Final report for the "Investigation of diabetes-related cardiovascular calcification and preclinical testing of a potential therapy" project

In this proposal my specific aims were:

Specific Aim 1: Evaluate the role of Abcc6 deficiency in vascular calcification in diabetes mellitus.
Specific Aim 2: Determine the efficacy of PPi supplementation in the prevention of diabetic vascular calcification.

In order to investigate these aims we first had to establish a diabetic mouse model suitable for the investigation of diabetes-related vascular calcification. Our first choice was the ob/ob mouse strain, which lacks the leptin gene (*Lep-/-*) resulting in excessive eating and obesity. This strain is a well-accepted model of obesity and Type II diabetes. I proposed to backcross this strain with Abcc6-decificent mice in order to investigate the role of Abcc6 in diabetes-related calcification. However, we had technical difficulties regarding the breading of that strain and developing and maintaining double knockout (*Lep-/-;Abcc6-/-*) mice. Therefore after a thorough search in the literature, I have changed the work plan to use a mouse model where Type I diabetes and diabetic nephropathy is induced by streptozotocin (STZ) treatment.
We have successfully optimized the STZ treatment on our mouse strains (C57/Bl6, *Abcc6+/-* and *Abcc6-/-*), using a repeated low-dose treatment. As 1.25% of the human population carries an ABCC6 mutation, the investigation of reduced ABCC6 expression (*Abcc6+/-*) as a risk factor in DM-related calcification has important health relevance.
In order to investigate which are the first changes in the pathology of diabetes, we decided to analyse the experimental groups of mice at 4 different time points after the induction of diabetes: 8, 12, 16, and 20 weeks after diabetes has developed. Identifying the first steps of the pathological changes in diabetes may help to develop preventive therapies targeting the initiator molecules, and may also point to early biomarkers in the progression of the disease.

Our results show that the setup of this diabetic model was successful: blood glucose levels of STZ-treated mice increased to hyperglycemic range 1-2 weeks after STZ injection (Fig. 1A). Diabetic mice showed polyuria, a typical symptom in diabetes (Fig. 1B). We have also measured albumin, creatinine and urea levels in serum as indicators of kidney function: serum albumin may be decreased due to renal loss of albumin while plasma creatinine and urea can be up-regulated in renal disease, indicating a reduction

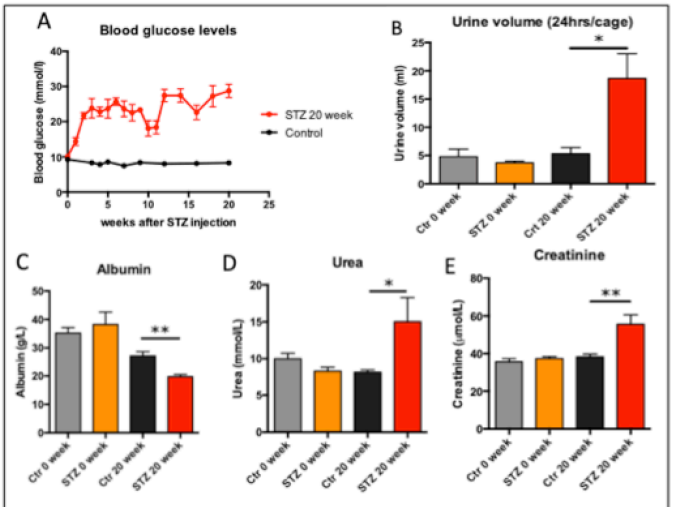

Figure 1. Characteristic symptoms of diabetes in our STZ-induced Type I diabetes *Abcc6-/-* mouse model: hyperglycemia **(A)**, polyuria **(B)**, decreased plasma albumin **(C)** and increased plasma urea **(D)** and creatinine **(E)**.
(Ctr: n=7; STZ: n=5)

in the filtration capacity of the glomeruli. Our mouse model recapitulated these phenotypes (Fig 1C-E).

