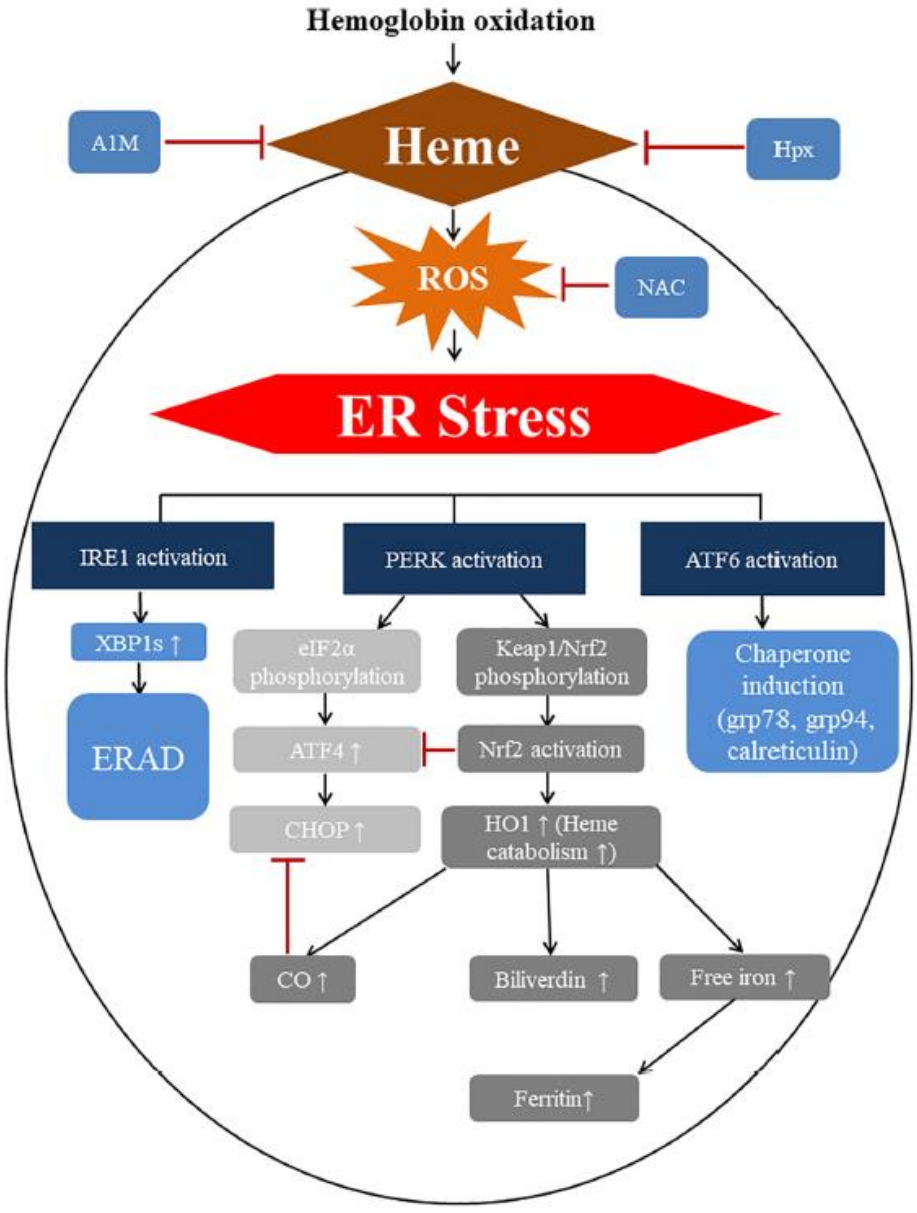
Project closing report (final report)

Title: Athero-heme and vascular calcification (NKFI-ID: 112333)

