GRANT CLOSING REPORT

1. Background and aims

Numerous disease states are associated with hemolysis or hemorrhage. Extracellular hemoglobin (Hb) is scavenged by haptoglobin, the acute phase protein present in plasma. When the scavenging capacity of hatoglobin is exceeded, Hb accumulates in the plasma. Outside of the protective environment of RBCs Hb is prone to oxidation, leading to the formation of oxidized Hb species i.e. metHb (Fe^{3+}) and ferrylHb ($Fe^{4+}= O^{2-}$). The ferryl state of iron is quite unstable and during its decomposition globin centered radicals and covalently crosslinked Hb species can form. Oxidized Hb forms bind the heme prostetic group quite weakly, therefore laible heme accumulates in the plasma.

The central hypothesis of our proposal was that RBC-derived extracellular Hb and/or its oxidation products act as damage associated molecular patterns (DAMPs) and trigger inflammasome activation in diverse immune and non-immune cells.

The aim of our project was to characterize the inflammatory nature of different oxidized Hb forms and to study the involvement of these Hb forms in sterile and non-sterile inflammatory conditions.

2. Major published results

2.1. Heme and ferrylHb act as DAMPs and induce NLRP3 inflammasome activation in macrophages

It has been shown that free heme is a prototypical alarmin which is implicated in hemolysis-induced lethality via triggering NLRP3 activation in macrophages.

Using the mouse model of phenylhydrazine (PHZ)-induced sterile hemolysis and cellular models our aim was to investigate the role of different Hb forms in the induction of NLRP3 inflamamsome.

In agreement with previous observations, acute intravascular hemolysis induced marked splenomegaly and decreased hematocrit levels in PHZ-treated C57BL/6 mice. PHZ treatment triggered elevation of total heme content of plasma. More detailed examination of plasma heme content revealed that most of the heme moiety can be found in hemichrome, a denatured form of oxidized Hb. A lesser amount of heme was present in Hb and metHb, and at later time points we could detect heme that was dissociated from the globin. Also, we could identify covalently crosslinked Hb dimers in plasma samples of PHZ-injected mice, which is an indirect evidence of the formation of the highly reactive ferryl species. We showed that PHZ-induced intravascular hemolysis is associated with increased plasma and liver IL-1β levels and the formation of active caspase-1 in the liver. When exposed in vitro to FHb, LPS-primed macrophages markedly up-regulate IL-1β mRNA. To a lesser extent, both Hb and MHb increase the level of IL-1β mRNA. Similarly to that of heme, FHb is able to induce processing of IL-1β in macrophages. On the contrary, naïve Hb and MHb lack the ability to induce IL-1β secretion in vitro.

In vivo administration of FHb through intraperitoneal injection provokes peritonitis, characterized by massive infiltration of neutrophils and monocytes into the peritoneal cavity. Heme, to a lesser extent, also induces neutrophil and monocyte recruitment. In contrast, injection of naïve Hb does not trigger leukocyte infiltration, and MHb provokes only minor neutrophil recruitment. We also showed that as a response to intravascular hemolysis caspase-1 is activated and IL-1β is processed in the liver, therefore we used this approach to further investigate the pro-inflammatory role of different Hb forms. We found that intraperitoneal administration of FHb and heme induced caspase-1 activation and processing of IL-1β.

Dutra et al. showed that survival rates of mice lacking NLRP3 inflammasome components such as NLRP3, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) or Caspase-1 are remarkable higher than WT mice upon intravascular hemolysis. To further investigate this phenomenon first we confirmed the survival advantage NLRP3 deficient mice in our acute PHZ-induced hemolysis model. Theoretically survival advantage of these knock-out mice strains can rely on their increased resistance to PHZ-induced RBC lysis, or their increased tolerance to lysis. Measurement of hematocrit levels of WT and Nlrp3-/- mice subjected to PHZ-induced hemolysis revealed that NLRP3 deficiency does not impair RBC lysis therefore this mechanism cannot explain increased survival rate of Nlrp3-/- mice compared to WT. Moreover, we found no difference between plasma concentrations of Hb, MHb or hemichrome in WT and Nlrp3-/- mice suggesting that NLRP3 deficient mice have increased tolerance to lysis. Tissue damage control in response to infection as well as sterile inflammation can improve survival of the host in diverse disease conditions. In response to hemolysis NLRP3 inflammasome is activated leading to the production of the pro-inflammatory cytokine IL-1β in the liver of WT mice. In contrast, we found no active IL-1β production in the liver of PHZ-, or FHb-treated Nlrp3-/- mice. We think that lack of the pro-inflammatory response in NLRP3 deficient mice might contribute to the improved survival of those mice under hemolytic conditions (Fig.1).

