently underway in France (https://clinicaltrials.gov/ct2/show/NCT04868578). However, despite of the various attempts of basic researchers, clinicians, and even pharma companies, the reliable quantification of PPi for clinical diagnostics is yet unsolved. Hence the field is in the urgent need of a robust PPi determination. Anticoagulants and preanalytical steps are critical for PPi determination in biological fluids, especially in blood. The biological matrix also heavily influences PPi determination, resulting in high individual variability in patient cohorts and animal models. To tackle these unsolved problems, in a co-authored paper, we reported the development of a **novel PPi determination method** that applies an internal ATP standard to correct for sample-specific interference and outperforms available methods due to its high sensitivity, accuracy, and precision (***Lundkvist et al.*** **Anal Bioanal Chem**).

1.2. To further improve the PPi determination and to make it applicable for high-throughput methodologies suitable for clinical applications, we recently developed an **advanced PPi determination,** in addition to a **new method to assess calcification propensity of serum samples.** Both methods were considered suitable for patenting by **Danubia Patent and Law Office**. In its expert opinions, Danubia considered both IPs to be "new and industrially applicable technical solution to a technical problem". *The documentation is currently being prepared for patents' filing; results will be submitted for publication concomitantly, not to hamper IP originality.*

2. (Patho)physiological aspects

2.1. According to the broadly accepted view based mainly on a study published in Science in 2000, Ankylosis Homologue (ANKH) was for decades considered a PPi transporter preventing calcification of the joint capsule/synovial fluid. In contrast, we demonstrated that **ANKH is not capable of PPi transport** neither in vitro nor in vivo. Instead, **ANKH is involved in the transcellular efflux of ATP** and other NTPs to the extracellular *milieu* (***Szeri et al.*** **JMBR**), similarly to ABCC6, another key player of the extracellular ATP and PPi homeostasis. Extracellularly transported ATP is rapidly converted to PPi by ENPP1 activity. We applied Crispr/Cas9 technology to knock out the ENPP1 gene in HEK293 cells. ENPP1 is the sole extracellular enzyme capable of converting extracellular ATP into PPi, hence in its absence ATP cannot be converted to PPi. HEK293 cells were then tested for ANKH-dependent extracellular ATP and PPi accumulation in the presence/absence of ENPP1. Overexpression of the wild-type and a catalytically inactive ANKH in ENPP1-deficient cells resulted in cellular ATP release for any ANKH proficient cells irrespective of their ENPP1 status. However, no extracellular PPi accumulation was detected in any ENPP1-deficient cells, irrespective of the presence of the wild-type ANKH. However, if the cells were proficient for both proteins, ANKH-dependent ATP efflux and high PPi levels appeared. These experiments clearly showed that ANKH is not a PPi transporter but transports ATP, and ENPP1 produces PPi in the extracellular space, like in the case of ABCC6. In vivo, we found that Ank activity is responsible for 75% of PPi in the bones of wild-type mice and Abcc6 to a much lesser extent. However, the bones of Enpp1 KO mice contained only 2% of the PPi detected in the bones of wild-type mice. Thus, Enpp1 activity is a prerequisite for both Ank- and ABCC6-dependent PPi incorporation into the mineralized bone matrix, which

confirms our in vitro results. We also showed that the effect of Ank on the physiological plasma PPi level is much smaller (30%), while the effect of Abcc6 is about 70%. **In summary,** our *in vitro* and *in vivo* data concordantly show that ANKH does not transport PPi, but ATP. Based on our data, **the function of ANKH in bones and ABCC6 in plasma is decisive in setting the physiological PPi level (Fig 1)**. *This 2022 D1 first author publication obtained 29 independent citations (MTMT)*. Interestingly, we also found in our in vitro and animal model experiments, that the **Krebs cycle intermediates are also substrates of ANKH**, furthermore TCA metabolites appear extracellularly in humans in vivo (***Szeri et al.* PLOS Genetics**). *This 2020 D1 first author publication obtained 42 independent citations (MTMT).*

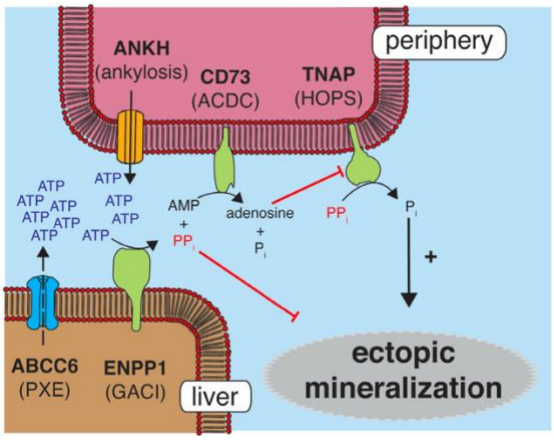


***Figure 1** ANKH transports ATP and is a key player of PPi homeostasis (Szeri et al., 2022, JMBR)*

2.2. Our second goal was to assess the pathophysiologic role of PPi homeostasis in rare/common disorders.

2.2.1. In collaboration with Annika Keller (University of Zurich), we investigated the first loss-of-function mouse model of the rare neurodegenerative disease, **primary familial brain calcification** (PFBC). In this newly generated mouse model, we showed that heterozygous deficiency of the phosphate exporter XPR1 leads to reduced Pi levels in cerebrospinal fluid. This Pi imbalance is in concordance with the age- and sex-dependent appearance of vascular calcification we observed in the thalamus of these mice. Interestingly, heterozygous XPR1deficiency did not lead to altered pro- or anti-calcification metabolites in the blood circulation (***Maheshwari et al.,* Brain Pathology**). However, in aged mice (18 months), metabolite levels were dysregulated (unpublished data).

