SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

THE ROLE OF HYPOXIA INDUCIBLE FACTOR (HIF) SIGNALING PATHWAY ACTIVATION IN THE OSTEOGENIC DIFFERENTIATION AND CALCIFICATION OF VALVULAR INTERSTITIAL CELLS

by Máté Dávid Csiki

Supervisor:

Viktória Jeney, PhD, DSc

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNBIOLOGY

DEBRECEN, 2023

The role of hypoxia inducible factor (HIF) pathway activation in the osteogenic differentiation and calcification of valvular interstitial cells

By Máté Dávid Csiki, Bioengineer MSc

Supervisor: Viktória Jeney, PhD, DSc

Doctoral School of Molecular Cell and Immunbiology, University of Debrecen

Head of the **Defense Committee**:	István Balogh, PhD, DSc
Reviewers:	Ágnes Gyetvai, PhD
	Péter Bencsik, PhD
Members of the Defense Committee:	Kitti Pázmándi, PhD
	Márta Sárközy, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. "A", Department of Internal Medicine, Faculty of Medicine, University of Debrecen in 19th of December 2023 from 13:00

Introduction

Vascular calcification is the deposition of calcium and phosphate rich hydroxyapatite in the vessel wall. Studies revealed that vascular calcification is an active, regulated process with the involvement of vascular smooth muscle cells (VSMCs). The major cellular mechanism of vascular calcification is the osteochondrogenic transition of vascular smooth muscle cells (VSMCs) into osteoblast-like cells.

Vascular calcification and valvular heart disease are highly prevalent in patients with chronic kidney disease (CKD) and these conditions are responsible for the high risk of cardiovascular events and mortality. Hyperphosphatemia is a critical player in CKD-associated vascular and valvular calcification. The prevalence of valve calcification (VC) is eight times higher in end stage renal disease patients undergoing hemodialysis than in the general population. Aortic and mitral valves are affected most frequently, and calcification of both valves arises 10–20 years sooner in CKD patients compared with subjects with normal kidney function. Heart valves are avascular but metabolically active tissues, composed of an outer monolayer of valve endothelial cells and several internal layers of valve interstitial cells (VICs). Many lines of evidence suggest that VC is an actively regulated process in which VICs differentiate into osteoblast-like cells and myofibroblasts. Studies indicated that excessive formation of reactive oxygen species (ROS) play a critical role in the initiation and progression of these processes. The osteogenic transition of VICs is characterized by elevated expression of osteogenic markers including runt-related transcription factor 2 (Runx2), bone morphogenetic protein 2, alkaline phosphatase (ALP), osteopontin and osteocalcin (OCN). Importantly, these osteogenic markers are found to be upregulated along with increased ROS production in calcified human aortic valves.

Most of the healthy human heart valves are avascular, therefore adequate nutrition and oxygenation of VICs are ensured via diffusion from the circulating blood. On the other hand, valve thickening compromise the diffusional oxygen transfer, and additional blood supply is required to support the needs of active metabolism of valve cells. In line of this notion, a large body of evidence show the presence of neovasculature in thickened and stenotic valves. Formation of neovessels is found to be associated with increased expression of hypoxia inducible factor (HIF) alpha subunits HIF-1α and HIF-2α, activation of the HIF pathway and upregulation of vascular endothelial growth factor. Furthermore, studies revealed that neovessel density correlates with valve calcification

HIF-1 pathway is the master regulator of cellular and systemic homeostatic response to hypoxia. The HIF-1 pathway is regulated by the oxygen-sensitive α subunit (HIF-1α) that is stabilized upon hypoxia. Recently, a direct role of HIF-1 activation has been shown in phosphate-induced VSMC calcification that could be relevant in mineral imbalance-induced calcification in patients with chronic kidney disease. Hypoxia and sustained HIF activation have been shown to promote vascular smooth muscle cells (VSMCs) phenotype switch towards osteoblast-like cells, and accelerate vascular calcification. Therefore, in this work we have investigated whether hypoxia and HIF signaling are actively participating in osteogenic trans-differentiation of VICs and subsequent VC.

Chronic kidney disease (CKD) is an irreversible and progressive disease associated with alteration of the renal structure and decline of kidney functions. CKD is frequently associated with other chronic diseases, including anemia, metabolic bone diseases and cardiovascular diseases. CKD patients have five to ten times higher risk of premature death than the general population, which is largely attributed to death from cardiovascular diseases.