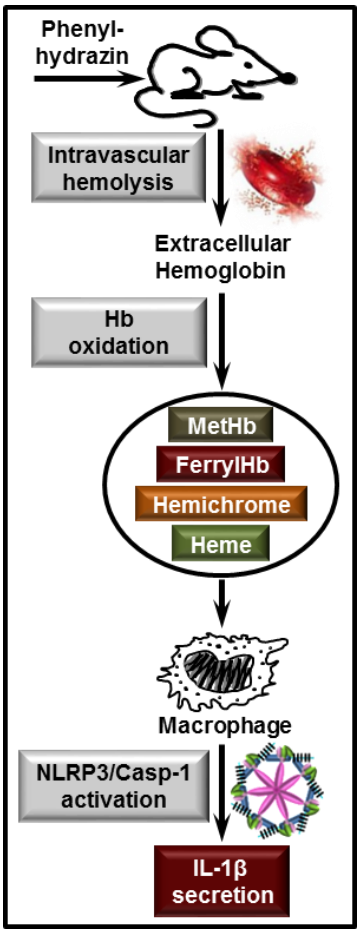


Figure 1. Proposed model of extracellular Hb-driven NLRP3 inflammasome activation and IL-1β production following intravascular hemolysis.

This work has been published: Nyakundi BB, Tóth A, Balogh E, Nagy B, Erdei J, Ryffel B, Paragh G, Cordero MD, Jeney V. Oxidized hemoglobin forms contribute to NLRP3 inflammasome-driven IL-1β production upon intravascular hemolysis. ***Biochim Biophys Acta Mol Basis Dis.*** 2019 Feb 1;1865(2):464-475. doi: 10.1016/j.bbadis.2018.10.030. (IF: 5.108).

2.2. Heme triggers NLRP3 inflammasome activation in human umbilical vein endothelial cells

Endothelial cells provide a barrier between blood and tissue and therefore play a fundamental role in the inflammatory response. Upon intravascular hemolysis, these cells are the first to get exposed to extracellular Hb and its oxidized forms. Endothelial cells when exposed to DAMPs, such as ATP, activate NLRP3 inflammasome and produce active IL-1β.

Our aim in this project was to investigate whether endothelial cells sense and activated by different oxidation forms of extracellular Hb and of free heme.

Heme induced a ~50-fold upregulation of IL-1β mRNA level in LPS-primed human umbilical vein endothelial cells (HUVECs). We found active IL-1β in the HUVECs supernatant, suggesting that heme triggered IL-1β maturation and secretion as well. Along with IL-1β, heme increased mRNA levels of the NLRP3 inflammasome complex, NLRP3 and ASC. When injected into C57BL/6 mice, heme induced Caspase-1 activation and cleavage of IL-1β in the liver, which effect was not observed in $NLRP3^{-/-}$ mice. This suggests that heme triggers IL-1β production via NLRP3 inflammasome activation.

Reactive oxygen species (ROS) play a critical role in NLRP3 inflammasome activation, therefore we tested whether ROS are involved in heme-mediated NLRP3 inflammasome activation and subsequent IL-1β production. We showed that heme induces ROS production in HUVECs. Inhibition

of ROS production with N-acetyl cysteine (NAC) attenuated heme-mediated increase in IL-1β mRNA and protein levels.

