

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

**ROLE OF IRON-FERRITIN/FERROXIDASE AND HYDROGEN SULFIDE
IN THE DEVELOPMENT OF THE VALVULAR MINERALIZATION:
IMPLICATIONS FOR CALCIFIC AORTIC VALVE DISEASE**

by Katalin Éva Sikura

Supervisor:
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UNIVERSITY OF DEBRECEN
KÁLMÁN LAKI DOCTORAL SCHOOL

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Head of the **Examination Committee:** László Muszbek MD, PhD, DSc

Members of the Examination Committee: Mariann Harangi MD, PhD
Szilvia Mészáros MD, PhD

The Examination takes place at the Library of Division of Division of Clinical Laboratory Science (8th floor), University of Debrecen, 8th November, 2019, at 11:00 AM

Head of the **Defense Committee:** László Muszbek MD, PhD, DSc

Reviewers: Harjit Pal Bhattoa MD, PhD
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 8th November, 2019, at 1:00 PM
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Introduction

Vascular and valvular calcification

Cardiovascular calcification is a complex disease involving the major and medium-sized arteries along with the aortic valves and is accompanied with chronic kidney disease. Rudolf Ludwig Karl Virchow, the ‘father of cellular pathology’, was the first who described the phenomenon of vascular calcification with the presence of stiff, ‘bone-like’ consistency in atheroma as a degenerative process in 1858. Today it is evident that cardiovascular calcification is a well-regulated and dynamic process, implicated with an increased risk of cardiovascular morbidity and mortality. It is also well known, that the pathological progression of cardiovascular mineralization is much more pronounced in patients with diabetes and in chronic kidney disease (CKD) (Libby, Ridker et al. (2002), Davignon and Ganz (2004), Stocker and Keane (2004), Rajamannan, Evans et al. (2011)).

Calcific aortic valve disease (CAVD) is one of the most common valvular heart disease in developed countries, and is a well defined disease process (Yutzey, Demer et al. (2014)). In addition to calcification, lipid accumulation and inflammation result in an atherogenous lesion within the heart valve tissue. As demonstrated by the National Heart Lung and Blood Institute, in valvular disease inflammation is a critical initiation step (Rajamannan, Evans et al. (2011) and other research groups (Mohler, Gannon et al. (2001); Mazzone, Epistolato et al. (2004); Freeman and Otto (2005); Dweck, Boon et al. (2012); Yutzey, Demer et al. (2014)). The ratio of the calcified and non-calcified regions could guide us to determine the state of calcification (Chester (2011); Lusic, Mar et al. (2004); Mohler (2004); Speer and Giachelli (2004); Mohler, Gannon et al. (2001)). In stenotic aortic valve calcification, VIC can transdifferentiate into myofibroblast-like cells, which are identified by markers of contractility such as alpha smooth muscle actin (α -SMA), or into osteoblast-like cells: identified by upregulation of ALP activity, and increased levels of osteocalcin expression, and RUNX2 nuclear translocation into the nucleus in later stages.

In CKD patients, the elevated plasma phosphate level is one of the most potent inducer of the initiation of vascular calcification (Hruska, Mathew et al. (2008); Giachelli (2009); (Adeney, Siscovick et al. 2009)). High phosphate provokes calcification of vascular cells in a process mediated by a sodium-dependent phosphate co-transporter (Pit channels), that facilitates entry of phosphate into the cells. In calcified valves, VIC is also shown to transdifferentiate into osteoblast-like cells, determined by the increasing of osteocalcin levels, ALP activity and other osteogenic factors expression/translocation (Rajamannan, Subramaniam et al. (2003)) such as RUNX2, the osteogenic transcription factor and its nuclear translocation (Ducy (1997)). Phosphate uptake occurs via phosphate carriers Pit1 and Pit2 (Crouthamel 2013); Li, Yang et al. (2006)).

Ferritin/Ferroxidase

Iron is essential in several cellular functions. Iron overload can be potentially toxic, and is mainly modulated by the ferritin molecules. Ferritin is an iron storage protein that has antioxidant properties, and it is known to protect the endothelium/smooth muscle

cells/valvular interstitial cells against the damaging effects of inflammation and calcification (Balla, Jacob et al. 1992); Sikura, Potor et al. (2019), Zarjou, Jeney et al. (2009)). Ferritin is a large molecule (450 kDa), and its function is to store up to 4500 Fe atoms in a safe, spherical shell in a nontoxic form. Ferritin has 24 subunits of two types (heavy [H] and light [L] chain) the proportions of which depend on the iron status of the cell, the tissue, and the organ (Theil (1990)). The H-chain of ferritin has ferroxidase activity that is essential for iron incorporation and also in controlling the potentially toxic Fe (II) ions via reduction in oxidative damage (Arosio and Levi (2002)). Additionally, ferritin has various functions apart from iron storage, i.e., it has been described as having immunomodulatory effects as summarized in a review by Wang, Knovich et al. (2010). Additionally, levels of serum ferritin are widely utilized by clinicians as a mirror of iron stores, and may signal abnormality in different diseases, e.g., infections, inflammation, cardiovascular disorders (Zarjou, Black et al. (2019)). While the connotation of iron/ferritin role in inflammation and cardiovascular diseases is not well understood and is extensively debated, in this part of this work we attempt to highlight a possible mechanism and its role in CAVD and CKD.

Role of H₂S

Hydrogen sulfide is a water soluble, colorless gas which was described first time in the 17th century (Wang (2010); Wang (2012)). Hydrogen sulfide is the novel endogenous gasotransmitter, along with nitric oxide and carbon monoxide (Wang (2002)). In mammalian tissues H₂S is mainly produced by CSE (cystathionine gamma-lyase) and CBS (cystathionine beta-synthase) from L-cysteine and homocysteine (Zhang, Wang et al. (2018)). Beltowski (2015) previously demonstrated that H₂S levels may be enhanced in vivo by conventional inorganic sulfide salts and Kang, Neill et al. (2017) have described that reaction between Lawesson's reagent and morpholine results in a new generation slow H₂S release compound GYY4137. Protonation reaction of GYY4137 results more stable H₂S releasing compounds, such as AP67 and AP72. AP72 has excellent water solubility and very slow generation of H₂S compared to the fast H₂S releasing donors such as NaSH and Na₂S (Chitnis, Njie-Mbye et al. (2013); Kang, Neill et al. (2017)); Nagy (2013)). Previously, we demonstrated that NaSH is a significant inhibitor of mineralization of vascular smooth muscle cells (Zavaczki, Jeney et al. (2011)). CSE is important for normal heart function and physiological functioning as Jiang et al, and others have concluded (Chen, Xin et al. (2007)). The development of heart disease is manifested by the disorder of H₂S production as indicated by the lower levels of plasma H₂S in patients with coronary heart disease (Jiang, Wu et al. (2005); Shen, Shen et al. (2015)). Additionally, the endogenous H₂S production by CBS also ameliorates the normal function of the brain (Abe and Kimura (1996)). Furthermore, elevation of H₂S levels is now achievable. Rose, Moore et al. (2017) recently demonstrated that H₂S play a functional role in cell-signaling and post-translational-modifications in cardiovascular system. Moreover, using siRNA or genetic animal (mouse) models to demonstrate the role of H₂S during mineralization, a mimic loss of function of genes (CSE; CBS) involved in the biosynthesis and degradation of H₂S was observed within the affected cells/tissue (Rose, Moore et al. (2017)). In this work we would like to reveal new insights into the biology of H₂S within the cardiovascular system and in cell signaling.

