

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

**THE ROLE OF HIF-1 PATHWAY ACTIVATION IN THE PATHOGENESIS OF
VASCULAR CALCIFICATION**

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The role of HIF-1 pathway activation in the pathogenesis of vascular calcification

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Introduction

Vascular calcification is the deposition of calcium and phosphate rich hydroxyapatite in the vessel wall, which is associated with high risk of cardiovascular events and mortality. Research of the last two decades revealed that vascular calcification has multiple forms and is an active, regulated process with the involvement of vascular smooth muscle cells (VSMCs). Diverse pathologies including chronic kidney disease, diabetes mellitus and atherosclerosis are associated with accelerated calcification.

Osteochondrogenic differentiation of vascular smooth muscle cells (VSMCs) is the major cellular mechanism underlying vascular calcification. Upon osteochondrogenic differentiation, VSMCs upregulate master transcription factors of osteogenesis and chondrogenesis, such as Runx2 and Sox9; parallel with this process, they lose VSMC contractile markers such as α -SMA.

Various inhibitors and inducers of vascular calcification have been identified, and recent studies highlighted the potential role of hypoxia and the HIF-1 (hypoxia-inducible factor 1) pathway activation in vascular calcification. For example, arterial calcification is increased in patients with asthma, chronic obstructive pulmonary disease, and obstructive sleep apnea, but whether hypoxia plays a contributory role in triggering arterial calcification in these conditions has not been addressed.

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-1 α) that is stabilized upon hypoxia. Some studies associated HIF-1 α levels and vascular calcification. For example, a positive correlation between plasma HIF-1 α levels and arterial calcification has been shown in patients with type 2 diabetes mellitus. 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. Moreover, pulmonary arterial hypertension was found to be associated with increased pulmonary arterial calcification and elevated expression of Runx2 in the lungs of patients with pulmonary arterial hypertension. These works proposed that sustained Runx2 expression promotes HIF-1 α activation and that Runx2/HIF-1 α axis triggers proliferative and osteogenic phenotype switch of VSMCs.

It is generally accepted that intracellular reactive oxygen species (ROS) levels also change during hypoxia, but the direction of this change and the origin of ROS remained controversial. Recent evidence suggests that upon hypoxia mitochondria-derived elevated ROS production stabilizes HIF-1 α ; and therefore, elevated ROS production plays a critical role in hypoxia-driven cellular responses. Elevated ROS formation was detected in animal models of vascular calcification and in human sclerotic and stenotic aortic valves. A recent study showed that inhibition of mitochondrial ROS generation attenuates phosphate-mediated osteogenic differentiation of VSMCs, suggesting a causative role of ROS in vascular calcification. Because hypoxia is a common feature in diverse pathologies when cardiovascular calcification occurs, in the first part of my thesis we investigated the role of hypoxia, HIF-1 activation, and ROS formation in osteochondrogenic differentiation of human aortic SMCs.

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-associated anemia is characterized by decreased erythropoietin (EPO) production, blood loss during dialysis and decreased gastrointestinal iron absorption due to chronic inflammation.

Accordingly, anemia in patients with advanced CKD was targeted with EPO or erythropoiesis-stimulating agents (ESAs), along with oral or intravenous iron supplementation. Red blood cell transfusion remained a treatment option only for blood loss or severe hyporesponsiveness for ESAs. The treatment of CKD-associated anemia was revolutionized by the introduction of EPO and ESAs, but safety concerns of ESA use have lately been emerged. Trials showed that ESA treatment increases the risks for cardiovascular events and probably increases risk for death, serious cardiovascular events, and the development of end-stage renal disease.

An alternative therapeutic strategy has emerged to treat CKD-associated anemia that relies on the modulation of the hypoxia-inducible factor (HIF) pathway. The activation of the HIF pathway leads to transcriptional activation of numerous genes, including EPO, and a subsequent increase in erythropoiesis. Therefore, small-molecule stabilizers of the HIF pathway have been developed. Recently four, clinically tested PHIs are available to treat CKD-associated anemia: Roxadustat, Vadadustat, Daprodustat and Molidustat. The Daprodustat (DPD, GSK1278863) is a small-molecule PHIS, developed by GlaxoSmithKline.

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 reduced hepcidin level, 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.