CKD is frequently associated with anemia, which is characterized by decreased eritropoetin (EPO) production, blood loss during dialysis. Anemia treatment in CKD patients was targeted with EPO or erythropoiesis-stimulating agents (ESAs), along iron supplementation. The treatment of CKD-associated anemia was revolutionized by the introduction of EPO and ESAs, but safety concerns of ESA have lately been reported. Clinical trials showed that ESA treatment increases the risks for cardiovascular events and may increases risk for death. Therefore, an alternative therapeutic strategy has been emerged to treat CKD-associated anemia, which is based on the activation of the hypoxia-inducible factor (HIF) pathway by prolyl-hydroxylase domain inhibitors (PHIs). Recently four, clinically tested PHIs are available to treat CKD-associated anemia: Roxadustat, Vadadustat, Daprodustat and Molidustat. Since 2020 Daprodustat (DPD) is approved to treat anemia of CKD patients in Japan. The effect of DPD was investigated in non-dialyzed and dialyzed CKD patient in vary of doses and they revealed that DPD is well-tolerated and hemoglobin level was significantly increased. Moreover, DPD increased EPO level and induced erythropoiesis, which suggest that DPD is a promising drug that can replace ESAs in the treatment of anemia in CKD patients. A longer clinical study revealed that 10 mg daily dose is adequate to reach the target hemoglobin values. Because PHIs target the HIF pathway and hypoxia-mediated activation of HIF-1 induces the calcification of VSMCs, here, we investigated the effect of DPD on valve calcification *in vitro* and *in vivo* conditions.

Aims

According to the findings in the literature we put the following hypothesis:

1. **Hypothesis**: Elevated calcium and phosphate (Pi) induces osteogenic differentiation and calcification of VICs through HIF pathway activation *in vitro.*
2. **Hypothesis**: Hypoxia increases OM-induced osteogenic differentiation and calcification of VICs via HIF pathway activation *in vitro*.
3. **Hypothesis**: Increased ROS production upon hypoxia increases osteogenic differentiation of VICs *in vitro*.
4. **Aim**: DPD accelerates CKD-induced valve calcification *in vivo*.
5. **Aim**: Hypoxia increases Pi-induced calcification of human aortic vascular smooth muscle cells (HAoSMCs) in a synergistic manner.

Materials and methods

- Human VICs were cultured in DMEM at 37°C in a humidified atmosphere containing 5% CO_2. Hypoxic environment was provided for the cells in a modular incubator chamber, in which we injected a pre-made gas mixture (1% O_2, 5% CO_2 and 94% N_2). Normoxic condition was maintained with a pre-made gas mixture (21% O_2, 5% CO_2 and 74% N_2). In some experiments we applied hypoxia mimetics cobalt-chloride (200 µmol/L), deferoxamine (DFO, 40 µmol/L) and DPD (20 µmol/L), HIF-1 inhibitor chetomin (6 nmol/L) and N-acetly-cysteine (NAC, 1 mmol/L). To induce calcification we applied 2% FBS containing DMEM supplemented with inorganic phosphate (Pi, 1.5-2.5 mmol/L, pH 7,4). In some of the experiments the calcification medium was completed with calcium (Ca, 0.3 mmol/L $CaCl_2$) as well.
- Calcification was detected by Alizarin Red staining. Cells were fixed, then stained with 2% alizarin red solution (pH 4.2). After removal of the dye, the plate was washed with distilled water. After the photos were taken, Ca deposits were dissolved in 100 µl of hexadecylpyridinium-chloride solution (100 mmol/L) and measured the absorbance of samples at 562 nm.
- At the end of the experiments, the extracellular matrix (ECM) of cells were decalcified in 0.6 mol/L HCl for 30 mins at room temperature. The Ca content of HCl-containing supernatant was determined by QuantiChrom Calcium Assay kit.