2.2.2. The role of PPi shortage is well documented in the pathogenesis of a few rare Mendelian disorders, such as pseudoxanthoma elasticum (PXE). However, its contribution to the etiology of common multifactorial disorders with manifested ectopic calcification is largely unknown. In collaboration with Dr. Anikó Ilona Nagy (Semmelweis University, Városmajor Heart and Vascular Centre), we investigated the correlation between circulatory mineralization inhibitors (e.g., PPi) and the extent of cardiovascular calcification, a clinically relevant question did not studied in depth so far. We collected clinical samples from patients with **aortic valve (AV) stenosis** and **coronary artery disease (CAD)**. In addition, we included the Hungarian PXE cohort. We investigated the correlation between coronary artery, and AV calcification and patients' circulating pro- and anti-mineralization factors including PPi. We prospectively enrolled patients with a wide age span (38-90 years of age) as PPi has been controversially reported to be dependent on age. Detailed demographic, clinical, and lifestyle data were collected in addition to high-resolution CT images of all patients, from which the extent of calcification was determined for the aortic and mitral valves and the 4 distinguished coronary artery beds. Blood sampling of patients was also performed, and a wide range of blood chemistry clinical parameters were determined. Plasmas were separated specifically for PPi determination *via* our specific preanalytical method finetuned in previously, and the biological samples were stored in our biobank. All samples were determined using the validated high-sensitivity PPi determination methods.

A shared last author manuscript entitled "Association between plasma phosphate/pyrophosphate ratio and aortic valve calcification in an unselected cohort of cardiovascular patients" is **currently under review**. This is the first study to investigate the association between PPi homeostasis and AV calcification. In univariate analysis, plasma PPi level did not show association with AV calcification, however, the Pi/PPi ratio, the key determinant of ectopic calcification, was positively associated with the degree of AV calcification, along with age, hypertension, plasma lipoprotein(a) (Lp(a)) concentration and statin treatment, whereas eGFR and LDL level showed significant negative associations. In multivariate analysis only age and Pi/PPi ratio remained significant determinant of the AV calcification. Furthermore, plasma PPi showed a significant positive association with plasma Pi and low-density lipoprotein (LDL) concentration and was inversely related to alkaline phosphatase activity. When controlled for age, female patients had higher PPi levels. Figure 2 shows the proposed role of PPi in AV calcification.

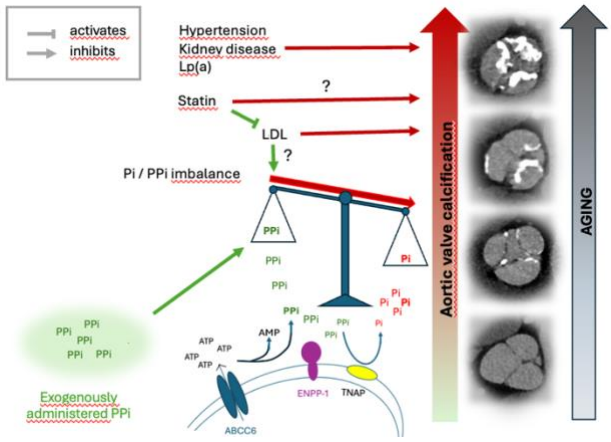


Figure 1 *Mechanisms of AV calcification and PPi substitution. Pro-calcific mechanisms are shown in red, anti-calcific mechanism are shown in green. Manuscript under revision.*

2.2.3. In another study, in 137 prospectively enrolled coronary artery disease (CAD) patients, by applying multiple linear regression analysis, we detected a similar positive correlation between plasma PPi levels and serum Pi concentration and inverse association of plasma PPi levels with alkaline phosphatase activity. Moreover, plasma PPi levels positively associated with body mass index (BMI) and female sex, while negatively associated with diabetes mellitus (DM) status. In addition, ordinary logistic regression analysis revealed a significant positive correlation of coronary artery calcium load with BMI, Lp(a), gamma-glutamyl transferase (GGT), age, and statin use, while female sex showed an inverse correlation. However, no correlation was found between coronary artery calcification and plasma PPi levels. *The manuscript on plasma PPi and coronary artery calcification is in preparation.*

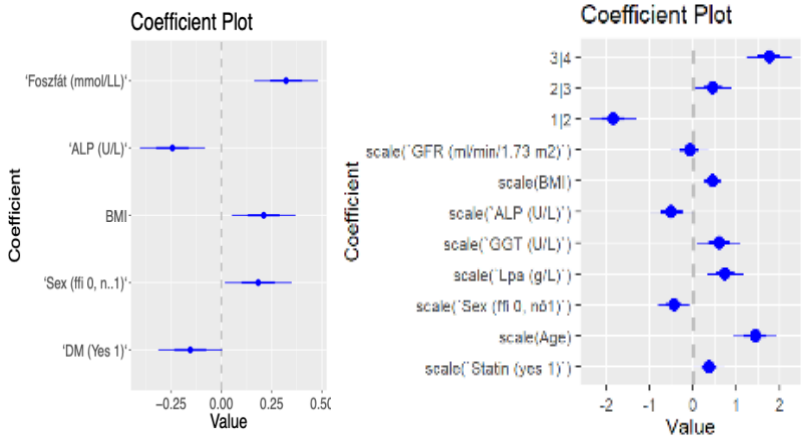


Figure 2 *Coefficient plots depicting significant association of plasma PPi level in multiple linear regression analysis (left panel) and coronary artery calcification in ordinary logistic regression analysis (right panel). Manuscript in preparation.*

2.2.4. We have an *additional, unpublished result* in the patient cohort. 22 patients had **zero coronary artery calcification** (CAS), fortunately with a wide age distribution (45-78 years). In this subpopulation, PPi has a strong positive correlation with age (Fig4 A), furthermore, PPi shows a strong negative correlation with alkaline phosphatase (AP) activity (Fig4 B). Furthermore, the Pi/PPi ratio, the key determinant of ectopic calcification,

decreases with age (Fig4 C), but positively correlates with AP activity (Fig4 D). These findings are corroborated by our analysis of **77 Belgian PXE patients** (Fig4 E, F). The samples from these rare-disease PXE patents were obtained in collaboration with Prof. Oliver Vanakker (Ghent University Hospital).