Aims of the Study

Part 1

CAVD is a complex disease mainly in the elderly. It is one of the leading causes of mortality and morbidity especially in CKD patients and in the developed countries. The progression of CAVD include the osteoblastic transdifferentiation of valvular interstitial cells (VIC) that is accompanied by bone specific gene expression, and its progression is manifested by the deposition of hydroxyapatite minerals in the affected tissue of the valve. Although, we have improved knowledge of the pathomechanisms of CAVD, the only therapeutic approach is heart valve replacement surgery. Previously, in our research laboratory demonstrated that ferritin/ferroxidase has an important role is vascular calcification. However, we have little knowledge on the role of ferritin/ferroxidase in cardiovascular mineralization.

Aims:

- Investigate the role of ferritin/ferroxidase in high phosphate induced CAVD progression in vitro and in vivo.
- Iron/ferritin role/possible mechanism in the inhibition of CAVD

Part 2

H₂S level has a crucial role in the progression of CAVD. Nonetheless, it is not known, what causes the progression of CAVD in H₂S deficiency. In this study, based on the results of the experiments outlined in Part 1 of our aims, we investigate the role of H₂S in CAVD.

Aims:

- Investigate the role of H₂S in CAVD progression.
- Examine role of exogenous exposure of H₂S donor molecules in the inhibition of CAVD.

Materials and Methods

Materials

All chemicals were analytical grade reagents or higher and obtained from Sigma-Aldrich, (St Louis, MO, USA). The sulfide donor molecules used in this work – GYY4137 (P-(4-Methoxyphenyl)-P-4-morpholinylphosphinodithioic acid morpholine salt), AP67 (4-methoxyphenyl) (pyrrolidin-1-yl) phosphinodithioic acid) and AP72 (4-methoxyphenyl) (piperidin-1-yl) phosphinodithioic acid) – were synthesized in-house (Kulkarni-Chitnis, Njie-Mbye et al. (2015); Li, Whiteman et al. (2008); Whiteman, Perry et al. (2015)). Sulfide stock solutions were prepared fresh daily.

Human tissue samples and cell isolation

Human aortic valve leaflets were obtained from patients undergoing valve replacement for stenosis with calcification (AS; N=52) and patients who had severe insufficiency without calcification (AI; N=28). The specimens were collected from Jan 2015 to Dec 2017 (80 patients) (Regional Research Ethical Committee, Project No.: 61538-2/2017/EKU and 4699-2016). VIC were isolated from human heart valves using collagenase (600 U/ml) (Worthington Biochemical Corp.). Cells isolated from donors were employed at passage 2 to 4. All experiments were performed on cells derived from 5 different donors.

Flow Cytometry

Cells were fixed and stained with FITC conjugated mouse anti-human CD31 antibody (Abcam; ab27333) and run on a FACSscan flow cytometer. Cell population was identified and gated based on size (forward scatter, FSC) and complexity (side scatter, SSC). Isotype sample was used as control.

Animals

All the in vivo experiments adhered to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and by the ARRIVE guidelines: British Journal of Pharmacology, 160: 1577–1579. Animal experiments performed in this study were approved by the Scientific and Research Ethics Committee of the Scientific Council of Health of the Hungarian Government (registration number DE MÁB/157-5/2010) and are reported in accordance with the ARRIVE guidelines. C57BL/6 ApoE^{-/-} mice were maintained at the University of Debrecen under specific pathogen-free conditions in accordance with guidelines from the Institutional Ethical Committee. Mice were randomly divided into four groups. Non-high fat diet group (N=5) received a standard chow diet. To induce aortic valve calcification, mice were kept on atherogenic diet (15% fat, 1.25% cholesterol, ssniffSpezialdiäten GmbH, Soest, Germany) till the age of 8 weeks. Parallel with the atherogenic diet mice were injected intraperitoneally with AP72 (266 µmol/kg body weight; N=5) or vehicle (saline; N=9) on every other day as previously described. Aortas were collected after 8 weeks of treatment. All mice were euthanized by slow-fill compressed CO₂ asphyxiation. Atherogenic food composition (high-fat diet) was as follows: Crude Nutrients (%): Crude protein 19%; crude fat 15.2%; crude fiber 3.4%; crude ash 6.3%; starch

25.6%; sugar 11.2%; Additives (per kg): vitamin A 15,000 IU; vitamin D3 1,000 IU; vitamin E 110 mg; vitamin K3 5 mg; vitamin C 0 mg; copper 13 mg.

Induction of calcification, calcium measurement, Alizarin Red S staining

VIC were cultured in calcification medium (2.5 mmol/L inorganic phosphate and 1.8 mmol/L calcium-chloride) with or without phenol red for 5 days. Calcium content of the supernatants was determined by QuantiChrome Calcium Assay Kit (Gentaur), normalized to protein content and expressed as $\mu\text{g}/\text{mg}$ protein. Alizarin Red S staining was used to visualize the calcium deposition. Plates were fixed with 3.7 % formaldehyde for 10 minutes followed by staining with a 2 % solution of Alizarin Red S. Photographs of the stained cells were taken with a light microscope (x10 magnification; Leica DMIL LED microscope).

Quantification of osteocalcin

ELISA kit was used (Bender MedSystem) for the quantification of osteocalcin from EDTA-solubilized extracellular matrix.

Alkaline phosphatase staining

To visualize alkaline phosphatase activity, cells were cultured in 24 well plates and fixed in Citrate-acetone solution (2:3) followed by staining with Naphtanol AS-MX –Fast Violet B solution (Sigma). Light microscope photographs were taken during the different treatments (Leica DMIL LED microscope, Leica DMC4500 camera with Leica application suite LAS Software 4.9.0).

Cell viability assay

Cells were cultured on 0.2 % collagen type I coated coverslip. NUCLEAR-ID[®] Blue/Red cell viability reagent was added to the cells at dilution 1:1000 for 30 minutes at 37 °C. Subsequently, the cells were fixed with Fluorescent Mounting Medium (Dako) on Superfrost Ultra Plus Microscope Slide (Thermo Scientific). Images were obtained with an immunofluorescence microscope (Leica DM2500 microscope, Leica DFC480 camera).