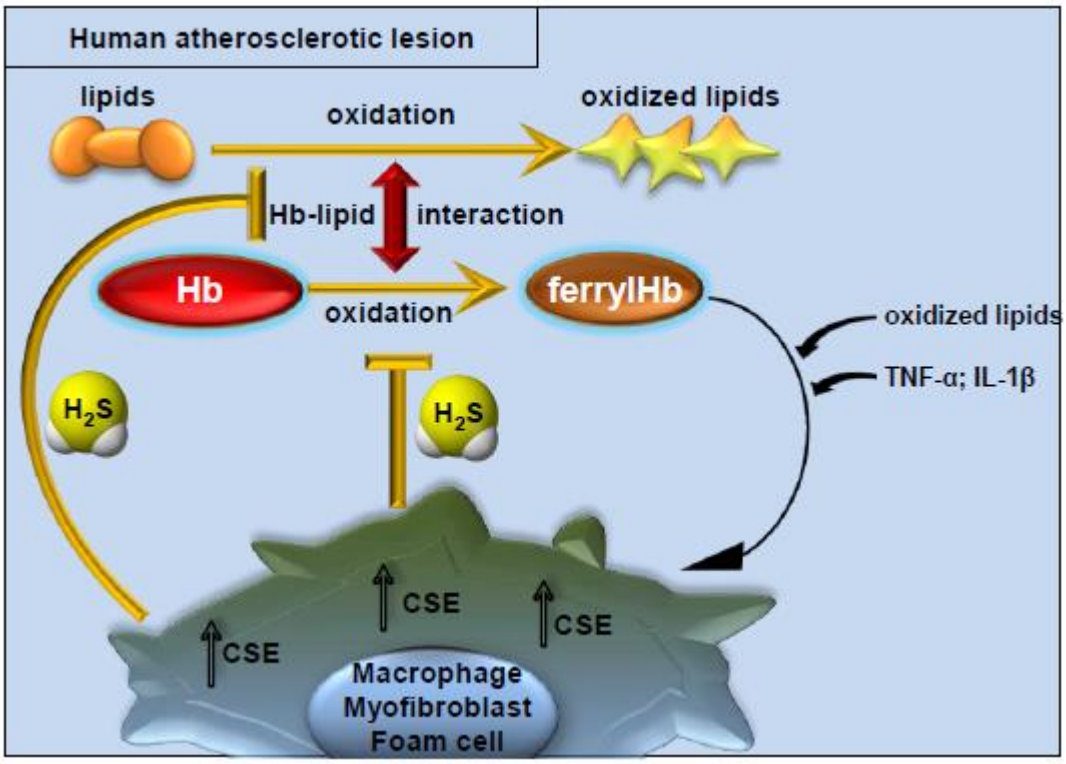
Since our laboratory previously revealed that in the progression of atherosclerosis exposure of arteries by heme derived from hemoglobin occur we examined their fate within the lesion.

Accumulation of damaged or misfolded proteins resulted from oxidative protein modification induces endoplasmic reticulum (ER) stress by activating the pathways of unfolded protein response (UPR). In pathologic hemolytic conditions, extracellular free hemoglobin is submitted to rapid oxidation causing heme release. It has been shown that resident cells of atherosclerotic lesions, after intraplaque hemorrhage, are exposed to heme leading to oxidative injury. Therefore, we raised the question whether heme can also provoke ER stress. Vascular smooth muscle cells are one of the key players of atherogenesis; thus, human aortic smooth muscle cells were selected as a model cell to reveal the possible link between ER stress and heme stress. Using Western blotting, quantitative polymerase chain reaction and immunocytochemistry, we quantitated the protein and mRNA levels of markers involved in ER stress. These were: phosphorylated eIF2α, Activating transcription factor 4 (ATF4), DNA-damage-inducible transcript 3 (also known as C/EBP homology protein, termed CHOP), X-box binding protein-1 (XBP1), Activating transcription factor-6 (ATF6), GRP78 (glucose-regulated protein, 78kDa) and a heme responsive gene (heme oxygenase-1, HO1 and an iron responsive protein, ferritin). Additional immunohistochemistry was performed on human carotid artery specimens from patients who had undergone carotid endarterectomies. We demonstrate that heme increases the phosphorylation of eiF2α [Ser51] in human aortic smooth muscle cells and, subsequently, the expression of ATF4. Heme also enhances the splicing of XBP1 and the proteolytic cleavage of ATF6. Consequently, there is up-regulation of target genes increasing both mRNA and protein levels of CHOP and GRP78. The activation of these pathways for

canonical ER stress occurs in a time and dose-dependent manner. When the heme binding proteins, alpha-1-microglobulin (A1M) and hemopexin (Hpx) are present in cell media, the ER stress provoked by heme is inhibited. Consistent with these findings, elevated expression of the ER stress marker GRP78 and CHOP were observed in vascular smooth muscle cells of complicated lesions compared to either atheromas or healthy arteries. We concluded that heme triggers ER stress in a time and dose-dependent manner in vascular smooth muscle cells. A1M and Hpx hamper heme-induced ER stress, suggesting a potential therapeutic approach to reverse such a deleterious effect. This work was published in Frontiers in Physiology in 2018.