A plausible explanation for the data in 2.2.2 - 2.2.4 is that AP activity cleaves PPi to Pi, evidenced in the literature from decades ago *in vitro*, however these correlations has not yet been shown in humans so far, to the best of our knowledge.

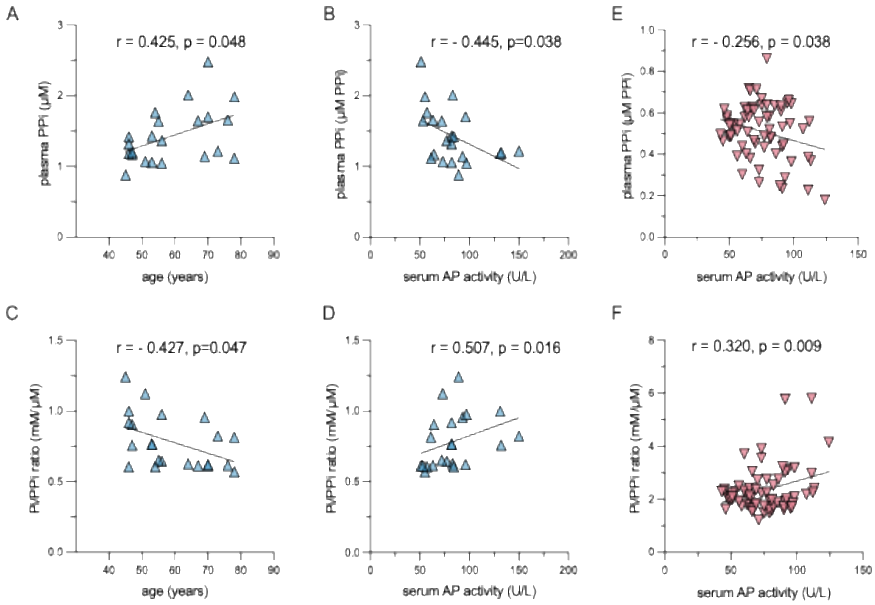


Figure 4 *Kinetics and correlations of PPi-AP-Pi axis in A-D: in zero coronary artery calcification patients; E-F: PXE patients r=Pearson/Spearman correlation*

The kinetics of the PPi-ALP-Pi axis has not been investigated in mice either. Therefore, we tested the age-dependence and cross correlation of these factors in Abcc6$^{-/-}$ and wild-type (C57BL6/J) mice. We found substantial changes in the players of the PPi-ALP-Pi axis with maturation and aging (Fig 5 A-C). Similarly to our results in zero CAS and PXE patients, there is a strong inverse correlation between PPi and AP activity, and a strong positive correlation of Pi/PPi with AP activity in mice as well. *From the above work a manuscript is currently in preparation.*

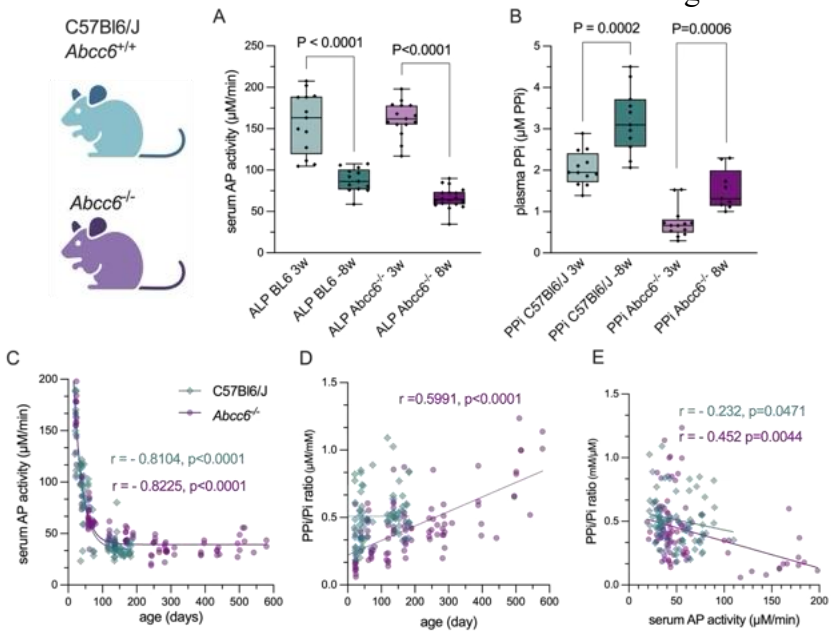


Figure 5 *PPi-ALP-Pi axis in Abcc6$^{-/-}$ and $^{+/+}$ mice; w=week old, C57BL6/J=Abcc6 $^{+/+}$ mice, ALP=AP= alkaline phosphatase activity*

2.3 An interesting *additional novel unpublished finding* in our PXE mouse model, is that **the hepatic expression levels of several PPi homeostatic genes are closely correlated**. The expression level of *Abcc6, Enpp1, Ank* and *Alpl*, the latter encoding tissue non-specific alkaline

phosphatase from which serum AP activity derives from, shows a significant correlation (Pearson r > 0.8) with each other (Fig 6 A). Notably, this phenomenon does not exist in kidney, where these genes are also expressed. This hints toward a potential liver specific co-regulation of these PPi homeostatic, calcification-related genes. Of note, these changes in the expression are independent of the major calcification regulator transcription factors, Runx2 and Sox9. Notably, AP activity inversely correlate with PPi levels in these mice, and both factors change dramatically from weaning to 2 months of age (Fig 5 A, B). Further investigations are currently ongoing to dissect the exact mechanism. Our data together with results obtained from the Hungarian and Belgian patients detailed in 2.2.1 - 2.2.24 suggest a direct regulation of PPi homeostatic calcification inhibitors/promoters both in mice and humans.