Immunofluorescence staining

Mouse monoclonal anti-human α -SMA (Santa Cruz; sc-3225; 400 ng/mL) and rabbit polyclonal anti-human von Willebrand factor antibody (Abcam; ab6994; 100 ng/mL) were used as primary antibodies to identify VIC and endothelium. Subsequently, samples were incubated with secondary antibodies, i.e., CY3-conjugated Streptavidin (Jackson ImmunoResearch; 016-160-084; 1000 ng/mL) and Biotin (Jackson ImmunoResearch; 715-065-150; 1000 ng/mL). For LAMP1 and H-ferritin double staining anti-human rabbit anti-human LAMP1 (Abcam; ab24170; 1000 ng/mL) and mouse anti-human H-ferritin (Santa Cruz; sc-376594; 400 ng/mL) were used with secondary antibodies anti-rabbit Alexa Fluor 488 against LAMP-1 (Thermo Fisher Scientific; A11070; 2000 ng/mL) and anti-mouse Alexa 647 (Thermo Fisher Scientific; A21244; 4000 ng/mL) against H-ferritin. Sox9 staining was performed with rabbit anti-human Sox9 (Abcam; ab26414; 2000 ng/mL) antibody followed

by the anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific; A11070; 2000 ng/mL) secondary antibody. Hoechst (0.5 ng/mL) was used to stain nuclei. Rabbit polyclonal anti-human RUNX2 (Proteintech, 20700-I-AP) was used (dilution 1:600) as a primary antibody to show RUNX2 localization in VIC. A primary antibody labeled with goat anti-rabbit Alexa 488 (Thermo Fisher Scientific, A11070) fluorophore at dilution 1:500 for 1 hour in dark at room temperature. Hoechst was used to stain nuclei. Multicolor STED imaging was acquired with STED (Stimulated Emission Depletion) Leica TCS SP8 gated STED-CW nanoscopy (Leica Microsystem Mannheim, Germany). Gated STED images were deconvolved using Huygens Professional (Scientific Volume Imaging B.V., Hilversum, Netherlands) software.

Nuclear and cytoplasmic protein extraction

Cells were cultured in growth medium and treated with or without calcification medium supplemented with 20 μ mol/L AP72. After treatment, cells were harvested with cell scraper and collected into a centrifuge tube. Pellets were washed twice with PBS followed by addition of ice-cold 1x cytoplasmic lysis buffer (20 mmol/L Tris-HCl pH 8.0, 100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L MgCl₂, protease inhibitor cocktail) to the pellets. Cell suspensions were incubated on ice for 15 minutes. After centrifugation, the supernatants were collected (contains cytoplasmic proteins), the pellets were washed with PBS and resuspended in ice-cold nuclear extraction buffer (20 mmol/L Tris-HCl pH 8.0, 300 mmol/L NaCl, 2 mmol/L EDTA pH 8.0, protease inhibitor cocktail). Next, the samples were drawn 5 times with 27 gauge needle for the extraction of the nuclear proteins followed by centrifugation at 8000 x g, 4°C for 20 minutes. The supernatant contains the nuclear fraction. The protein concentration of the samples was determined by the BCA Protein Detection Kit (Amersham).

Western Blot analysis

H-ferritin Western blotting was performed with rabbit anti-human H-ferritin antibody (Santa Cruz; sc-376594; 400ng/mL), followed by HRP-labeled anti-rabbit IgG antibody. To detect Pit-1, Pit2 and LAMP1 we used rabbit anti-human Pit1 antibody (Abcam; ab177147; 2000 ng/mL), rabbit anti-human Pit2 antibody (Proteintech; 12820-1-AP; 60 ng/mL) and rabbit anti-human-LAMP1 antibody (Abcam; ab24170; 1000 ng/mL), respectively. Western blot analysis for ENPP2 was performed using anti-human ENPP2 (Thermo Fisher Scientific; PA5-12478; 4000 ng/mL). Sox9 Western blot was performed with rabbit anti-human Sox9 (Abcam; ab26414; 2000 ng/mL). Western blots were performed with: rabbit anti-human Sox9 (Abcam; ab26414; 2000 ng/mL) and rabbit anti-human RUNX2 (Proteintech; 20700-I-AP; 400 ng/mL), rabbit anti-human TNF- α (Thermo Fisher Scientific; PA5-19810; 400 ng/mL), rabbit anti-human IL1- β (Invitrogen; 17h18116; 400 ng/mL), rabbit anti-human CSE at dilution 1: 600 (Proteintech, 12217-I-AP), rabbit anti-human CBS at dilution 1:600 (Proteintech, 14787-I-AP) and mouse anti-human Ankyrin G1 antibody at dilution 1:500 (Life Technologies, 338800). Complexes of antigen-antibody were visualized with a horseradish peroxidase chemiluminescence detection system (Amersham Biosciences; RPN2109). Membranes were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Quantitative Real-Time PCR (qRT-PCR)

VIC were cultured in growth media or calcification media supplemented with 20 $\mu\text{mol/L}$ AP72. Cells were harvested after 5 days. Total RNA was isolated using RNazol STAT-60 according to the manufacturer's instructions (TEL-TEST Inc., Friendswood, TX, USA). RNA concentration was measured with NanoDropTM 2000c spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). Subsequently, cDNA synthesis was performed using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA). We used real-time PCR technique for quantification of mRNA levels of ENPP2 and ANK1 (Thermo Fisher Scientific Inc.) and GAPDH (Thermo Fisher Scientific Inc.). TaqMan Universal PCR Master Mix was purchased from Applied Biosystems (Applied Biosystems, Foster City, CA). Finally, we performed TaqMan quantitative PCR (40 cycles at 95° C for 15 sec. and 60° C for 1 min.) on 96-well plates with the Bio-Rad CFX96 (Bio-Rad Laboratories Inc., Hercules, California, USA) detection system. Results were expressed as mRNA expression normalized to GAPDH.

Intracellular phosphate uptake measurement

Valvular interstitial cells were cultured on 12 well plates exposed to calcification medium in the presence or absence of phenol red using D-MEM supplemented with/without AP72 (20 $\mu\text{mol/L}$) for 5 days. Cells were lysed with 0.5 % NP40 and 1 % Triton-X100. Whole cell lysate centrifuged at 12000 x g for 15 min at 4°C. The supernatant was measured by QuantiChrom quantitative colorimetric phosphate assay kit (BioAssays System) on 96-well plates at 650 nm. Phosphate uptake was normalized to the protein content of the cells.

Pyrophosphate assay

VIC were cultured in phenol red-free growth medium (D-MEM; Sigma) or calcification medium and supplemented with AP72 (20 $\mu\text{mol/L}$). Heart valve tissue (AS N=3; AI N=3) and cells were lysed with EDTA free detergent. Inorganic pyrophosphate (PPi) was measured in the extracellular fluid of the VIC using PPLightTM inorganic pyrophosphate assay (Lonza; LT-07-610). The continuous kinetic assay was employed according to the manufacturer's instruction. The luminescence was monitored for 2 hours using SynergyTMMHTX Multi-Mode Microplate Reader from BioTek Instruments (USA) with 0.1 s integrated reading time. The relative luminescence (RLU) was normalized to the protein content of the cells.