These molecules inhibit the activity of HIF prolyl hydroxylase domain-containing (PHD) enzymes, which are responsible for hydroxylation of the oxygen-sensitive alpha subunits of HIF at conserved proline residues under normoxic conditions. PHD inhibitors (PHIs) mimic the effect of hypoxia and result in stabilization of the HIF α subunits, nuclear translocation, and eventually HIF activation with increased production of EPO.

Recent studies highlighted the potential role of hypoxia and the HIF-1 pathway activation in vascular calcification. 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 calcification *in vitro*, *ex vivo* and *in vivo* conditions.

Aims

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

- 1. Hypothesis:** Hypoxia increases osteochondrogenic differentiation of VSMCs via the activation of the HIF-1 pathway *in vitro*.
- 2. Hypothesis:** Increased ROS production plays a crucial role in hypoxia-induced acceleration of osteochondrogenic differentiation of VSMCs.
- 3. Hypothesis:** Hypoxia accelerates calcification of mice aortic rings *ex vivo* in a ROS-mediated way.
- 4. Aim:** DPD promotes osteochondrogenic differentiation of VSMCs via HIF-1 activation.
- 5. Aim:** DPD accelerates mice aortic calcification *ex vivo* and *in vivo*.

Materials and methods

- Human aorta VSMCs were cultured in DMEM at 37°C in a humidified atmosphere containing 5% CO₂. Hypoxic environment was provided for the cells in a modular incubator chamber, in which we injected a pre-made gas mixture (5% O₂, 5% CO₂ and 94% N₂). Normoxic condition was maintained with a pre-made gas mixture (21% O₂, 5% CO₂

and 74% N₂). In some experiments we applied hypoxia mimetics cobalt-chloride (200 µmol/L), deferoxamine (DFO, 20 µmol/L), 2,2'-bipyridyl (100 µmol/L) and DPD (1-100 µmol/L) and HIF-1 inhibitor chetomin (6 nmol/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, 0.6 mmol/L CaCl₂) 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-dimethyl-2-tiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide) assay. The viability of treated cells was compared to the viability of untreated cells.
- To isolate RNA, cells were lysed in Trizol reagent and RNA was purified according to the manufacturer's protocol. The absorption of RNA samples was measured by NanoDrop 2000 spectrophotometer. One µg RNA was reverse transcribed to cDNA by High Capacity cDNA RT kit. PCR reaction was performed by CFX96 Real-Time System with iTaq Universal Probes Supermix and validated FAM-konjugated TaqMan assay and pre-designed primers.
- 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.
- To determine VEGF (vascular endothelial growth factor) level, at the end of the experiment cellular supernatants were collected and the secreted VEGF was measured by ELISA according to the manufacturer's protocol.

- For OCN detection, the ECM of the cells grown on 6-well plates was dissolved in 100 μ L 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 μ mol/L 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester 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-1 α gene expressions we used Silencer® select siRNA constructs targeting HIF-1 α . 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 hypoxia and DPD, 8-12 weeks old C57BL/6 mice were used. For hypoxic treatment mice were placed into acryl chamber, in which pre-made gas mixture (10% O₂, 90% N₂) was injected with a 5-10 L/min flow rate. The hypoxia mimetic DPD was administered orally, suspended in 1% methyl cellulose.
- Renal and hematology parameters were determined from Na₃-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 aorta was examined by OsteoSense dye and analyzed *ex vivo* by an IVIS Spectrum In Vivo Imaging System.
- Aortic rings were fixed in 10% neutral-buffered formalin, embedded in paraffin blocks, and cut into 4- μ m-thick cross sections. After deparaffinization and rehydration, we performed von Kossa staining and hematoxylin eosin (H&E) counterstaining on the sections, according to manufacturer's protocol.
- C57BL/6 mice aorta was harvested and cleaned under aseptic conditions and cut into pieces. The Ca content was determined by using the QuantiChrom Ca-assay kit, as described earlier. In some of the experiments we applied N-acetyl-cystein and chetomin under hypoxic conditions.