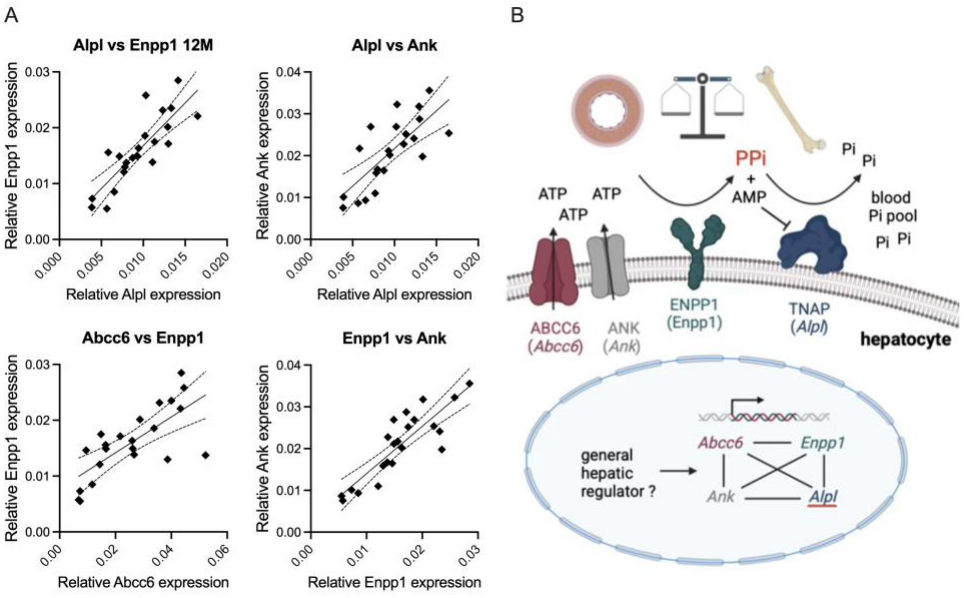


Figure 6 *Correlated hepatic expression of PPi homeostatic genes (A) and their role in determining the PPi-Pi balance (B)*

2.4 In collaboration with Magnus Back (Karolinska Institutet) we obtained media samples that were previously conditioned with coronary artery explants from ischemic or stented patients undergoing surgery and also from control healthy cadavers with no apparent calcification. We surprisingly found an elevated PPi concentration in the media samples from the calcified coronary arteries (Fig 7). This is counterintuitive for the first sight, but these results were corroborated with our following *in vitro* data.

We cultured A7r5 rat aortic cells with osteogenic and control media and found a significantly increased PPi accumulation in the media of calcified cells (Fig 8 D-E), along with epigenetic (histone methylation and acetylation) changes (Fig 8 A-C) detected by the Epigenetic group led by Tamás Aranyi (Semmelweis University). This is in line with our previously

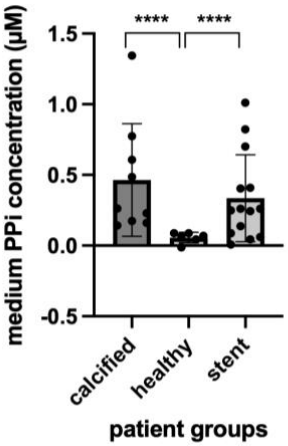
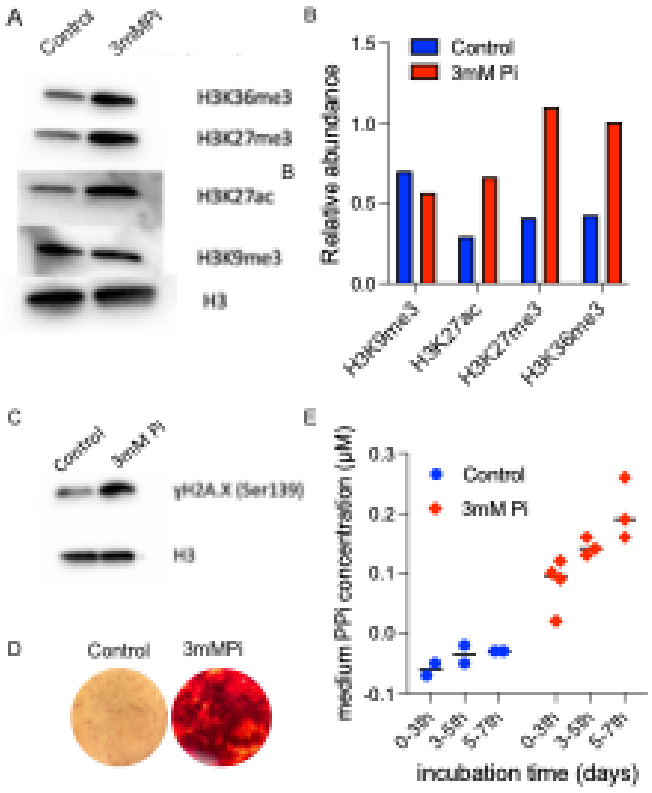


Figure 7 *PPi in coronary artery-conditioned media*



detailed data on the age-related increase in PPi concentration and the decline of the Pi/PPi ratio over age in mice and humans, hinting to a putative secondary compensatory mechanism that is acting against the well-known increase of ectopic (vascular) calcification via aging. This is an entirely novel concept; thus these (patho)physiological mechanisms are not fully explored in our work yet.