Isolation of lysosomes

To separate lysosomes, we used the Lysosome Enrichment Kit for Tissue and Cultured Cells (Thermo Fisher Scientific; 89839) and gradient ultracentrifugation. For protein analysis, lysosomes were lysed with 2 % CHAPS (Sigma Aldrich; C2632-25G) in Tris-buffered saline (TBS; 25 mmol/L Tris, 0.15 mol/L NaCl; pH 7.2), samples were centrifuged with Beckman ultracentrifuge at 38 000 x g and the lysosomal fractions were collected from each sample.

Determination of sulfide level from AS and AI valve tissue with zinc precipitation assay

Sulfide levels were measured with zinc precipitation method as developed by Gilboa-Garber (Gilboa-Garber, 1971) and improved by Ang et al. (Ang et al., 2012). The human valves were

homogenized under liquid nitrogen in PBS (pH 7.4) and were sonicated. After that the sample was centrifuged at 12 000 x g for 15 min and the lipids free clear supernatant was collected. 200 μ L of samples were mixed with 350 μ L 1% zinc acetate and 50 μ L of 1.5 mol/L sodium-hydroxide and incubated for 60 minutes on a shaker. Incubation step was followed by centrifugation at 2000 x g for 5 minutes to pellet the generated zinc sulfide. The supernatant was then removed, and the pellet washed with 1 mL of distilled water by vortexing extensively, followed by centrifugation at 2000 x g for 5 minutes. The supernatant was then aspirated off and the pellet reconstituted with 160 μ L of distilled water and mixed with 40 μ L of pre-mixed dye (20 μ L of 20 mmol/L dimethyl-p-phenylene-diamine-dihydro-chloride (NNDP) in 7.2 mol/L hydrochloric acid (HCl) and 20 μ L of 30 mmol/L Iron(III) chloride (FeCl₃) in 1.2 mol/L HCl). After 10 min the absorbance of the generated methylene blue (MB) was measured with a spectrophotometer at 667 nm. Since during the reaction 1 mol/L MB formed from 1 mol/L sulfide, the concentration was determined by the MB's extinction coefficient (30 200 M⁻¹cm⁻¹). Samples were normalized for protein concentration. Results were calculated for μ mol/L generated H₂S/ mg protein at 60 minutes.

H-ferritin, Cystathionine γ -lyase and cystathionine- β -synthase double gene silencing

H-ferritin, Cystathionine- γ -lyase (CSE) and cystathionine- β -synthase genes silencing using siRNAs (Ambion, s225998; 4392420; s3710) were performed. Briefly, valvular interstitial cells were cultured on 12 well plates in antibiotic-free medium (D-MEM, Sigma). At about 70 percent of confluence, cells were transfected with siRNA against H-ferritin, CSE and CBS (Ambion, s225998; 4390824; s289). Transfection occurred for 4 hours in minimal serum-content medium (Opti-MEM; Gibco). At the end of transfection 30% FBS containing, antibiotic free D-MEM was added. Next day, cells were washed and treated with AP72 every second day until 5 days. The sequences of the siRNAs were inserted into the Supplementary Methods.

Pharmacological inhibition of CSE and CBS

VIC were cultured in 12 well plates in growth medium or calcification medium. Inhibition of CSE, CBS and 3-MST were carried out employing pharmacological compounds: PPG; AOAA; KGA; AOAA+PPG; AOAA+KGA; PPG+KGA (20 μ mo/L of each inhibitor).

Immunohistochemistry

Heart valve tissues were fixed with formaldehyde for one day followed by TRIS buffer and embedded in paraffin wax. Subsequently, slides were deparaffinized in xylene for 5 minutes and then rehydrated. For immunohistochemistry, slides were subjected to a peroxidase-blocking reagent for 5 minutes (3% hydrogen peroxide was used to block endogenous peroxidase activity). Antigen retrieval was performed in an epitope retrieval solution (Leica RE-7113) at pH 6 using a pressure cooker (rice programs, IDA Avair 6 L pressure cooker). Double immunostaining of ALP-H-ferritin;ALP-CSE or α -SMA-CSE interaction were performed sequentially with the EnVision FLEX/HRP system. Following the first IHC staining terminating with the EnVision FLEX/HRP detection step, the incubation with the second primary monoclonal antibody was performed. In addition to DAB (brown color), the

chromogen VIP was used to highlight the second IHC reaction in a different color (dark violet). For double staining experiments, methyl-green counterstaining was performed. Samples were incubated with the following primary antibodies: rabbit anti-human ALP antibody (Abcam; ab65834; 1000 ng/mL) and mouse anti-human H-ferritin antibody (Santa Cruz; sc376594; 400 ng/mL). Other IHC stains were performed with the following antibodies: rabbit anti-human TNF- α (Thermo Fisher Scientific; PA5-19810; 400 ng/mL), rabbit anti-human IL1- β (Invitrogen; 17h18116; 400 ng/mL); rabbit anti-human CSE at dilution 1: 600 (Proteintech, 12217-I-AP), rabbit anti-human CBS at dilution 1:600 (Proteintech, 14787-I-AP). Antibody binding was visualized by the Super Sensitive TM One Step Polymer-HRP IHC Detection System. The intensity and distribution of antibodies expression were assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera and Leica Application Suite V3 software, Wetzlar, Germany). ALP and H-ferritin colocalization of the IHC samples were measured by Image J software.

Immunohistochemistry from mouse heart valves

Briefly, tissues were fixed in formaldehyde for one day followed by TRIS buffer and embedded in paraffin wax. Subsequently, slides were deparaffinized in xylene and then rehydrated. For immunohistochemistry, slides were subjected to the peroxidase-blocking reagent. Samples were incubated with the following primary antibodies: anti-CSE antibody at dilution 1:1000 (Proteintech, 12217-I-AP) and anti-SMA antibody at a dilution of 1:1000 (Santa Cruz; sc-32251). Antibody binding was visualized by the Super Sensitive TM One Step Polymer-HRP IHC Detection System. Liquid DAB chromogen (BG-QD630-XAKm BioGenex) was added for samples. The intensity and distribution of antibodies expression were assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera and Leica Application Suite V3 software, Wetzlar, Germany).

LDH cytotoxicity assay

The cytotoxicity of the treatment was assessed by Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed by GraphPad Prism 5.02 software (GraphPad Software Inc., 7825 Fay Avenue, Suite 230 La Jolla, CA 92037). All statistics data are expressed as mean \pm SEM. If data groups passed the normality test and equal variance test, we performed Student's t-test or One Way ANOVA followed by Bonferroni post hoc tests as indicated in figure legends. $P < 0.05$ was considered significant.