Results

Hypoxia and Daprodustat induces HIF-1 activation and osteochondrogenic differentiation of VSMCs

First, we investigated the activation of HIF-1 pathway in VSMCs under hypoxic conditions and in the presence of hypoxia mimetics, as well as DPD, and we found that hypoxia and hypoxia mimetics largely increased the activation of HIF-1 pathway and increased the expression of HIF-1 α regulated target proteins, Glut1 and VEGFA. Hypoxia and DPD increased calcification, Ca content and OCN level of ECM in VSMCs in a time-dependent manner. Protein expression of osteochondrogenic markers, Runx2, Sox9 and ALP were determined and the results suggest that hypoxia and DPD increases osteochondrogenic differentiation of VSMCs.

The role of HIF-1 pathway in hypoxia and DPD induced calcification of VSMCs

DPD acts via the stabilization of HIF-1 α . We aimed to investigate whether the HIF-1 activation and HIF-1 α play a role in the pro-calcifying effect of DPD. First, we applied the HIF-1 inhibitor chetomin and found that chetomin attenuated the calcification of VSMCs. Then HIF-1 α knock-down cells were generated with siRNA technique and we found that calcification was attenuated in HIF-1 knock-down cells compared to controls.

The involvement of ROS in HIF-1 activation and osteochondrogenic reprogramming in VSMCs

Next, we examined the complex interplay between hypoxia and ROS production in hypoxia-induced osteochondrogenic reprogramming of VSMCs. First, we measured intracellular ROS production of VSMCs under normoxic and hypoxic (5% O₂) conditions in the absence and presence of antioxidants. We found that hypoxia increased ROS production in VSMCs. Several antioxidants, such as N-acetyl cysteine (NAC), sodium-pyruvate and rotenone efficiently inhibited hypoxia-induced ROS production of VSMCs, which suggests that hypoxia-induced ROS originates from the mitochondria. Furthermore, we found that in the presence of antioxidants the hypoxia-induced expression of Runx2 and Sox9, as well as ECM calcification of VSMCs was abolished.

Hypoxia and DPD induces HIF-1 activation and calcification of mice aortas

The effect of hypoxia on aortic calcification was further examined in *ex vivo* tissue culture. We found that hypoxic treatment resulted a more than 6-fold increase in aortic calcification compared to normoxia. Furthermore, in the presence of osteogenic stimuli the calcification reached higher, near to 10-fold increase compared to normoxia. Next we investigated the effect of DPD in tissue culture. Aorta rings which were cultured in Ctrl, elevated Pi in the presence or absence of DPD (25 $\mu\text{mol/L}$) conditions. Calcium content of aortas was measured after 3, 5 and 7 days of treatment. The accumulation of Ca of Pi-treated aortas was increased at day 7, whereas the Ca level of Pi+DPD treated aortas was elevated at day 5. The result of Ca measurement was confirmed by von Kossa staining and we observed calcium deposition in Pi+DPD treated aortas.

DPD corrects anemia but increases aortic calcification in CKD mice model

We studied the effect of DPD on anemia and vascular calcification in a murine model of CKD. The renal parameters were determined in the presence or absence of DPD and we found that DPD treatment did not influence the CKD-induced elevation of serum Pi, urea and creatinine levels. Furthermore, we investigated the effect of DPD on hematological parameters in CKD mice and we found that 15 mg/kg/day dose of DPD successfully corrected CKD-induced decrease of hemoglobin, red blood cell and hematocrit values. Next, we determined the potential pro-calcifying effect of DPD with 15 mg/kg/day, which dose efficiently corrected CKD-associated anemia. Calcification was detected with near-infrared imaging technique by IVIS machine. The fluorescent intensity of the aorta of CKD mice was largely increased compared to control, whereas the accumulation of osteosense was further increased in aorta of DPD-treated mice.

Discussion

Vascular calcification is the most known and studied form of ectopic calcification. During calcification calcium and phosphate-rich hydroxyapatite deposited in the intima and media layer of the vessels. Calcification is characterized by the osteochondrogenic differentiation of

VSMCs which shares similarities with physiological bone formation. Vascular calcification was observed in various diseases associated with systemic or local hypoxia. Accelerated coronary artery calcification was observed in patients with chronic obstructive pulmonary disease that was strongly correlated with low arterial blood oxygenation. Plaque hypoxia contributes to the progression of atheroma through diverse mechanisms but the question whether hypoxia is directly linked to atherosclerotic plaque calcification has not been addressed.