Our results also show that STZ-induced diabetic *Abcc6-/-* mice, even though with high individual differences, developed substantial vascular calcification, while age-matched *Abcc6-/-* mice had only sporadic calcification in the aorta (Fig. 2A,B). Vascular calcification occurred in the media layer of the arteries (Fig. 2C,D), as expected both in PXE and diabetes. Therefore this mouse model is a useful tool to investigate the molecular mechanism of diabetes-related arterial calcification, as well as to test potential preventive treatments.

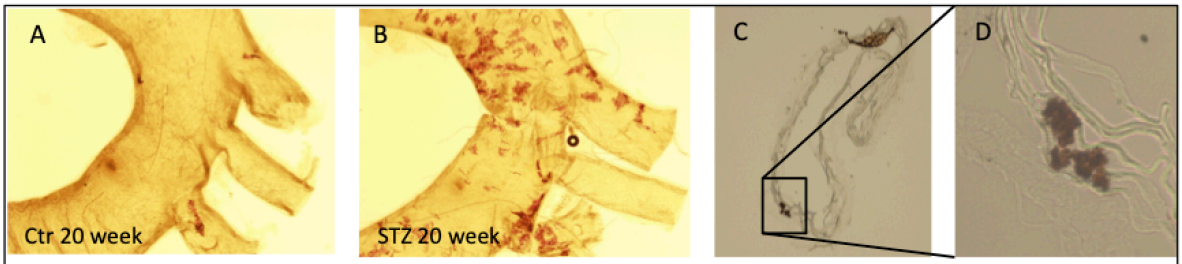

Figure 2. Vascular calcification visualized by Alizarin Red staining in *Abcc6-/-* mice 20 weeks after control (**A**) or streptozotocin treatment (**B**). Calcification develops in the media layer of the arteries (**C,D**).

Abcc6-deficiency clearly contributes to the calcification phenotype, therefore it is interesting to investigate what is the role of Abcc6 in calcification, what pathways are influenced by the function of this protein. Our results obtained in collaboration with the group of Dr. Olivier Le Saux at the University of Hawaii show that Abcc6-deficiency causes changes in lipoproteins, with decreased HDL cholesterol in both mice and humans, and induces atherosclerosis.

There is increasing evidence showing that inflammation can be both a stimulus and a result of calcification. IL-6 seems to play a key role in vascular calcification. Both in animal models and in humans with DM and kidney disease elevated IL-6 was detected.

Interestingly, we have found that the lack of Abcc6 also leads to increased pro-inflammatory cytokines, including IL-6, both in humans and mice. These changes are likely contributors for the calcification phenotype observed in our diabetic mouse model.

These results were published this year:

Brampton C, Pomozi V, Chen LH, Apana A, McCurdy S, Zoll J, Boisvert WA, Lambert G, Henrion D, Blanchard S, Kuo S, Leftheriotis G, Martin L, Le Saux O.: ABCC6 deficiency promotes dyslipidemia and atherosclerosis. *Sci Rep*. 2021. (IF: 4.379)

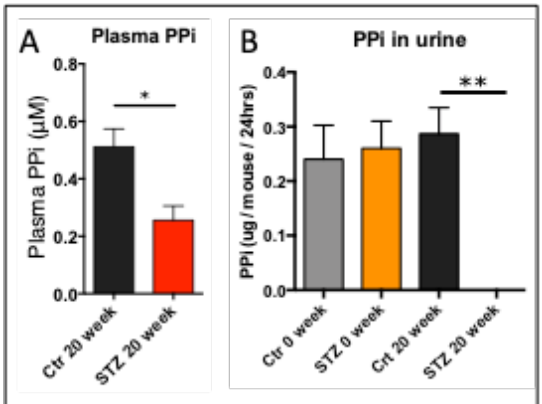

Figure 3. PPi level in plasma (**A**) and urine (**B**) of diabetic mice is significantly decreased (Ctr: n=7; STZ: n=5)

Our important novel finding with the STZ-induced diabetic mice was that PPi level significantly decreased both in the plasma and in the urine of diabetic mice compared to