Results

Part1

Based on our previous investigations that identified induction of H-ferritin as an inhibitory mechanism against osteoblastic transition of vascular smooth muscle cells (Zarjou, Jeney et al. (2009)), we tested whether elevation of intracellular level of H-ferritin would mimic these effects in VIC. Iron and D3T were utilized to induce the expression of H-ferritin in VIC. Calcification medium alone significantly increased the extracellular calcium content of cultured VIC. Iron and D3T significantly decreased the extracellular calcium in a dose-dependent manner and the expression of osteocalcin, respectively. Apo-ferritin (devoid of iron) also inhibited the extracellular calcium deposition and osteocalcin level of VIC. These observations were supported by Alizarin Red S staining after five days. Next, we investigated whether inhibition of the mineralization pathway of VIC also relies on H-ferritin/ferroxidase. Indeed, we found that upregulation of H-ferritin via iron or D3T or supplementation with apo-ferritin prevents calcium deposition as well as expression of osteocalcin. To further confirm the importance of the inhibitory role of H-ferritin, we transfected VIC with small interfering RNA (siRNA) specific to H-ferritin. In the presence of H-ferritin siRNA, iron or D3T failed to inhibit calcification as reflected by the accumulation of calcium and osteocalcin. To validate the ferroxidase activity's paramount role in inhibition of mineralization, we used another protein that possesses this activity, namely ceruloplasmin. Mineralization of VIC in calcifying condition was inhibited by ceruloplasmin or H-ferritin as reflected by decreased calcium content of extracellular matrix that was also confirmed by Alizarin Red S staining.

Induction of H-ferritin by iron or D3T in cells derived from healthy subjects, as well as administration of recombinant H-ferritin but not mutant H-ferritin²²² lacking ferroxidase activity, prevented mineralization of VIC as reflected by accumulation of calcium and osteocalcin in the extracellular matrix.

Calcification potential of VIC derived from isolated insufficient aortic valve and stenotic aortic heart valves

We investigated whether the origin of VIC (cells derived from the aortic valve with insufficiency (AI) or stenotic valve leaflets (AS)) may influence the calcification potential of these cells. Cells from stenotic valve tissue have greater ability to transdifferentiate into osteoblast-like cells as indicated by the higher level of extracellular calcium deposition and the accumulation of calcium binding protein, osteocalcin. Furthermore, we found that the inhibitory effect of iron and apo-ferritin on mineralization was more pronounced in AI valve cells compared to AS VIC. We also demonstrate that calcium accumulation and osteocalcin levels were higher in AS compared to AI VIC. Interestingly, iron exposure could not inhibit mineralization in the AS stage VIC compared to AI VIC. However, apo-ferritin treatment could inhibit calcification regardless of the degree of valvular disease. This is likely explained by the high concentration of apo-ferritin and may suggest that significantly higher levels of iron may exert similar effects. Osteocalcin, the marker of osteoblastic activity, also corroborated the above findings and its levels suggested that apo-ferritin had a paramount effect on its expression than iron.

Effect of iron, apo-ferritin and D3T in localization of RUNX2 under calcific condition of VIC

Runx2-related transcription factor 2 (RUNX2) is a key determinant of osteoblast activity (Komori, Yagi et al. (1997)) and implicated in the pathogenesis of vascular mineralization (Steitz, Speer et al. (2001)). Therefore, we investigated whether regulation of osteoblastic differentiation of VIC by ferritin occurs via modulation of RUNX2. We examined cultured VIC by immunocytochemistry and by Western blot and found nuclear translocation of RUNX2 in cells maintained in calcification medium. On the contrary, treatment of VIC with iron, apo-ferritin or D3T prevented translocation of RUNX2 into the nucleus. Furthermore, we also tested the location of RUNX2 by immunostaining in VIC derived from AS and its expression from whole protein lysates derived from AS valves as compared to AI valves. We found that RUNX2 is located in AS sample's nuclear region. Conversely, nuclear location of RUNX2 was not detected in AI derived VIC. Moreover, silencing of H-ferritin gene resulted in increased translocation of RUNX2 into the nucleus.

Sox9 in valvular mineralization

Since RUNX2 activity was previously revealed to be antagonized by Sox9 in VIC (Cheng and Genever (2010)), we investigated the location of Sox9 in cultured VIC by immunocytochemistry and Western blot analysis. In cells cultured in growth medium Sox9 existed in the nucleus, while under calcific conditions nuclear Sox9 was barely detectable. In contrast, nuclear location of Sox9 was maintained in cells cultured in calcification medium supplemented with iron, apo-ferritin and D3T. Moreover, we also collected human heart valves of AS exhibiting calcification for comparison to AI without calcification. Western blot analysis showed the nuclear presence of Sox9 in AI valve samples. On the contrary, nuclear Sox9 in AS valves was below the detection level.

Regulation of VIC phosphate uptake by iron, apo-ferritin, D3T and H-ferritin

Nuclear translocation of RUNX2 was shown to be regulated by intracellular phosphate level (Jono, McKee et al. (2000); Fujita, Izumo et al. (2001)). To test whether H-ferritin affects intracellular phosphate levels in VIC maintained in normal condition or calcification medium we treated VIC with iron, apo-ferritin or D3T. Significantly lower intracellular phosphate levels were found in cells exposed to iron, apo-ferritin or D3T compared to cells growing in calcific condition alone. Importantly, lysosomal phosphate level was also decreased to the control level in response to iron exposure in cells cultured in calcification medium. Accordingly, expression of Pit1 (the membrane-associated channels responsible for the phosphate transport) was attenuated after treatment of VIC with iron, apo-ferritin or D3T. In addition, Pit2 expression was diminished in response to ferritin induction. Exogenous H-ferritin also decreased Pit1 protein expression in VIC cells derived from AS tissues.

H-ferritin and inorganic phosphate association in the lysosome under calcific conditions

Apo-ferritin has a hollow internal cavity which can accommodate iron in a ferric oxyhydrophosphate complex (de Silva, Guo et al. (1993)). Through the action of ferroxidase, it is capable of storing iron and phosphate (2250 and 380 atoms, respectively). The amount of

iron and phosphate within the core of ferritin are not related to different subunit composition. Double immunostaining against LAMP1 (lysosome marker) and H-ferritin revealed maximal co-localization. Furthermore, inside the fused lysosomes (shown by arrows) a significant accumulation of ferritin is evident. To confirm this finding, we isolated lysosomes from cultured and treated VIC, and tested for H-ferritin/LAMP1 in different conditions: in the growth medium; calcification medium alone, or supplemented with iron. In iron-treated cells, we found a markedly increased H-ferritin level inside the lysosome, which is demonstrated by the co-localization rate of H-ferritin and LAMP1.

Role of extracellular pyrophosphate in the inhibition of valvular calcification

Pyrophosphate (PPi) is also a key inhibitor of mineralization through binding to nascent hydroxyapatite crystals (Fleisch and Bisaz (1962); Terkeltaub (2001)). Therefore, we analyzed the changes of the level of pyrophosphate compared with the calcification medium. Iron/apo-ferritin/D3T treatment significantly increased the level of pyrophosphate contrary to cultured cells in calcification medium. Since, the major pyrophosphate generating ectoenzyme is the ENPP2 we examined whether ENPP2 expression correlated with PPi level. Similarly to PPi, a decrease in ENPP2 expression was observed under calcification condition. Intriguingly, elevated expression of ENPP2 and higher PPi level were found in cells exposed to iron, apo-ferritin and D3T as compared to those cultured in calcification medium alone. Moreover, treatment of cells with H-ferritin also increased ENPP2 protein level in VIC derived from AS tissues. To confirm the importance of H-ferritin in controlling the pyrophosphate generation, we transfected VIC with small interfering RNA (siRNA) specific to H-ferritin. In the presence of H-ferritin siRNA, iron and also H-ferritin failed to enhance PPi level. Expression of Ankyrin G1 protein was not altered by ferritin.