- Cell viability was determined by MTT (3-(4,5-dimetyl-2-tiazolyl)-2,5-difenyl-2H-tetrazolium-bromide) assay. The viability of treated cells was compared to the viability of untreated cells.
- To analyze protein expression we performed Western blot. Lysates were separated on 7.5-10% SDS-PAGE, then proteins were transferred onto nitrocellulose membrane. Membranes were blocked in non-fat dry milk, then the membranes were incubated with primary antibody overnight. Next day, the membranes were washed and incubated in horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. After 1 hour, membranes were washed and a chemiluminescent substrate was used to detect the result, which were recorded both on x-ray film and digitally.
- For OCN detection, the ECM of the cells grown on 6-well plates was dissolved in 100 μL of EDTA (0.5 mol/L, pH 6.9). OCN content of the EDTA-solubilized ECM samples were measured by ELISA according to the manufacturer's protocol.
- For the measurement of intracellular ROS production, at the end of the treatment the cells were washed with HBSS and incubated with 10 μmol/L 5-(-6)-chlorometyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl esther reagent for 30 mins at 37 °C. After the incubation, cells were washed in HBSS and fluorescence intensity was measure for 4 hours (488 nm excitation/533 nm emission). In some of the experiments we applied antioxidants.
- To knock-down HIF-1α and HIF-2α gene expressions we used Silencer® select siRNA constructs targeting HIF-1α and HIF-2α. As a control we used negative control #1 construct. Lipofectamine® RNAiMAX reagent was used to transfect VSMCs according to the manufacturer's protocol.
- Animal experiments were performed in accordance to the institutional (Institutional Ethics Committee of University of Debrecen) and national guidelines. To examine the effect of DPD, 8-12 weeks old C57BL/6 mice (n=5/group) were used. First of all, we induced CKD in mice with a two-phase diet. The last 3 weeks of the experiment we orally administrered DPD (15 mg/kg/day), suspended in 1% methyl cellulose.
- Renal and hematology parameters were determined from Na_3-citrate anticoagulated whole blood murine samples. Plasma urea and creatinine levels measured by kinetic assays on a Cobas c502 instrument, the hematology parameters were analyzed by a Siemens Advia-2120i hematology analyzer with the 800 Mouse C57BL program of Multi Species software.
- Calcification of the mice hearts was examined by OsteoSense dye and analyzed *ex vivo* by an IVIS Spectrum In Vivo Imaging System.

- Mice hearts were fixed in 10% neutral-buffered formalin, embedded in paraffin blocks, and cut into 4-μm-thick cross sections. After deparaffinization and rehydration, we performed von Kossa staining, Alizarin red staining and hematoxylin eosin (H&E) counterstaining on the sections, according to manufacturer's protocol.

Results

Activation of osteogenic and hypoxia signaling in valve interstitial cells (VICs) exposed to high phosphate

Osteogenic differentiation and extracellular matrix (ECM) mineralization of VICs play a major role in the development of VC. We treated VICs with osteogenic medium and we observed time-dependent upregulation of Runx2 and Sox9, as well as ALP. OM triggered calcification, Ca and OCN content of VICs. OM also triggered a hypoxia response in VICs, characterized by elevated protein expression of HIF-1α, HIF-2α and Glut-1.

Hypoxia signaling is involved in high Pi-induced calcification of VICs

To address whether hypoxia signaling is implicated in osteogenic differentiation of VICs, we used siRNA to downregulate protein expressions of HIF-1α and HIF-2α, the regulatory subunits of the HIF complexes. Knockdown of either HIF-1α or HIF-2α was associated with decreased calcification of VICs, suggesting that HIF pathways are not only activated upon osteogenic stimulation, but they are actively participated in the calcification process.

Hypoxia enhances calcification of VICs in a HIF-1α- and HIF-2α-dependent manner

Next, we asked whether hypoxia influences OM-induced osteogenic differentiation and calcification of VICs. First, we exposed VICs to normoxia (21% O_2) or hypoxia (1% O_2) and evaluated protein expressions of HIF-1α, HIF-2α and Glut-1. Then we treated VICs with OM under normoxic and hypoxic conditions for 24 and 48 h. Compared to control, OM slightly increased Runx2 and Sox9 expressions under normoxic condition after 48 h of exposure but hypoxia strongly upregulated Runx2 expression even in the absence of OM stimulation. Compared to normoxia, Sox9 expression was elevated under hypoxia at each condition. These results suggest that hypoxia may influences osteogenic reprogramming of VICs. Next, we addressed the effect of hypoxia on ECM calcification in VICs. We induced VICs calcification with OM containing calcium and different amounts of excess Pi under normoxic and hypoxic conditions. As revealed by Alizarin Red staining and calcium measurement, hypoxia

potentiated the pro-calcification effect of Pi at each tested concentrations. Then we investigated time-dependency of VICs calcification under normoxic and hypoxic conditions. Alizarin Red staining showed positivity after 2 days of OM exposure under hypoxic condition, whereas under normoxia calcification became detectable only on day 6. Calcium measurement from HCl-solubilized ECM also supported the finding that hypoxia potentiates and accelerates Pi-induced calcification of VICs.