Figure 8 *Calcification in A7r5 cells. A-C: epigenetic changes, D: Alizarin red stained calcification, E: PPi increase in medium over time*

3. Molecular aspects

3.1 In addition to the (patho)physiological investigations, we also carried out projects at **the cell and molecular biology level.** To better understand the molecular mechanism of ABCC6 underlying PXE, we developed a novel homology model based on the latest CryoEM structures of the closely related ABCC1 protein. We investigated if the modelled substrate binding cavity of ABCC6 overlaps with that of the ABCC1 from the structures. We generated a total of 14 ABCC6 mutants by site-specific (USER) mutagenesis and studied the expression and intracellular localization of these single mutants in HEK293 cells. Functional characterization of these mutants in two independent biochemical assays (real-time ATP efflux and PPi accumulation assay) revealed that these mutations did not affect the function of ABCC6. The only exceptions were mutations of two highly conserved amino acid residues, which are also conserved in related ABCC proteins, thus presumably these residues don't play a role in the coordination of the specific physiological ABCC6 substrate but are important to ABCC protein activity in general. Additionally, we generated a multiple mutant chimeric ABCC6 that contains the exact same amino acid residues corresponding to those that mediate the coordination of the physiological substrate in ABCC1. Surprisingly, despite of the low expression level of this ABCC6 chimera, its localization and functional activity was fully maintained. Based on our results, we concluded that the binding site of the physiological substrate in ABCC6 is distinct from that of ABCC1 (***Szeri et al.*****, IJMS**).

3.2 Data is scarce on function of the over 300 genetic variants of ABCC6, hampering diagnosis and genetic counseling. Therefore, in a large international collaboration, we have assembled the largest, clinically well-characterized European patient cohort of PXE. In 590 patients, we determined the allele frequency of the 217 ABCC6 missense variants previously

characterized as pathogenic. We have shown that five variants, which were initially considered pathogenic, are, in fact, non-pathogenic, and two variants are incompletely penetrant (IP), i.e., they manifest the disease only under certain conditions. However, we have shown that when PXE manifests, the disease caused by IP variants is as severe as the complete loss-of-function phenotype. In search of an explanation at the molecular level, we excluded intra- and inter-allelic associating SNPs. We hypothesized that IP disrupts the critical functional interaction between ABCC6 and an unidentified protein. The interaction is perturbed by the IP mutation only if the interacting partner is also mutant. Accordingly, in *in vitro* studies, as expected, functional characterization of IP mutants did not reveal differences in expression, localization, and function of IP variants compared to the wild type (***Szeri et al.*, Human Mutation**).
Along this line, we performed whole-exome sequencing of all 18 patients carrying the most frequent IP variant p.(R391G) in the PXE cohort of 590 patients in a collaborative work. Together with Olivier Vanakker (University of Ghent) and Tamás Arányi (HUN-REN RCNS / Semmelweis University), we compiled a list of what other genes these patients' shared variants of. These genes are potential interaction partners of ABCC6. We currently investigate and validate these genes as interaction partners for ABCC6 in functional tests. Careful genetic analysis however not yet revealed the nature of the interaction partner, as no obvious candidates were identified in our analysis. At the moment, we are looking for deep intronic variants to identify the mechanistically vital interaction partner of ABCC6 as it would be of great therapeutic importance in PXE and potentially be a novel pharmacological target in a broad range of ectopic calcification disorders.

3.3. Experimental data are currently not available for the vast majority of pathogenic missense *ABCC6* variants. Therefore, we applied site-specific mutagenesis in human *ABCC6* to generate, in addition to the incomplete penetrant variants, the five most frequent pathogenic ABCC6 missense variants. Functional characterization of these variants indicates that one of the missense variants has a somewhat retained functional activity, while the others are either improperly folded, as reflected by a very low expression level, or are functionally inactive.

4. Translational aspects, a novel preclinical model for ectopic calcification

4.1. *Another significant and yet unpublished recent finding* of our laboratory is a first-of-its-kind **extremely early-onset calcification phenotype** in *Abcc6*$^{-/-}$ mice. The so-far earliest soft-tissue mineralization (STM) biomarker in *Abcc6*$^{-/-}$ mice is the calcification of the capsule lining the blood sinuses of the vibrissae, detectable faintly earliest at ~4 months of age and relatively well >8 months of age. Our early-onset calcification phenotype biomarker is well detectable at a significantly earlier age. It is currently under patenting for testing novel therapeutic approaches in translational research to circumvent ectopic mineralization. Therefore, the exact nature of model will not be revealed in this report.

However, normalized total Ca^{2+} content of the tissue is barely detectable on standard diet, however, is well pronounced if the knock-out or heterozygous animals are kept on special diet (Fig9A). We also assessed the early-onset calcification biomarker, via an independent method. The quantitative histomorphology analysis revealed similar results (data not shown). These results are consistent both in males and females independent of the detection methodologies and diet used (data not shown).

4.2. These data demonstrate that the early-onset calcification phenotype, with a significant difference between *Abcc6* wild-type and KO littermates on a special diet regime, present an outstanding alternative to test treatment efficacy compared to traditional late-onset calcification biomarkers. To exploite this potential we tested altogether 3 treatment modalities known to be effective to alleviate STM in preclinical models, via 2 different modes of administration (Fig 9 B) in *Abcc6-/-* mice on special diet. Both parenteral and oral treatments decreased calcification in the early-onset calcification phenotype significantly, on a dose dependent manner. Altogether these results demonstrate the robustness and feasibility of our novel early-onset calcification biomarker on special diet, for preclinical studies. The patenting of this model is currently ongoing.