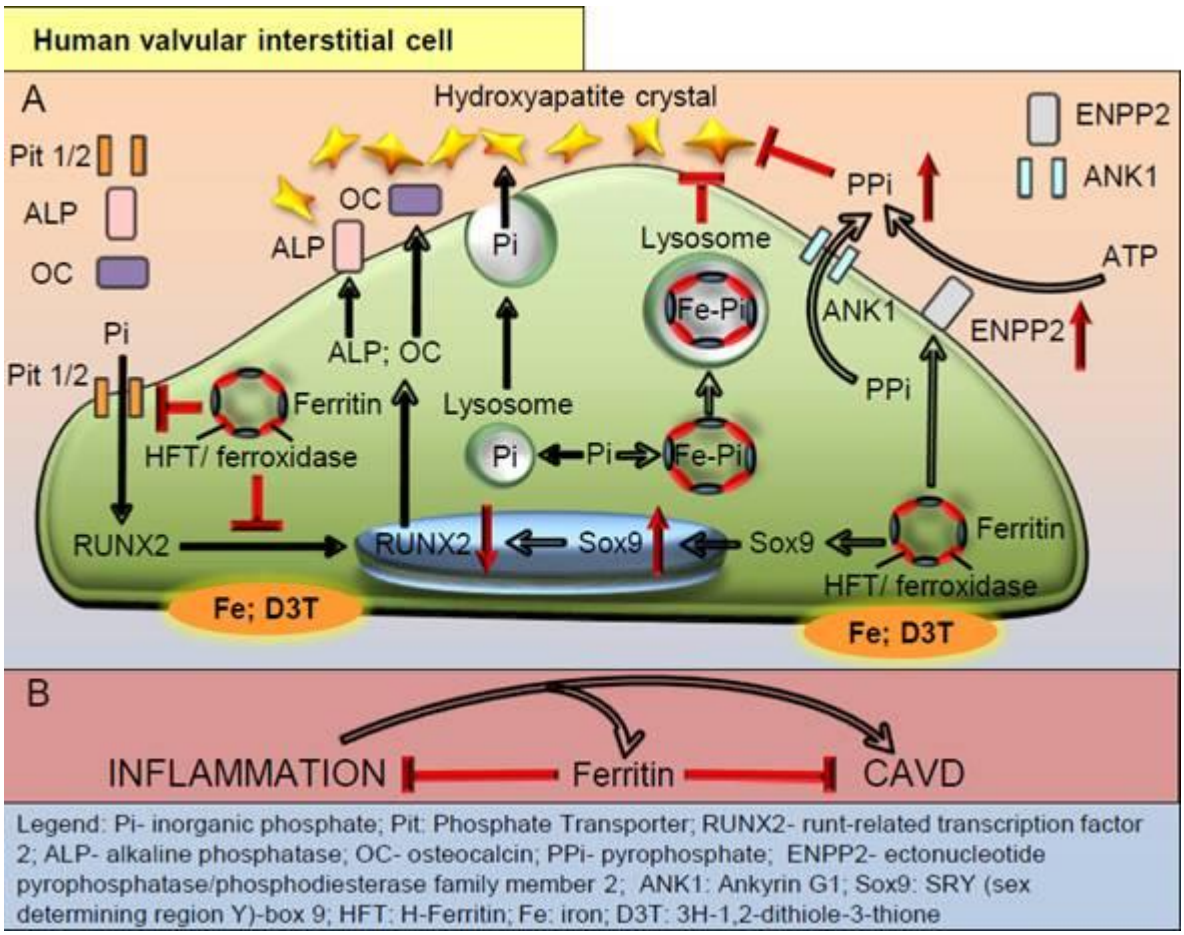


Inside the lesion hemoglobin (Hb) is oxidized to ferri- and ferrylHb which exhibit pro-oxidant and pro-inflammatory activities. Cystathione-gamma lyase (CSE)-derived H2S has been suggested to possess various anti-atherogenic actions. Expression of CSE was upregulated predominantly in macrophages, foam cells and myofibroblasts of human atherosclerotic lesions derived from carotid artery specimens of patients. Similar pattern was observed in aortic lesions of apolipoprotein E deficient mice on high-fat diet. We identified several triggers for inducing CSE expression in macrophages and vascular smooth muscle cells including heme, ferrylHb, plaque lipids, oxidized low-density lipoprotein, tumor necrosis factor-α and interleukin-1β. In the interplay between hemoglobin and atheroma lipids, H2S significantly mitigated oxidation of Hb preventing the formation of ferrylHb derivatives, therefore providing a novel function as a heme-redox-intermediate-scavenging antioxidant. By inhibiting Hb-lipid interactions sulfide lowered oxidized Hb-mediated induction of adhesion molecules in endothelium and disruption of endothelial integrity. Exogenous H2S inhibited heme and Hb-mediated lipid oxidation of human atheroma derived lipid and human complicated lesion. Our study suggests that the CSE/H2S system represents an atheroprotective pathway for removing or limiting the formation of oxidized Hb and lipid derivatives. These findings were published in Journal of Oxidative Medicine and Cellular Longevity in 2018.