The presence of hypoxia is also known in CKD patients, which firstly affects the kidneys, although Mokas et al. described increased HIF-1 α expression in the media layer of aorta of CKD rats. Based on these observations we hypothesized that hypoxia might influence osteochondrogenic differentiation and calcification of VSMCs.

The complex interplay between hypoxia and ROS production was examined in several studies, resulting controversial data. Certain publication showed decreased ROS production in hypoxia, while other studies demonstrated increased ROS production in hypoxic cells. Previous studies already suggested a causative role of excessive ROS production in cardiovascular pathophysiology including vascular calcification.

Studies showed that mitochondrial electron transport chain complexes I and III and NADPH oxidases are involved in hypoxia-mediated increase in ROS production. Moreover, the finding that mitochondria-targeted antioxidants abolish the hypoxia response proved that mitochondrial ROS production is essential for propagation of the hypoxic signal toward the activation of the HIF pathway. Based on this evidence we hypothesized that hypoxia-mediated elevation of mitochondrial ROS production plays critical role in hypoxia-mediated osteochondrogenic differentiation of VSMCs. In line of this notion here we confirmed that hypoxia triggers elevated ROS production in VSMCs. Inhibition of ROS production with NAC and the mitochondrial complex I inhibitor rotenone abolished hypoxia-induced HIF-1 α stabilization and inhibited hypoxia-induced upregulations of Runx2, Sox9, and OCN and attenuated calcification.

Previous studies already suggested a causative role of excessive ROS production in cardiovascular pathophysiology including vascular calcification. Osteogenic differentiation of VSMCs has been linked to enhanced ROS production. Importantly, Zhao et al. showed that inhibition of mitochondrial ROS generation attenuates phosphate-mediated osteogenic differentiation of VSMCs. Byon et al showed that hydrogen peroxide promotes osteogenic differentiation of VSMCs through the induction of Runx2. In agreement with these findings here we showed that hypoxia-mediated production of hydrogen peroxide plays an essential

role in the stabilization of HIF-1 α and in the promotion of osteochondrogenic response in VSMCs. We found that hydrogen peroxide is sufficient to stabilize HIF-1 α under normoxic condition in VSMCs which is in agreement with previous observations. Interestingly, we also found that inhibition of HIF-1 activity abolished hypoxia-mediated ROS production, suggesting a more complex interplay between ROS generation and HIF-1 activity.

Hypoxia and accelerated arterial calcification coincide in diverse diseases such as asthma, chronic obstructive pulmonary disease, and obstructive sleep apnea. Here we showed that hypoxia upregulates Runx2 expression in mice lung and aorta *in vivo*. In addition, using an *ex vivo* aorta tissue culture we showed that hypoxia triggers Ca accumulation in mouse aorta in a HIF-1–dependent and ROS-dependent manner.

To conclude our results we suggest that hypoxia triggers osteochondrogenic differentiation of VSMCs. We propose that unfettered generation of ROS during hypoxia may be the signal switching on the osteochondrogenic differentiation program of VSMCs that leads eventually to vascular calcification.

CKD is often accompanied by anemia. DPD is a new generation drug to treat anemia in CKD patients. DPD is a PHI which acts through the activation of the HIF pathways. Our study provides evidence of a role for DPD in the progression of vascular calcification during CKD. We described synergistic effects between DPD and high Pi during osteochondrogenic differentiation of VSMCs. We report that oral administration of DPD accelerates high Pi-induced calcification in a mouse model of CKD. We also established HIF-1 α as major functional contributor of DPD-driven calcification. Our results revealed that DPD upregulates Glut-1 and VEGF, which are important target genes of HIF, proving that DPD potentially activated the HIF pathway in human VSMCs.

Growing evidence suggests that diseases with hypoxemia and/or hypoxia are associated with increased vascular calcification. Moreover, Mokas et al. showed that hypoxia synergizes with high Pi to enhance osteochondrogenic transdifferentiation of VSMCs. Furthermore, we reported recently that hypoxia itself is a pro-calcifying factor and is able to induce osteochondrogenic transdifferentiation and ECM calcification of VSMCs. These observations warranted us to test the pro-calcifying potential of DPD.