Hypoxia enhances OM-induced osteogenic trans-differentiation of VICs through HIF-1 signaling

To see whether HIF signaling was involved in hypoxia-induced acceleration of VICs calcification, first we applied the HIF inhibitor chetomin and investigated OM-induced calcification under hypoxic condition. As shown by Alizarin Red staining and calcium measurement, chetomin inhibited calcification of VICs. Then we knocked-down HIF-1α, HIF-2α or both with target-specific siRNAs under hypoxia. Silencing of either HIF-1α or HIF-2α resulted partial, whereas silencing of both HIF-α subunits caused complete inhibition of hypoxia-induced calcification, supporting the involvement of HIF signaling in hypoxia-induced VICs calcification.

The involvement of ROS in hypoxia-mediated potentiation of VICs calcification

To explore whether increased ROS production is implicated in VICs calcification under hypoxia we measured ROS production in control and OM-stimulated VICs under normoxic and hypoxic conditions. Osteogenic stimulation increased ROS production under normoxia, which was further increased by hypoxia in both control and OM conditions. However, antioxidant N-acetyl-cysteine (NAC) attenuated excessive ROS production and prevented OM-induced cell death under both normoxia and hypoxia.

Hypoxia mimetic drugs enhance VICs calcification

We investigated different hypoxia mimetic drugs, cobalt-chloride (CoCl2), desferrioxamine (DFO) and Daprodustat (DPD), to see whether they influence Pi-induced VICs calcification under normoxic condition. We treated VICs with CoCl2, DFO or DPD and we evaluated protein expressions of HIF-1α and HIF-2α. Hypoxia mimetics increased both HIF-1α and HIF2α levels markedly in VICs. Next, we investigated the effects of hypoxia mimetic drugs on OM-induced calcification of VICs. We observed that all the three tested hypoxia mimetic drugs enhanced OM-induced calcification in VICs. These results suggest

that not only real hypoxia but also chemical activation of the HIF pathways enhances calcification of VICs. Silencing of either HIF-1α or HIF-2α resulted in partial inhibition of OM + DPD-induced calcification as assessed by Alizarin Red staining.

DPD enhances aortic VC in CKD mice

DPD enhances VICs calcification in vitro, therefore we addressed its effect on VC in an adenine-induced CKD mice model. CKD was induced with a diet containing adenine and elevated phosphate. Then we increased phosphate content of the diet, and started to administer DPD (15 mg/body weight kg/day orally) in the next 4 weeks of the experiment. DPD efficiently corrected CKD-associated anemia resulting in normalized Hb concentration, red blood cell count and hematocrit levels, similar to the controls with normal renal function. To address the effect of DPD on heart calcification we performed OsteoSense staining and detected higher amount of hydroxyapatite deposition in the hearts derived from DPD-treated CKD mice compared to vehicle-treated mice. Then we performed histological analysis of hearts derived from Ctrl, CKD and CKD + DPD mice to detect VC. We found stronger von Kossa and alizarin red staining in heart valves of CKD + DPD mice compared to CKD. These results suggest that DPD may accelerate VC in mice with CKD.

Hypoxia Accelerates Pi-Induced ECM Calcification in VSMCs

A previous study by Mokas et al. showed that hypoxia amplifies the pro-calcification effect of elevated inorganic phosphate (Pi). To confirm this finding, we set up an in vitro model of vascular calcification with cultured human VSMCs maintained in a calcification medium supplemented with different concentrations of Pi under normoxic and hypoxic conditions. We found increased intensity of AR staining at all tested Pi concentrations under hypoxia in comparison to normoxia. Measurement of Ca levels confirmed the result of AR staining, having more Ca in the ECM of VSMCs under hypoxic condition than normoxic groups. In a time course experiment, we investigated the kinetics of calcification in the presence of 2.5 mmol/L excess Pi under normoxic and hypoxic conditions. Results show that calcification is significantly higher on both days 4 and 6 under hypoxia than under normoxia. These results confirm the previously established pro-calcifying effect of hypoxia under normal and high Pi conditions.