Because heme stress provokes up-regulation of ferritin we examined whether H-ferritin affects remodeling of the vasculature. Calcific aortic valve disease (CAVD) is a prominent finding in elderly and patients with chronic kidney disease, therefore we investigated the potential role of iron metabolism in the pathogenesis of CAVD. Cultured valvular interstitial cells (VIC) of stenotic aortic valve with calcification from patients undergoing valve replacement exhibited significant susceptibility to mineralization/osteoblastic transdifferentiation in response to phosphate. This process was abrogated by iron via induction of H-ferritin as reflected by lowering ALP and osteocalcin secretion, and preventing extracellular calcium deposition. Cellular phosphate uptake and accumulation of lysosomal phosphate were decreased. Accordingly, expression of phosphate transporters Pit1 and Pit2 were repressed. Translocation of ferritin into lysosomes occurred with high phosphate binding capacity. Importantly, ferritin reduced nuclear accumulation of RUNX2, and as a reciprocal

effect it enhanced nuclear localization of transcription factor Sox9. Pyrophosphate generation was also increased via up-regulation of ENPP2. 3H-1, 2-dithiole-3-thione (D3T) mimicked these beneficial effects in VIC via induction of H-ferritin. Ferroxidase activity of H-ferritin was essential for this function, as ceruloplasmin exhibited similar inhibitory functions. Histological analysis of stenotic aortic valve revealed high expression of H-ferritin without iron accumulation and its relative dominance over ALP in non-calcified regions. Increased expression of H-ferritin accompanied by elevation of TNF-α and IL1-β levels, inducers of Hferritin,corroborates the essential role of ferritin/ferroxidase via attenuating inflammation in CAVD. Our results indicate that H-ferritin is a stratagem in mitigating valvular mineralization/osteoblastic differentiation. Utilization of D3T to induce ferritin expression may prove a novel therapeutic potential in valvular mineralization. These studies are published in ATVB, in 2019.

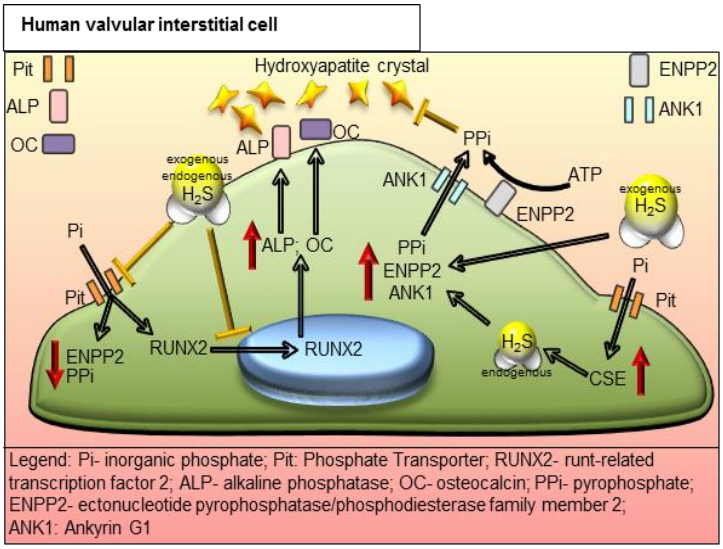


Legend: Pi- inorganic phosphate; Pit: Phosphate Transporter; RUNX2- runt-related transcription factor 2; ALP- alkaline phosphatase; OC- osteocalcin; PPi- pyrophosphate; ENPP2- ectonucleotide pyrophosphatase/phosphodiesterase family member 2; ANK1: Ankyrin G1; Sox9: SRY (sex determining region Y)-box 9; HFT: H-Ferritin; Fe: iron; D3T: 3H-1,2-dithiole-3-thione