H-ferritin and ALP expression in stenotic aortic valve

The above findings prompted us to examine whether the expression of ferritin is altered in VIC in CAVD. Therefore, Western blot analysis was performed for H-ferritin from patients' tissue lysate samples of AS and AI. Significantly higher level of H-ferritin was present in AS when compared to AI valves. To localize H-ferritin in CAVD dual immunohistochemistry investigation (ALP and H-ferritin) was carried out. ALP⁺ and H-ferritin⁺ cells were present in the affected valve from the endothelial surface to the calcific core. In the distant surface area more H-ferritin and less ALP staining was observed in cells, the ratio was 2.3:1 ±0.41, respectively. Expression of ALP and H-ferritin progressively increased toward the core and the ratio of H-ferritin to ALP decreased to 0.6 ±0.15 at the zone close to the calcific core of AS. In AI valves the staining for ALP and H-ferritin was even and moderate, the ratio of H-ferritin to ALP was 2.2:1±0.65.

Association between increased level of H-ferritin and inflammation in AS valves

Our previous findings highlight the question as to whether the elevated H-ferritin expression is due to iron exposure. To answer this question, we stained AS valves with Prussian blue (specific iron staining) and iron was absent in the highly calcified region. Torti et al. previously demonstrated that inflammatory cytokines upregulate the expression of H-ferritin in the absence of iron (Torti, Kwak et al. (1988); Tsuji, Miller et al. (1991)). Moreover, inflammation is implicated in the progression of CAVD (Rajamannan, Evans et al. (2011)). Therefore, we performed immunohistochemistry and Western blot analysis for inflammatory markers, TNF- α and IL1- β on human healthy and AS tissue. Importantly, elevated H-ferritin expression corroborated the increased protein level of TNF- α and IL1- β in AS tissues as compared to healthy aortic valves.

Function of H-ferritin in the development of inflammation of heart valve

To test whether ferritin/ferroxidase system has the potential to act against inflammation in CAVD, we treated VIC cells derived from AS tissue with H-ferritin. We found that H-ferritin exposure inhibited the expression of TNF- α and IL1- β .

Part2

H₂S function in the calcification of valvular interstitial cells *in vitro*

The potential of different H₂S donors for inhibition of calcification of valvular interstitial cells isolated from human aortic valves were investigated. Cells were cultured in calcific condition containing 2.5 mmol/L inorganic phosphate and 1.8 mmol/L calcium-chloride. VIC were treated with H₂S: the simple sulfide salts NaSH and Na₂S that instantaneously generates H₂S via pH-dependent salt dissociation and novel slow-release sulfide donors (AP67 and AP72) and the more commonly used donor GYY4137 (synthesized in-house). As expected, the osteoblastic transition of VIC occurred in calcifying environment, which is reflected by the extracellular accumulation of calcium, and the increased expression of osteocalcin and ALP. Particularly, all H₂S donors decreased calcium deposition in a dose-responsive fashion. NaSH reached the maximum inhibition at 150 μmol/L, Na₂S and GYY4137 attenuated calcification at 100 μmol/L, while AP67 suppressed calcification at 50 μmol/L concentration compared to calcification medium without H₂S supplementation. Among the H₂S donors, AP72 fully abrogated calcium deposition in the extracellular matrix of VIC at 20 μmol/L concentration. Moreover, osteocalcin accumulation and expression of ALP in VIC along with calcium deposition were also prevented by AP72. Similarly, other fast (NaSH, Na₂S) and slow (GYY4137, AP67) sulfide releasing molecules significantly attenuated the osteocalcin secretion. ALP and Alizarin Red S staining showed pronounced osteoblastic transformation of VIC in the calcific environment and this effect was prevented by AP72. AP72 did not exhibit any cytotoxic effects on VIC at the applied dose. We observed a “U” shape curve in the inhibition of mineralization. Use of H₂S donors at concentrations in excess of that stated above resulted in a concentration-dependent decline in protection. Then, we selected from these studies the most effective H₂S donor (AP72) for further investigation to explore the mechanism by which H₂S regulates the calcification processes.

Phenol was shown to capture H₂S (Huang, Zhang et al. (2017)), so we tested if AP72 affects calcification at lower concentrations in phenol red-free medium as compared to calcification medium with phenol red. In phenol red free condition, AP72 significantly inhibited calcification of VIC at concentration of 2.5 nmol/L to 5 μmol/L. Respectively, osteocalcin accumulation was prevented and phosphate uptake was also decreased by AP72 in condition without phenol red. Alkaline phosphatase and Alizarin Red S staining indicated the inhibitory effect of AP72 at a concentration of 2 μmol/L. In phenol red containing medium AP72 exhibited inhibitory effect on calcification in VIC at one order magnitude higher concentration. At the most effective concentrations of the sulfide donor molecules did not caused cytotoxicity in VIC.

Association of AP72 source and RUNX2 localization under calcification of VIC

RUNX2 is the key transcription factor of the early osteoblastic differentiation of vascular smooth muscle cells and VIC. We were curious whether cultured VIC in calcification medium affected by AP72 treatment how determined the localization of RUNX2. Immunofluorescence staining demonstrated that RUNX2 was located in the cytoplasm of VIC

cultured in growth medium (control medium). Phosphate exposure of VIC triggered the translocation of RUNX2 from the cytoplasm to the nucleus. AP72 prevented the appearance of RUNX2 in the nucleus of VIC maintained in calcification medium. To support our immunofluorescence observation, we examined RUNX2 translocation by Western blot analysis using from cytoplasm and nucleus fractions of VIC. We found that RUNX2 appeared in the nucleus in response to calcification medium while its level was decreased in the cytoplasmic fraction. Moreover, AP72 treatment prevented the translocation of RUNX2 into the nucleus of VIC exposed to phosphate.

We also examined the nuclear location of the RUNX2 in VIC derived from human valves with visible calcification (AS) and without calcification (AI). Confocal microscopy and Western blot analysis revealed that RUNX2 was mainly located in the nucleus of VIC derived from AS human valve tissue. On the contrary, RUNX2 was detected in the cytoplasm of VIC derived from AI tissue. Importantly, exposure of cells to 200 $\mu\text{mol/L}$ of AP72 did not restrain the nuclear translocation of RUNX2 from the cytoplasm to the nucleus.