Discussion

Our study is the first, reporting that HIF-1 activation is critically implicated in phosphate-induced calcification of VICs. We found elevation of osteogenic and hypoxia markers in the heart tissue of CKD mice, as well as high phosphate-treated VICs. Knock-down of HIF-1α or HIF-2α and HIF inhibitor chetomin resulted attenuation of Pi-induced calcification of VICs, suggesting a causative role of HIF-1 pathway activation in this process. Further activation of the HIF-1 pathway by either hypoxia or hypoxia mimetics intensified high-phosphate induced calcification of VICs in a HIF-1α, HIF-2α and ROS-dependent manner. The hypoxia mimetic drug DPD increased osteogenic activity in the heart tissue and intensified aortic valve calcification in adenine-induced male CKD mice. Previous studies showed that HIF-1α is upregulated in stenotic valves and co-localize with areas of angiogenesis and calcification. Moreover, neovessel density positively correlates with the extent of valve calcification. A recent integrated proteomic and metabolomic profile analyses of cardiac valves identified HIF-1 signaling as a key pathway in calcific aortic valve disease. Previous works linked HIF-1 activation and valve calcification. For example, non-hypoxic activation of HIF-1α has been shown to play a causative role in lipopolysaccharide and interferon gamma-induced calcification of VICs. Our study provided evidence that HIF-1α and HIF-2α are not only upregulated but taking a regulatory part in the calcification process of VICs.

Tissue hypoxia is implicated in the pathomechanism of many human diseases including kidney disease. Hypoxia accelerates the progression of CKD via promoting fibrogenesis of renal fibroblasts, and triggering epithelial-mesenchymal transformation of renal tubular cells. Due to CKD-associated anemia, tissue hypoxia in CKD is not limited to kidney but affects other organs as well. In line of this notion, here we showed increased mRNA and protein expression of HIF-1α and HIF-2α in heart derived from CKD mice. Despite the growing evidence that VICs are exposed to hypoxia in certain disease conditions the effect of hypoxia on VICs remained mostly undiscovered. The effect of hypoxia on osteogenic differentiation potential was studied on diverse cells. In contrast, hypoxia has been reported to decrease the expression of osteogenic markers in MG63 osteoblast-like cells. According to another study, hypoxia does not influence osteogenic differentiation of primary osteoblasts and mesenchymal precursors, but quick exposure to anoxia inhibits bone nodule formation and calcification through the downregulation of Runx2. Overall, these results suggest that the effect of hypoxia on osteogenic differentiation is finely regulated and cell specific. Unfettered ROS production plays an important causative role in vascular calcification and in the

pathophysiology of calcific aortic valve disease. The relation between hypoxia and ROS production is controversial, but a majority of the evidence suggests that hypoxia stimulates ROS formation in most types of eukariotic cells. Hypoxia impairs the function of the mitochondrial electron transport chain complexes leading to increased ROS signals that play critical role in initiating hypoxia response. Additionally, a study on pulmonary artery smooth muscle cells revealed that hypoxia-induced mitochondrial ROS activates NADPH oxidases which provides a positive feedback loop of increased ROS formation upon hypoxia. Our results revealed that hypoxia increases ROS formation in VICs. Phosphate-induced calcification of VICs was abolished by the antioxidant NAC under both hypoxic and normoxic conditions, suggesting a causative role of ROS in the phosphate-induced calcification process.

Under physiological conditions, HIF α subunits are hydroxylated by prolyl hydroxylase domain proteins (PHDs) and eliminated via the proteasomal degradation. Here we showed that non-hypoxic activation of the HIF pathway hypoxia mimetics cobalt chloride, DFO, and DPD promoted OM-induced calcification of VICs under normoxic condition. CKD is frequently associated with anemia. Anemia of patients with CKD was treated with recombinant erythropoietin or erythropoiesis-stimulating agents (ESAs). Unfortunately, studies showed that ESAs increase the risks for major cardiovascular events and accelerate disease progression. In this study we used DPD to investigate the effect of HIF-1 pathway activation on valve calcification in the adenine-induced CKD model. The basis of our choice of the experimental model was that DPD is a new-generation drug and approved in Japan since 2020 for the treatment of patients with CKD-associated anemia. Here we showed that DPD corrected anemia, but promoted CKD-induced aortic VC in vivo. Previously we found similar effect of DPD on aorta calcification. Although the clinical relevance of this model is clear, the conclusions are limited to DPD-driven HIF-1 activation. Therefore further studies are needed to investigate the effect of functional hypoxia and other hypoxia mimetic drugs on vascular and aortic valve calcification.