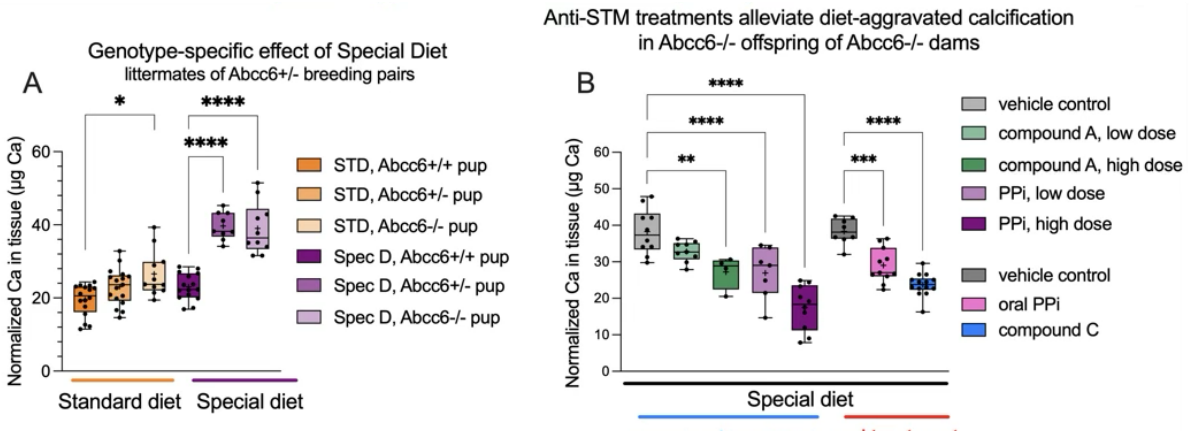


Figure 9 *Diet-aggravated early calcification phenotype is an outstanding preclinical model. Special diet induces genotype-specific calcification (A). Known anti-STM treatments rescue the calcification phenotype in a concentration dependent manner (B). Unpublished due to patenting.*

5. Aspects on gestational PPi homeostasis

5.1 Another important line of research was the project aiming to reveal the prenatal mechanisms leading to the later-in-life ectopic mineralization. We hypothesized that **PPi plasma levels may be decreased in pregnancy.** To test this hypothesis, we longitudinally monitored the plasma PPi levels of sheep throughout gestation and during lactation. We found that plasma PPi levels decreased gradually but drastically in gestation. To find out whether plasma PPi levels also decline in pregnant humans, we recruited healthy pregnant women and found that plasma PPi levels at term were significantly lower compared to aged-matched non-pregnant women. The gestational PPi shortage of mothers at least partially accounts for the ectopic mineralization of knock-out offspring in the generalized arterial calcification of infants (GACI) and PXE mouse models. We analyzed data from CT images of a large cohort of patients with PXE, a rare disease with chronically low PPi levels. We found that the lower limb arteries of PXE patients with multiple childbirths showed significantly higher calcification compared to age matched PXE patients with one or no childbirths. These results are consistent with epidemiological studies showing a higher cardiovascular risk in multiparous women, a yet unexplained phenomenon. Based on our hypothesis, **gestational PPi shortage result to increased vascular calcification, and thus to increased cardiovascular morbidity risk** in multipara. A prophylactic treatment during pregnancy restoring PPi levels may normalize the cardiovascular risk (***Veiga-Lopez et al.* Front Cell and Dev Biol**).

5.2. We sought to test if multiple pregnancies result in increased ectopic calcification in the *Abcc6*$^{-/-}$ mouse model of PXE and if prenatal PPi treatment rescues the maternal and offspring mineralization. However, in our *Abcc6*-/- mouse colony, during the PPi prenatal treatment experiments a novel lethal hydronephrosis phenotype appeared. Whole-exome sequencing of these mice with our subsequent analysis yielded a loss-of-function mutation in *Mus81* gene, which we assumed to be the cause of the kidney disease, as it resulted to a truncated *Mus81* gene product missing functionally crucial conserved domains. According to our hypothesis, a simultaneous mutation in *Mus81* and *Abcc6* was necessary for the appearance of the new phenotype. Nonetheless, the expression of the two proteins overlaps in the renal proximal tubules. Later on, we disproved this hypothesis, as by generating homozygote *Mus81* wild-type mice on the same genetic background with crossings heterozygous carriers, we found that these mice still developed hydronephrosis. After a second round of WES not yielding suitable candidates, we concluded that WGS would be necessary to reveal the genetic alteration underlying hydronephrosis. However, due to lack of additional external funding, we discontinued this line of investigation.

In the meantime, we have imported *Abcc6*$^{-/-}$ mice from an independent source from abroad, devoid of both the kidney phenotype and the loss-of-function mutation in the Mus81 gene. After backcrossing these *Abcc6*$^{-/-}$ mice to the C57Bl6 strain we restarted the pregnancy experiments aiming to delineate whether multiple pregnancy, *via* gestational PPi shortage, results in higher ectopic calcification. As we found in the preliminary experiments that our mice show a limited ectopic mineralization, we boosted the phenotype by keeping the mice on a high phosphate diet from weaning to the age of 60 days old. After this period, we put our mice on standard diet and initiated the breading experiment. We currently have 12 mice between 4-6 pregnancies and will terminate these long-term experiments at the age of 12 months in the following 2 months. As controls we have 11 matching littermates that were never bred but had the same high phosphate diet between 21 and 60 days of age. Analysis of the calcification in various tissues will begin after sacrifice of the animals. We hypothesize that *this project will lead to conclusive results to abrogate our previous findings in PXE patients on the long-term maternal consequence of gestational PPi shortage.* If the results will be as expected, it will raise the question of PPi supplementation during gestation as prophylactic treatment. This experiment is critical for the potential translation of the gestational PPi shortage to (multiple) human pregnancies, and the prophylactic PPi supplementation to circumvent increased maternal cardiovascular risk reported in women with multiple childbirths.