PPi production changes in the absence/presence of H₂S

PPi is a well-known anti-calcification molecule, regulated by ENPP2 and ANK1. Accordingly, we tested whether H₂S may control PPi production by regulating the expression of ENPP2 and ANK1 in VIC. Expression of ENPP2 was decreased in VIC cultured in calcification medium as compared to control. Exposure of cells with AP72 abolished such an effect. ENPP expression was significantly elevated above the level observed in control cells. Furthermore, the level of ANK1 protein in VIC maintained under calcifying conditions did not change compared to cells grow in growth medium. In contrast, AP72 induced ANK1 expression at both mRNA and protein levels in VIC. Consequently, the amount of generated PPi decreased in cells grown under calcifying conditions. Using AP72 supplementation PPi generation increased significantly in VIC compared to both cells cultured in control growth medium or calcification medium without any treatment. GYY4137 and AP67 were also tested for affecting PPi level in VIC. These H₂S donors enhanced the PPi level lesser than AP72. The fast sulfide releasing molecules (NaSH and Na₂S) were able to enhance the PPi level only to the baseline. Additionally, our measurements in human heart valve tissue samples indicated significantly lower ENPP2 protein levels and lower PPi content in AS valve specimens as compared to AI valves samples.

CSE and CBS level changes in VIC calcification

To investigate potential anti-calcification effects of endogenously produced H₂S we silenced CSE production in VIC using small interfering RNA (siRNA). We found that CSE silencing did not significantly enhance the calcium accumulation under calcifying conditions. Intriguingly, we observed that CSE silencing increased the expression of CBS, which is another pyridoxal 5'-phosphate (PLP or vitamin B6) dependent transsulfuration enzyme involved in endogenous sulfide production. Thus, we performed double silencing against CSE and CBS to test whether calcium deposition in extracellular matrix was affected. We observed an increase in mineralization after CSE and CBS were concomitantly silenced in VIC suggesting that transsulfuration pathways are likely to control calcification. Next, we

performed experiments with pharmacological inhibitors of CSE (PPG) together with CBS (AOAA) in calcifying condition in VIC. We found that pharmacological inhibition of CSE with CBS increased the amount of extracellular calcium deposition. Moreover, we tested all three synthetic inhibitors for CSE (PPG), CBS (AOAA) and 3-MST (KGA) alone, or in combination. Calcium depositions of CSE/CBS double silenced VIC are shown inhibition of CSE and CBS altered calcification in VIC. Furthermore, AOAA+PPG; AOAA+LKGS and PPG+LKGS pharmacological inhibitors significantly reduced the H₂S production in VIC. To our surprise, nuclear translocation of RUNX2 was not influenced by double silencing of CSE and CBS in calcification medium. Moreover, we monitored the progression of calcification in extracellular matrix on the first and third days. On the first day we did not find significant alteration in the calcium content of VIC maintained in calcifying condition compared to control. In contrast, we detected a significantly increased extracellular calcium content in VIC silenced with CSE/CBS siRNA. Mineralization was more robust in the double silenced VIC by day three. H₂S production was lowered in calcifying condition compared to cells cultured in growth media, and that was further decreased by double silencing for CSE and CBS. Finally, we examined the expression of 3-MST in VIC treated with CSE/CBS siRNA. We found that siRNA specific to CSE and CBS decreased 3-MST protein level in VIC.

Hydrogen sulfide interaction by the functions of phosphate channels inhibits phosphate uptake

Cellular phosphate uptake is a key event in the process of mineralization; therefore next we measured intracellular phosphate levels in VIC and observed a significant elevation in cells cultured in calcification medium compared to cells kept in control medium. Exposure of VIC to AP72 diminished this increase in phosphate content to the level observed in control cells. In order to explain the inhibition of phosphate uptake, we measured the expression of phosphate channels (Pit1; Pit2) in VIC maintained in calcification medium in the presence or absence of AP72. We found that AP72 did not affect the expression of Pit1 and Pit2 channels. Thus, we hypothesize that sulfide induced posttranslational modification of these channels might affect phosphate uptake. Measurements to support or disprove this hypothesis are underway.

H₂S generation and CSE expression in human aortic valves

In the human body, CSE was claimed to be one of the main endogenous hydrogen sulfide producing proteins. By Western blot analyzes we investigated the expression of CSE in tissue lysates of human AS and AI valves. We found higher expression of CSE in AS valves with massive calcification as compared to AI valves known to lack calcification. In contrast, sulfide levels that can be precipitated by Zn²⁺ under alkaline conditions from tissue lysates of valves were markedly, and significantly lower in calcified AS specimens compared to not calcified AI specimens. Next, we performed dual immunohistochemistry analyses (CSE-SMA and CSE-ALP) on human AI and AS valves to localize CSE. Less SMA+ and more CSE+ cells were present in calcified AS tissue than in AI tissue. ALP-CSE double staining revealed the appearance of ALP+ cells expressing high levels of CSE protein in AS valve samples, while ALP+ cells were not detected in AI valve.

The potential of osteoblastic differentiation was dependent upon the origin of VIC. Under the same calcifying condition VIC derived from AS exhibited earlier mineralization than AI. The higher CSE level found in AI was accompanied by delayed calcification.

Role of H₂S in valvular calcification of apolipoprotein E deficient mice

Von Kossa staining demonstrates that calcific nodules appeared in aortic heart valves of ApoE^{-/-} mouse, which were kept on a high-fat diet as opposed to ApoE^{-/-} mouse on standard chow diet, where no valvular calcification was observed. ApoE^{-/-} mouse on a high-fat diet exhibited an expansion of extracellular matrix in aortic valve compared to those received standard diet. To demonstrate the benefit of H₂S in vivo, we administered AP72 intraperitoneally (266 μmol/kg body weight) and assessed valvular calcification in ApoE^{-/-} mice on a high-fat diet. AP72 significantly inhibited the development of calcific nodules in aortic valves and decreased the expansion of extracellular matrix. Similarly, to human aortic stenosis the amount of CSE⁺ cells in calcified aortic valves of mice fed with the high-fat diet was increased as compared to in aortic valves of mice on a regular diet. Consequently, the administration of exogenous H₂S lowered the total measured expression of CSE in aortic valves of mice fed with a high-fat diet. Alpha-SMA staining demonstrated that AP72 maintained the phenotype of valvular interstitial cells in the aortic valve.

Summary

The studies that are presented in this thesis focused on two different but very important molecules in the development of CAVD. In Part 1 we focused on our previous findings to understand the molecular mechanism of the inhibitory role ferritin/ferroxidase of the high phosphate induced development of CAVD. Our results suggested that ferritin/ferroxidase is not just a potent inhibitor of the cardiovascular mineralization; it is inducible exogenously by different drugs such as D3T. In Part 2 we sought to investigate the role of H₂S in valvular mineralization induced by high phosphate. We provide strong evidence that H₂S level changes during the progression of CAVD caused by the possible structural deficiency (sulphydration of Serin residues) (Bibli, Hu et al. (2019)) of H₂S producing enzyme CSE. Moreover, the lack of H₂S could be replaced by H₂S releasing donor molecules and perhaps be possible a therapeutic approach in the future (Sikura, Potor et al. (2019)). These studies were the first to provide two different mechanistic insights in the same clinical condition (CAVD) and offer novel pharmaceutical avenues that may lead to innovative therapeutic modalities.