In conclusion, here we showed that hypoxic or pharmacological activation of the HIF pathway accelerates phosphate-induced calcification of VICs, in a HIF-1α, HIF-2α and ROS-dependent manner. The new generation PHD inhibitor DPD increased aortic VC in vivo in the adenine-induced mice model of CKD with high plasma phosphate level. Further studies are needed to investigate the potential involvement of this mechanism to the occurrence of major cardiovascular events which was reported to happen in 25.2% of hemodialysis-dependent CKD patients on DPD treatment during a 2.5-year follow-up period, and in 19.5% of non-dialyzed CKD patients on DPD treatment during a 1.9-year follow-up period.

Summary of the new scientific results

1. Calcium and phosphate, as double osteogenic stimulus induces osteogenic differentiation and calcification of VICs via HIF pathway activation.

2. Hypoxia increases OM-induced osteogenic differentiation and calcification of VIC in a HIF-1α and HIF-2α-dependent manner.

3. Hypoxia-induced ROS production accelerates osteogenic transition and calcification of VICs, which process can be inhibited by antioxidants.

4. DPD efficiently corrected CKD-associated anemia but increases calcification of heart and heart valves via HIF pathway activation *in vivo* in an adenine-induced CKD mice model.

List of publications

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/332/2023.PL
Subject: PhD Publication List

Candidate: Dávid Máté Csiki
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Csiki, D. M.**, Ababneh, H., Tóth, A., Lente, G., Szöőr, Á., Tóth, A., Fillér, C., Juhász, T., Nagy, B. J., Balogh, E., Jeney, V.: Hypoxia-inducible factor activation promotes osteogenic transition of valve interstitial cells and accelerates aortic valve calcification in a mice model of chronic kidney disease.
   Front. Cardiovasc. Med. 10, 1-15, 2023.
   DOI: http://dx.doi.org/10.3389/fcvm.2023.1168339
   IF: 3.6 (2022)
2. Tóth, A., **Csiki, D. M.**, Nagy, B. J., Balogh, E., Lente, G., Ababneh, H., Szöőr, Á., Jeney, V.: Daprodustat Accelerates High Phosphate-Induced Calcification Through the Activation of HIF-1 Signaling.
   Front. Pharmacol. 13, 1-12, 2022.
   DOI: http://dx.doi.org/10.3389/fphar.2022.798053
   IF: 5.6

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

3. Szabó, L., Balogh, N., Tóth, A., Angyal, Á., Gönczi, M., **Csiki, D. M.**, Tóth, C., Balatoni, I., Jeney, V., Csernoch, L., Dienes, B.: The mechanosensitive Piezo1 channels contribute to the arterial medial calcification.
   Front. Physiol. 13, 1037230, 2022.
   DOI: http://dx.doi.org/10.3389/fphys.2022.1037230
   IF: 4

4. Balogh, E., Chowdhury, A., Ababneh, H., **Csiki, D. M.**, Tóth, A., Jeney, V.: Heme-Mediated Activation of the Nrf2/HO-1 Axis Attenuates Calcification of Valve Interstitial Cells.
   Biomedicines. 9 (4), 1-17, 2021.
   DOI: http://dx.doi.org/10.3390/biomedicines9040427
   IF: 4.757

Total IF of journals (all publications): 17,957
Total IF of journals (publications related to the dissertation): 9,2

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

06 July, 2023

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

Acknowledgements

I would like to thank everyone who took part in the preparation of my thesis.

I am grateful to my supervisor, *Viktória Jeney*, who gave me opportunity 4 years ago to make my thesis. Furthermore, I am so thankful for all her help, supporting advice and coordinating my project.

I would like to thank to my colleague and my partner, *Andrea Tóth*, to share her knowledge and endless support.

I would like to thank to my present and former colleagues for their support in lab works in the Vascular Pathophysiology Research Group, especially to *Enikő Balogh*, *Judit Erdei*, *Gréta Lente*, *Bogonko Benard Nyakundi*, *Haneen Ababneh* and *Arpan Chowdhury*.

I truly appreciate the hard work and support of our collaborators.

Finally, I am so grateful to *my mother* to her endless support during my studies.