5.3 We used our novel early-onset calcification biomarker detailed in 4.1 to test if the maternal genotype influences the phenotype of the offspring in the *Abcc6*-/- mouse model. We strikingly detected a **significant difference in the calcification of the *Abcc6*-/- offspring depending on the maternal genotype** (Fig 10). *Abcc6* KO offspring born from *Abcc6*-/- dams (light purple) had increased calcification compared to KO offspring born from *Abcc6* heterozygous dams (dark purple) when kept on special diet. A similar trend, however, not significant, was also found on standard diet (Fig 10 orange colors). It is known, that *Abcc6* heterozygous mice has higher PPi levels compared to that of *Abcc6* KO mice. It is plausible to hypothesize, that PPi levels also decrease during gestation in mice, similarly to human and sheep. The experiment shown in Fig 10 raises the possibility that the *Abcc6* KO offspring has increased ectopic calcification as a consequence of the insufficient gestational PPi levels of the *Abcc6* KO mothers. Hence, **gestational PPi shortage may affect ectopic calcification in the offspring** as well, a remarkable hypothesis that we plan to investigate experimentally in the close future.

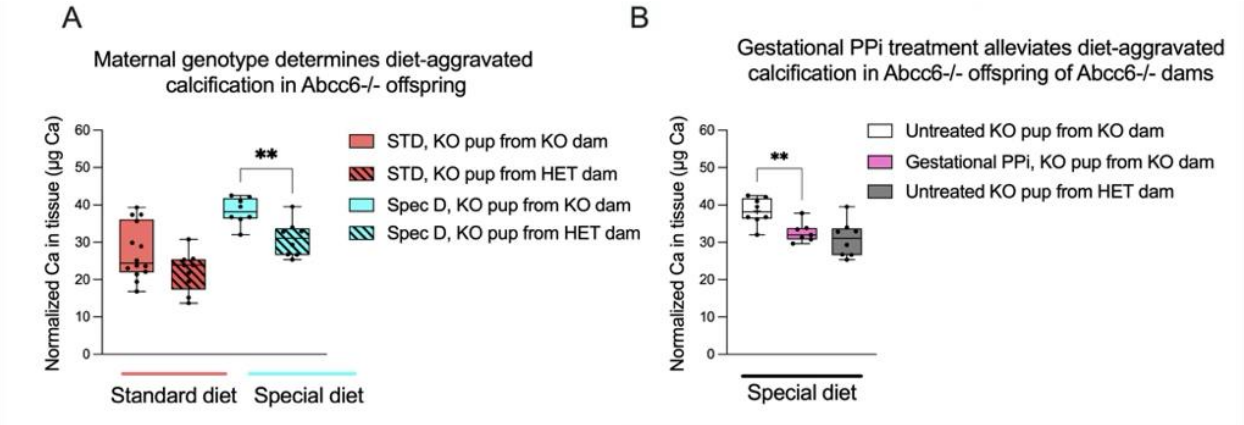


Figure 10 *Maternal genotype determines diet-aggravated calcification of Abcc6 KO offspring (A). PPi supplementation to the mother during gestation alleviates the calcification phenotype in the offspring (B). Unpublished due to patenting.*

6. Reviews

We published a **co-authored review** on vascular aging (**Roth et al, Aging Research Reviews**). A **shared first author review** entitled "Tissue-specific roles of de novo DNA methyltransferases" has been published in Epigenetics & Chromatin (**Toth et al, Epigenetics & Chromatin**). A co-authored manuscript entitled "REACT-PXE A Consensus on Diagnosis and Future Research of Pseudoxanthoma Elasticum (PXE)" has been **accepted for publication and is currently in press** in Ann Dermatol Venereol, the Journal of the French Society of Dermatology.

7. Publications

7.1. Publications directly linked to FK OTKA grant:

1. Tóth DM*, **Szeri F***, Ashaber M, Muazu M, Székvölgyi L, Arányi T. Tissue-specific roles of de novo DNA methyltransferases. Epigenetics Chromatin. 2025 Jan 17;18(1):5. doi: 10.1186/s13072-024-00566-2. PMID: 39819598 **Q1, IF: 4.2, number of citations: 1**
2. Roth L, Dogan S, Tuna BG, Aranyi T, Benitez S, Borrell-Pages M, Bozaykut P, De Meyer GRY, Duca L, Durmus N, Fonseca D, Fraenkel E, Gillery P, Giudici A, Jaisson S, Johansson M, Julve J, Lucas-Herald AK, Martinet W, Maurice P, McDonnell BJ, Ozbek EN, Pucci G, Pugh CJA, Rochfort KD, Roks AJM, Rotllan N, Shadiow J, Sohrabi Y, Spronck B, **Szeri F**, Terentes-Printzios D, Tunc Aydin E, Tura-Ceide O, Ucar E, Yetik-Anacak G. Pharmacological modulation of vascular ageing: A review from VascAgeNet. Ageing Res Rev. 2023 Dec;92:102122. doi: 10.1016/j.arr.2023.102122. Epub 2023 Nov 11. PMID: 37956927. **D1, IF: 13.100, number of citations: 9**
3. Maheshwari U, Mateos JM, Weber-Stadlbauer U, Ni R, Tamatey V, Sridhar S, Restrepo A, de Jong PA, Huang SF, Schaffenrath J, Stifter SA, **Szeri F**, Greter M, Koek HL, Keller A. Inorganic phosphate exporter heterozygosity in mice leads to brain vascular calcification, microangiopathy, and microgliosis. Brain Pathol. 2023 Nov;33(6):e13189. doi:

10.1111/bpa.13189. Epub 2023 Jul 28. PMID: 37505935; PMCID: PMC10580014. **D1, IF: 7.611, number of citations: 7**

4. Lundkvist S, Niaziorimi F, **Szeri F**, Caffet M, Terry SF, Johansson G, Jansen RS, van de Wetering K A new enzymatic assay to quantify inorganic pyrophosphate in plasma. Anal Bioanal Chem. 2023 Jan;415(3):481-492. **Q2, IF: 4,478, number of citations: 3**
5. **Szeri F**, Miko A, Navasiolava N, Kaposi A, Verschuere S, Molnar B, Li Q, Terry SF, Boraldi F, Uitto J, van de Wetering K, Martin L, Quaglino Q, Vanakker OM, Tory K, Aranyi T The pathogenic c.1171A>G (p.Arg391Gly) and c.2359G>A (p.Val787Ile) ABCC6 variants display incomplete penetrance causing pseudoxanthoma elasticum in a subset of individuals. Hum Mutat. 2022 Dec;43(12):1872-1881. **Q1, IF: 4,878, number of citations: 2**
6. **Szeri F.,** Niaziorimi F., Donnelly S., Fariha N., Tertyshnaia M., Patel D., Lundkvist S., van de Wetering K. The Mineralization Regulator ANKH Mediates Cellular Efflux of ATP, Not Pyrophosphate. J Bone Miner Res. 2022 May;37(5):1024-1031.**D1, IF: 6.390, number of citations: 32**
7. **Szeri F**, Corradi V, Niaziorimi F, Donnelly S, Conseil G, Cole SPC, Tieleman DP, van de Wetering K. Mutagenic Analysis of the Putative ABCC6 Substrate-Binding Cavity Using a New Homology Model. Int J Mol Sci. 2021 Jun **D1, IF: 6.208, number of citations: 8**
8. Veiga-Lopez A, Sethuraman V, Navasiolava N, Makela B, Olomu I, Long R, van de Wetering K, Martin L, Aranyi T*, **Szeri F***. Plasma Inorganic Pyrophosphate Deficiency Links Multiparity to Cardiovascular Disease Risk. Front Cell Dev Biol. 2020 Dec 9. **Q1, IF: 6.684 , number of citations:11**

* shared first or last authors

7.1. Publications not directly linked to FK OTKA grant:

1. Pagliaroli L, Fothi A, Nespoli E, Liko I, Veto B, Devay P, **Szeri F**, Hengerer B, Barta C, Aranyi T. Riluzole Administration to Rats with Levodopa-Induced Dyskinesia Leads to Loss of DNA Methylation in Neuronal Genes. Cells. 2021 Jun **Q1, IF:7.660, number of independent citations: 4**
2. **Szeri F**, Lundkvist S, Donnelly S, Engelke UFH, Rhee K, Williams CJ, Sundberg JP, Wevers RA, Tomlinson RE, Jansen RS, van de Wetering K. The membrane protein ANKH is crucial for bone mechanical performance by mediating cellular export of citrate and ATP. PLoS Genet. 2020 Jul 8. 1. **D1, IF: 6.020, number of citations: 50**
3. Novais EJ, Tran VA, Miao J, Slaver K, Sinensky A, Dyment NA, Addya S, **Szeri F**, van de Wetering K, Shapiro IM, Risbud MV. Comparison of inbred mouse strains shows diverse phenotypic outcomes of intervertebral disc aging. Aging Cell. 2020 May **D1, IF: 11.005, number of independent citations: 33**
4. **Szeri F**, Niaziorimi F, Donnelly S, Orndorff J, van de Wetering K. Generation of fully functional fluorescent fusion proteins to gain insights into ABCC6 biology. FEBS Lett. 2020 Oct 15. **Q1, IF: 4.124, number of independent citations: 7**
5. Farkas K, Kiss N, Szabó V, Resch M, Vámos R, Borbándy Á, Nagy A, Apor A, Arányi T, **Szeri F**, Wikonkál N, Nagy Z, Merkely B, Medvecz M. Pseudoxanthoma elasticumban szenvedő betegek multidiszciplináris ellátása. Orv Hetil. 2022 May Q4, **IF: 0.707, number of independent citations: 0**

6. Kiss N, Fésűs L, Bozsányi S, **Szeri F**, Van Gils M, Szabó V, Nagy AI, Hidvégi B, Szipőcs R, Martin L, Vanakker O, Arányi T, Merkely B, Wikonkál NM, Medvecz M. Nonlinear optical microscopy is a novel tool for the analysis of cutaneous alterations in pseudoxanthoma elasticum. Lasers Med Sci. 2020 Oct **Q2, IF 2.555, number of independent citations: 20**
7. Bäck M, Aranyi T, Cancela ML, Carracedo M, Conceição N, Leftheriotis G, Macrae V, Martin L, Nitschke Y, Pasch A, Quaglino D, Rutsch F, Shanahan C, Sorribas V, **Szeri F**, Valdivielso P, Vanakker O, Kempf H. Endogenous Calcification Inhibitors in the Prevention of Vascular Calcification: A Consensus Statement From the COST Action EuroSoftCalcNet. Front Cardiovasc Med. 2019 Jan 18 **Q1, IF: 5.846, number of independent citations: 76**

* shared first or last authors

Patent

"Oral Pyrophosphate for Use in Reducing Tissue Calcification" Int. Application Number PCTNL2017050601, EPO 17 781 568.5-1112, Ref 73034EP, 2020.06.16