Considering that calcification is a major life-threatening complication of atherosclerosis, we also investigated the osteoblastic trans-differentiation of smooth muscle cells (SMC). Human aortic SMC were cultured using β-glycerophosphate with activated vitamin D_3, or inorganic phosphate with calcium, and induction of alkaline phosphatase (ALP) and osteocalcin as well as accumulation of calcium were used to monitor osteoblastic transformation. In addition, to examine the role of vitamin D_3 analogues, plasma samples from patients on haemodialysis who had received calcitriol or paricalcitol were tested for their tendency to induce calcification of SMC. Addition of exogenous ferritin mitigates the transformation of SMC into osteoblast-like cells. Importantly, pharmacological induction of heavy chain ferritin by 3H-1,2-Dithiole-3-thione was able to inhibit the SMC transition into osteoblast-like cells and calcification of extracellular matrix. Plasma samples collected from patients after the administration of activated vitamin D_3 caused significantly increased ALP activity in SMC compared to the samples drawn prior to activated vitamin D_3 and here, again induction of ferritin diminished the osteoblastic transformation. Our data suggests that pharmacological induction of ferritin prevents osteoblastic transformation of SMC. Hence, utilization of such agents that will cause enhanced ferritin synthesis may have important clinical applications in prevention of vascular calcification. Our observation was published in Journal of Cellular and Molecular Medicine in 2015.

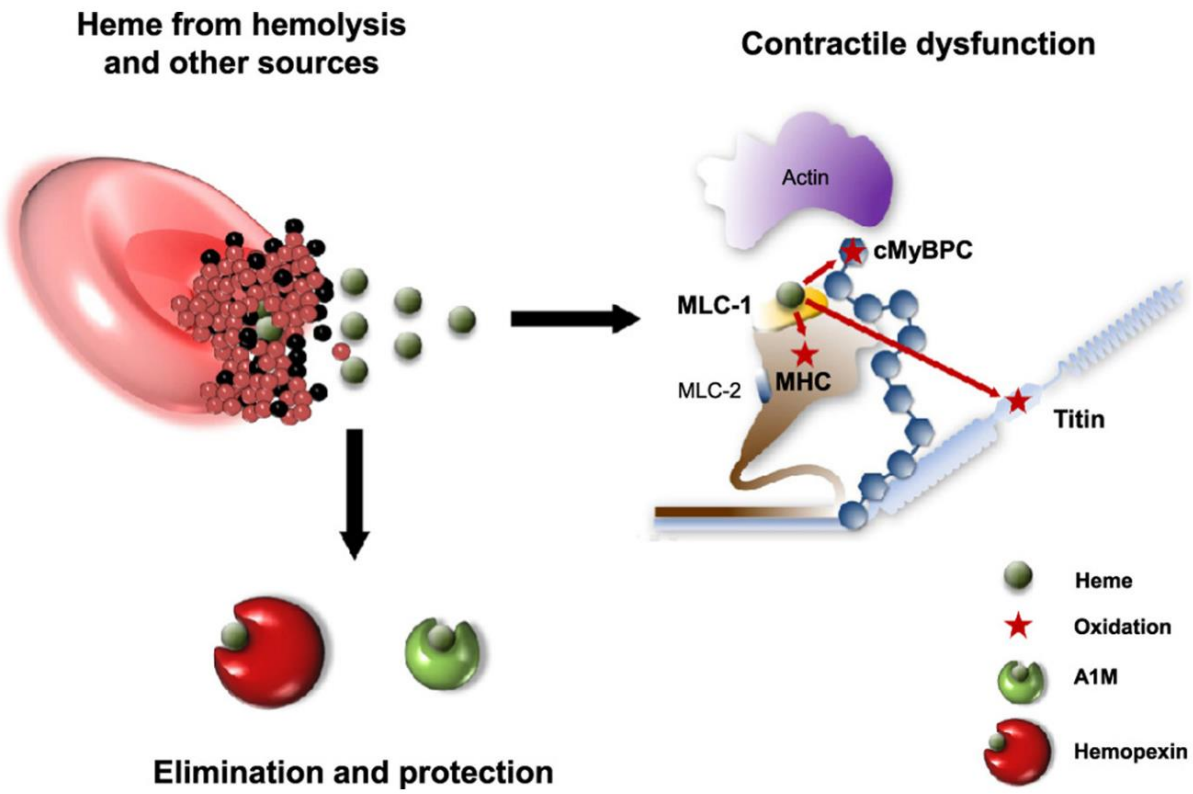
Hydrogen sulfide (H_2S) has been previously suggested by us to possess various anti-calcific actions. We now aimed to investigate H_2S as a potential therapeutic in valvular calcification and to identify its targets in the pathogenesis. In human VIC H_2S treatment employing donors (NaSH, Na_2S, GYY4137, AP67, AP72) inhibited mineralization/osteoblastic transdifferentiation in a dose-responsive manner in response to phosphate. Accumulation of