We chose a cellular model of vascular calcification in which we induced calcification of VSMCs with high Pi because DPD is a drug intended to be used in progressive CKD patients who develop positive phosphate balance. Here, we reported that similar to hypoxia, DPD intensifies high Pi-induced osteochondrogenic transdifferentiation, ECM calcification of VSMCs *in vitro*, and aorta calcification *ex vivo*. Similar results were obtained in previous

studies with another PHI, roxadustat (FG-4592), that also enhanced VSMCs calcification under high phosphate conditions.

After seeing the pro-calcifying action of DPD in elevated phosphate condition *in vitro*, we aimed to test the effect of DPD on anemia and calcification *in vivo*. We applied an adenine-induced CKD mice model, in which high-phosphate condition was approached by a diet rich in phosphorous. We tested three doses of DPD (5, 10, and 15 mg/kg/day) and found that DPD at the dose of 15 mg/kg/day corrected anemia of CKD mice completely. Here, we reported that besides its beneficial effect in correcting anemia, DPD at the dose of 15 mg/kg/day accelerated aorta calcification in CKD mice with high plasma phosphate levels. We showed that DPD stabilized both HIF-1 α and HIF-2 α in VSMCs. Chetomin that blocks the interaction of HIF α subunits with transcriptional co-activators, thereby attenuating hypoxia-inducible transcription, inhibited calcification triggered by DPD + Pi. Therefore, focusing on HIF-1 α , here, we showed that the pro-calcifying effect of DPD is dependent on HIF-1 α stabilization. Our study provides evidence of a role for DPD in the progression of vascular calcification during CKD.

To our knowledge, this is the first study that addressed the potential pro-calcifying effect of DPD. We found that DPD efficiently corrected anemia but DPD treatment accelerates phosphate-induced vascular calcification *in vitro* in primary VSMCs, *ex vivo* in mouse aorta rings, and *in vivo* in a murine CKD model with a high plasma phosphorous level. We assumed that administration of DPD in CKD patients with hyperphosphatemia could increase the risk of vascular calcification.

A recent study compared the effect of one of the ESAs, and DPD on the risk of cardiovascular events in dialyzed patients with a 8-11.5 g/dl hemoglobin level. 2964 patients were participated in a 2.5 year follow up period of this study and the results showed that 25.2% of DPD-treated patients and 26.7% of epoetin- α -treated patients have suffered from severe cardiovascular events.

Based on this knowledge, prior to the application of new generation hypoxia mimetics, the cost/benefit ratio should have been considered based on the severity of anemia and cardiovascular status of CKD patients.

Conclusion of the new scientific results

1. Both hypoxia and DPD triggers osteochondrogenic differentiation and extracellular matrix calcification of VSMCs.
2. Both hypoxia and DPD-induced osteochondrogenic differentiation of VSMCs is HIF-1 dependent.
3. Hypoxia induces mitochondrial ROS production, which plays a critical role in hypoxia-induced osteochondrogenic differentiation and calcification of VSMCs.
4. Both hypoxia and DPD accelerate aorta calcification *ex vivo* mice aorta tissue culture.
5. Hypoxia-induced calcification can be abolished by antioxidants and HIF-1 inhibitor *ex vivo*.
6. Hypoxia triggers osteogenic reprogramming of mice aorta *in vivo*.
7. DPD (15 mg/kg) corrects CKD-associated anemia, but as a side-effect, accelerates CKD-induced aortic calcification *in vivo*.

List of publications



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PhD Publication List

Candidate: Andrea Tóth

Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Tóth, A.**, Csiki, D. M., Nagy, B. J., Balogh, E., Lente, G., Ababneh, H., Szöör, Á., Jeney, V.:
Daprodustat Accelerates High Phosphate-Induced Calcification Through the Activation of
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Front. Pharmacol. 13, 1-12, 2022.
DOI: <http://dx.doi.org/10.3389/fphar.2022.798053>
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2. Balogh, E., **Tóth, A.**, Méhes, G., Trencsényi, G., Paragh, G., Jeney, V.: Hypoxia Triggers
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DOI: <http://dx.doi.org/10.1161/ATVBAHA.119.312509>
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List of other publications

3. Chowdhury, A., Balogh, E., Ababneh, H., **Tóth, A.**, Jeney, V.: Activation of Nrf2/HO-1 Antioxidant
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Biochim. Biophys. Acta. Mol. Basis Dis. 1862 (9), 1724-1731, 2016.
DOI: <http://dx.doi.org/10.1016/j.bbadis.2016.06.012>
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