The relationship between iron homeostasis/H₂S balance and valvular calcification has never been explored and their role in mineralization is poorly understood. Although most patients with CKD or CAVD, may require renal or heart valve (or both) replacement, complications in these cases include cardiovascular calcification, deranged iron homeostasis and deficiency of H₂S. In patient with CKD iron accumulation can be observed in reticuloendothelial cells that are accompanied by higher levels of ferritin in plasma. This increase results from the iron sequestration of reticuloendothelial cells and its availability to the other cells is significantly lower. This mechanism results in depletion of intracellular ferritin and subsequent anemia of chronic, inflammatory disease. In the first part of our observations we suggest that the derangement of iron metabolism possibly facilitated by high phosphate induced valvular calcification may be controlled (induction of ferroxidase) by oral administration of D3T to prevent mineralization of the cardiovascular system. In the second part, we propose that CAVD is a condition of disturbed H₂S bioavailability. The observed deficiency in the generation of H₂S in human calcified stenotic valves is accompanied with elevated expression of CSE. The development of heart disease is manifested by the disorder of H₂S production as indicated by the lower levels of plasma H₂S in patients with coronary heart disease (Jiang, Wu et al. (2005);(Shen, Shen et al. 2015)). The replacement of the absence H₂S is achievable by a reaction between Lawesson's reagent and morpholine resulting in a new generation slow H₂S release compound (GYY4137). Protonation reaction of GYY4137 results more stable H₂S releasing compounds such as AP67 and AP72. Although the link between valvular calcification and H₂S homeostasis is not characterized, our results demonstrate a therapeutic approach against CAVD from a new perspective. In conclusion, improved insight and better understanding of the pathogenesis of CAVD may help develop effective strategies to prevent or decrease iron and/or H₂S deficiency manifested clinical cardiovascular conditions.

Novel findings

1. H-ferritin induces by iron and 3H-1, 2-dithiole-3-thione inhibits mineralization and osteoblastic differentiation of human valvular interstitial cells.
2. H-ferritin enhances nuclear localization of transcription factor Sox9, and as a reciprocal effect it reduces nuclear accumulation of RUNX2.
3. H-ferritin inhibits cellular phosphate uptake and lysosomal phosphate accumulation via decreasing the expression of phosphate channels and localization of lysosomal H-ferritin with high phosphate binding capacity, as well as enhances pyrophosphate generation via up-regulating ENPP2.
4. Histological analysis of stenotic aortic valve revealed high expression of H-ferritin and its relative dominance over ALP in the intact non-calcified regions.
5. H-ferritin decreases the expression of inflammatory markers (TNF- α ; IL1- β) in VIC derived from human AS valve.
6. CSE and CBS derived H₂S and H₂S releasing donors inhibit mineralization of aortic valve.
7. Anti-calcification function occurs via inhibiting phosphate uptake, preventing nuclear translocation of RUNX2, increasing pyrophosphate level.
8. H₂S releasing donors and CSE/CBS derived H₂S has therapeutic potentials in calcific aortic valve disease.

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List of publications related to the dissertation

1. **Sikura, K. É.**, Potor, L., Szerafin, T., Oros, M., Nagy, P., Méhes, G., Hendrik, Z., Zarjou, A., Agarwal, A., Posta, N., Torregrossa, R., Whiteman, M., Fürtös, I., Balla, G., Balla, J.: Hydrogen sulfide abrogates heart valve calcification: implications for calcific aortic valve disease.
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3. Becs, G., Zarjou, A., Agarwal, A., **Sikura, K. É.**, Becs, Á., Nyitrai, M., Balogh, E., Bányai, E., Eaton, J. W., Arosio, P., Poli, M., Jeney, V., Balla, J., Balla, G.: Pharmacological induction of ferritin prevents osteoblastic transformation of smooth muscle cells.
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Abstracts and presentations

1. Magyar Szabadgyök-Kutató Társaság VII. Kongresszusa, 2013; Debrecen, Hungary. *Kovács Katalin Éva, Jeney Viktória, Szerafin Tamás, Balla József, Humán szívbíbillentyűk kalcifikációjának gátlása a ferritin rendszeren keresztül.*
2. Magyar Szívsebészeti Társaság XXII. Kongresszusa; 2015.; Szeged, Hungary. *Kovács Katalin Éva, Szerafin Tamás, Hendrik Zoltán, Balla György, Balla József, Humán valvuláris interstitialis sejtek mineralizációs folyamatai.*
3. Scientific Congress of the Society of Hungarian Cardiologists; May, 2015; Balatonfüred, Hungary. *Katalin Éva Sikura, Tamás Szerafin, Zoltán Hendrik, György Balla, József Balla, Mineralization process of human heart valve interstitial cells.*
4. 9th International Conference on Heme Oxygenase. November, 2016; Prague, Czech Republic. *Katalin Éva Sikura, Tamás Szerafin, Abolfazl Zarjou, Anupam Agarwal, Gerardo Alvarado, Zoltán Hendrik, György Balla, József Balla, Targeting H-ferritin to mitigate valvular mineralization.*
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7. Magyar Szívsebészeti Társaság XXV. Kongresszusa. 2018. Budapest, Hungary. *Katalin Éva Sikura, László Potor, Tamás Szerafin, Abolfazl Zarjou, Anupam Agarwal, Paolo Arosio, Maura Poli, Zoltán Hendrik, Gábor Méhes, Melinda Oros, Niké Posta, Livia Beke, Ibolya Fürtös, György Balla, József Balla, HYDROGEN SULFIDE ABROGATES HEART VALVE CALCIFICATION: IMPLICATIONS FOR CALCIFIC AORTIC VALVE DISEASE.*
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9. Magyar Osteológiai és Osteoarthrológiai Társaság Osteológiai Kongresszusa, 2019. Balatonfüred, Hungary. *Katalin Éva Sikura, László Potor, Tamás Szerafin, Abolfazl Zarjou, Anupam Agarwal, Paolo Arosio, Maura Poli, Zoltán Hendrik, Gábor Méhes, Melinda Oros, Niké Posta, Livia Beke, Ibolya Fürtös, György Balla, József Balla, POTENTIAL ROLE OF H-FERRITIN IN MITIGATING VALVULAR MINERALIZATION.*
10. Magyar Szabadgyök-Kutató Társaság X. Kongresszusa, 2019. Szeged, Hungary. *Katalin Éva Sikura, László Potor, Tamás Szerafin, Abolfazl Zarjou, Anupam Agarwal, Paolo Arosio, Maura Poli, Zoltán Hendrik, Gábor Méhes, Melinda Oros, Niké Posta, Livia Beke, Ibolya Fürtös, György Balla, József Balla, HYDROGEN SULFIDE ABROGATES HEART VALVE CALCIFICATION: IMPLICATIONS FOR CALCIFIC AORTIC VALVE DISEASE.*

Membership in National and International Societies

- Hungarian Society of Cardiology (Magyar Kardiológusok Társasága (MKT))
- Hungarian Society of Cardiac Surgery (Magyar Szívsebészeti Társaság)
- Magyar Szabadgyök-Kutató Társaság (MSZKT)
- European Society of Cardiology