calcium in the extracellular matrix and expression of osteocalcin and alkaline phosphatase was abrogated. Nuclear translocation of the RUNX2 did not occur, and phosphate uptake was lowered. We also found that pyrophosphate generation was increased via up-regulating ENPP2 and ANK1. Lowering endogenous production of H_2S by concomitant silencing of CSE and CBS favored VIC calcification. IHC and western blot analysis of human specimens revealed higher levels of CSE expression in aorta stenosis valves with calcification (AS) compared to valves of aorta insufficiency (AI). In contrast, tissue H_2S generation was lower in AS valves compared to AI valves. We observed an inhibition of valvular calcification by H_2S in $ApoE^{-/-}$ mouse on high-fat diet. We concluded that the CSE-CBS/H_2S system exhibits an anti-calcification function in heart valves providing a novel therapeutic approach to prevent hardening of valves. These studies have been resubmitted to British Journal of Pharmacology as we were encouraged to do that.



Considering that intracellular free heme predisposes to oxidant-mediated tissue damage, we hypothesized that free heme also causes alterations in myocardial contractility via disturbed structure and/or regulation of the contractile proteins. Isometric force production and its Ca^{2+}-

sensitivity (pCa_{50}) were monitored in permeabilized human ventricular cardiomyocytes. Heme exposure altered cardiomyocyte morphology and evoked robust decreases in Ca^{2+}-activated maximal active force (F_o) while increasing Ca^{2+}-independent passive force ($F_{passive}$). Heme treatments, either alone or in combination with H_2O_2, did not affect pCa_{50}. The increase in $F_{passive}$ started at 3 μM heme exposure and could be partially reversed by the antioxidant dithiothreitol. Protein sulfhydryl (SH) groups of thick myofilament content decreased and sulfenic acid formation increased after treatment with heme. Partial restoration in the SH group content was observed in a protein running at 140 kDa after treatment with dithiothreitol, but not in other proteins, such as filamin C, myosin heavy chain, cardiac myosin binding protein C, and α-actinin. Importantly, binding of heme to hemopexin or alpha-1-microglobulin prevented its effects on cardiomyocyte contractility, suggesting an allosteric effect. In line with this, free heme directly bound to myosin light chain 1 in human cardiomyocytes. Our observations suggest that free heme modifies cardiac contractile proteins via posttranslational protein modifications and via binding to myosin light chain 1, leading to severe contractile dysfunction. This may contribute to systolic and diastolic cardiac dysfunctions in hemolytic diseases, heart failure, and myocardial ischemia–reperfusion injury. These findings were published in FRBM in 2015.


Heme from hemolysis
and other sources
Contractile dysfunction
Actin
cMyBPC
MLC-1
MLC-2
MHC
Titin
Heme
Oxidation
A1M
Hemopexin
Elimination and protection

Decision letter from British Journal of Pharmacology

Dear Professor Balla,

Re 2018-BJP-0393-RP.R1: "HYDROGEN SULFIDE ABROGATES HEART VALVE CALCIFICATION: IMPLICATIONS FOR CALCIFIC AORTIC VALVE DISEASE"

Your paper has been seen by an editor and expert referees. I enclose below the comments. As you will see the referee 2 set out a number of points which will need your attention before we can consider the submission further. I would urge you to give these points your careful attention.

I hope that you will be prepared to make the necessary amendments and submit a revised manuscript within three months. This should be accompanied by a statement of how you have responded to the criticisms raised, preferably numbered point by point. Should you decide that you do not wish to submit a revised manuscript to BJP, please contact the Editorial Office so we may withdraw your manuscript from the system.

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Thank you for submitting your work to the British Journal of Pharmacology.

Yours sincerely,

Dr Giuseppe Cirino PhD, FBPhS, FRBS
Senior Editor, British Journal of Pharmacology
cirino@unina.it

Jozsef Balla, MD, PhD, DSc
Professor of Medicine
Division of Nephrology, Department of Medicine
Faculty of Medicine, University of Debrecen
Hungary