Abcc6-/- controls (Fig 3 A,B). As PPi is an inhibitor of calcification, decreased PPi may cause or contribute to the development of the observed vascular calcification.
It is important to note that even though plasma PPi level is decreased both in PXE and in diabetic patients, it may not directly correlate with the severity of the calcification phenotype. Investigating a cohort of PXE patients we have found that the level of PPi was not associated with the severity of the disease:
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 Dermatol* (IF: 8.851)

During the previous years we have published that PPi supplementation prevents ectopic calcification in *Abcc6-/-* mice when given intraperitoneally or orally. (Pomozi *et al*, 2017; Dedinszki *et al*, 2017.) As part of my second aim, we have started PPi supplementation both in control and diabetic groups. Unfortunately due to the pandemic we had to decrease the size of our mouse colony in 2020 and therefore some of these experiments were started with delay and are still ongoing. However, so far we have promising preliminary results: PPi-treated diabetic mice did not develop arterial calcification while the parallel diabetic groups had vascular calcification (Fig 4).

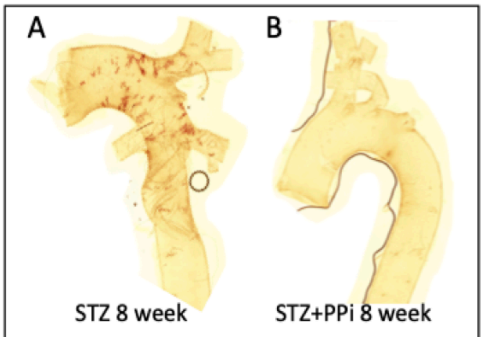

Figure 4. Calcification in the aorta visualized by Alizarin Red staining in diabetic mouse with no treatment (A) and in diabetic mouse who got 1 mM PPi in drinking water (B)

These preliminary results support our hypothesis that PPi treatment is a potential therapy to prevent diabetes-related vascular calcification.

Meanwhile we have been investigating the potential therapeutic effects of PPi supplementation under different pathological conditions as well, and published a paper about how PPi therapy could prevent trauma-induced calcification:
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. (IF: 5.310)
These results further support our hypothesis that PPi supplementation may be effective in the prevention of diabetic vascular calcification as well.

Based on our promising results of PPi treatment using PXE, diabetic 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)

For the potential use of PPi as a regular treatment, it is important to find the least harmful and most effective salt form. Currently we are using disodium-pyrophosphate, however, too much sodium may lead to high blood pressure and may increase the risk of stroke. In collaboration with Pyrogenix, an American company, we have tested different salt forms to replace sodium. We have found safer salt forms with higher bioavailability, and also tested different formulation options (gelatine versus collagene capsules).
Our manuscript summerizing these finding is under editorial review:
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 Dermatol* (under review)

We also published a review paper summarizing therapeutic options for ectopic calcification, including pyrophosphate treatment:
Shimada BK, Pomozi V, Zoll J, Kuo S, Martin L, Le Saux O.: ABCC6, Pyrophosphate and Ectopic Calcification: Therapeutic Solutions. *Int J Mol Sci*. 2021 (IF: 4.556)

Publications related to this project:

- Brampton C, **Pomozi V**, Chen LH, Apana A, McCurdy S, Zoll J, Boisvert WA, Lambert G, Henrion D, Blanchard S, Kuo S, Leftheriotis G, Martin L, Le Saux O.: ABCC6 deficiency promotes dyslipidemia and atherosclerosis. *Sci Rep*. 2021. (IF: 4.379)
- 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*. (IF: 5.310)
- Shimada BK, **Pomozi V**, Zoll J, Kuo S, Martin L, Le Saux O.: ABCC6, Pyrophosphate and Ectopic Calcification: Therapeutic Solutions. *Int J Mol Sci*. 2021 (IF: 4.556)

Manuscript under revision:

- 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 Dermatol* (IF: 8.851)

Manuscript under editorial review:

- 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 Dermatol* (IF: 3.368)

Budapest, 2021.09.30. Pomozi Viola