We also tested whether Hb forms are capable of inducing NLRP3 inflammasome activation and IL-1β secretion in HUVECs. Exposure of HUVECs to oxidized Hb forms (metHb, ferrylHb) resulted upregulation of heme oxygenase-1 mRNA and protein expressions, suggesting that heme release from these oxidized Hb forms readily occurs. Both ferrylHb and metHb induced ROS production in HUVECs. To our surprise this was associated with a minor upregulation of IL-1β mRNA in naïve HUVECs upon exposure to ferrylHb but not metHb. We did not see upregulation of IL-1β mRNA in LPS-primed HUVECs exposed to oxidized Hb forms. On the other hand, ferrylHb is a strong inducer of the expressions of cell surface adhesion molecules Icam-1, Vcam-1 and E-selectin, suggesting that oxidized Hb forms and in particularly ferrylHb act as a pro-inflammatory agonist toward endothelial cells, but not involved in NLRP3 inflammasome activation.

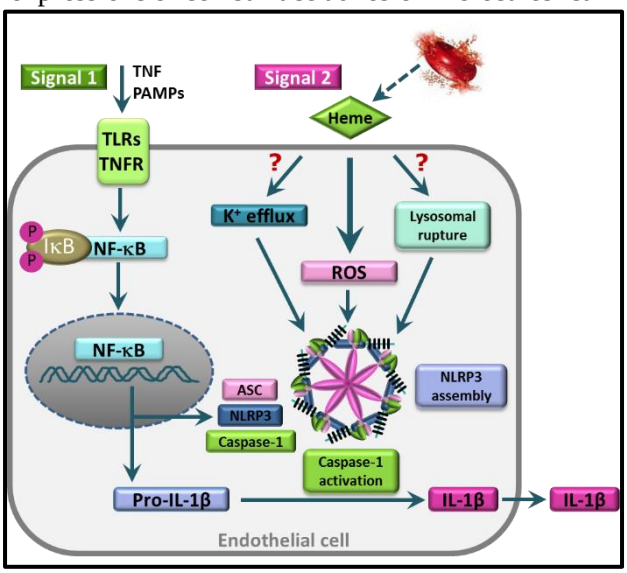


Figure 2. Working model of heme-induced NLRP3 inflammasome activation in endothelial cells. Pathogen-associated molecular patterns (PAMPs), such as LPS or TNF (Signal 1), bind to toll-like receptors (TLRs) or TNF receptor and prime endothelial cells to activate NF-κB to induce the expression of NLRP3, caspase-1, and IL-1β. Heme (signal 2) that can derive from Hb released form damaged RBCs induces ROS generation, caspase-1 activation, and cleavage of pro-IL-1β. Mature form of IL-1β is secreted from the cell.

This work has been published: Erdei J, Tóth A, Balogh E, Nyakundi BB, Bányai E, Ryffel B, Paragh G, Cordero MD, Jeney V. Induction of NLRP3 Inflammasome Activation by Heme in Human Endothelial Cells. ***Oxid Med Cell Longev.*** 2018 Mar 20;2018:4310816. doi: 10.1155/2018/4310816. (IF: 4.936).

2.3. Hydrogen sulfide abrogates hemoglobin-lipid interaction in atherosclerotic lesion

The infiltration of red blood cells into atheromatous plaques is implicated in atherogenesis. Inside the atherosclerotic lesion, Hb is oxidized to ferri- and ferrylHb which exhibit prooxidant and proinflammatory activities. Cystathione gamma-lyase (CSE) derived hydrogen sulphide (H_2S) has been suggested to possess various antiatherogenic actions.

We aimed to investigate whether H_2S inhibits hemoglobin-lipid interactions in atherosclerotic lesions and alter subsequent endothelial cell reactions. We also determined how atherogenesis influenced the vascular expression of CSE and identified pathophysiological modulators of CSE expression.

We found that expression of CSE was upregulated predominantly in macrophages, foam cells, and myofibroblasts of human atherosclerotic lesions derived from carotid artery specimens of patients. A 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 Hb and atheroma lipids, H_2S 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 H_2S inhibited heme and Hb-mediated lipid oxidation of human atheroma-derived lipid and human complicated lesion. Our study suggests that the CSE/H_2S system

represents an atheroprotective pathway for removing or limiting the formation of oxidized Hb and lipid derivatives in the atherosclerotic plaque.

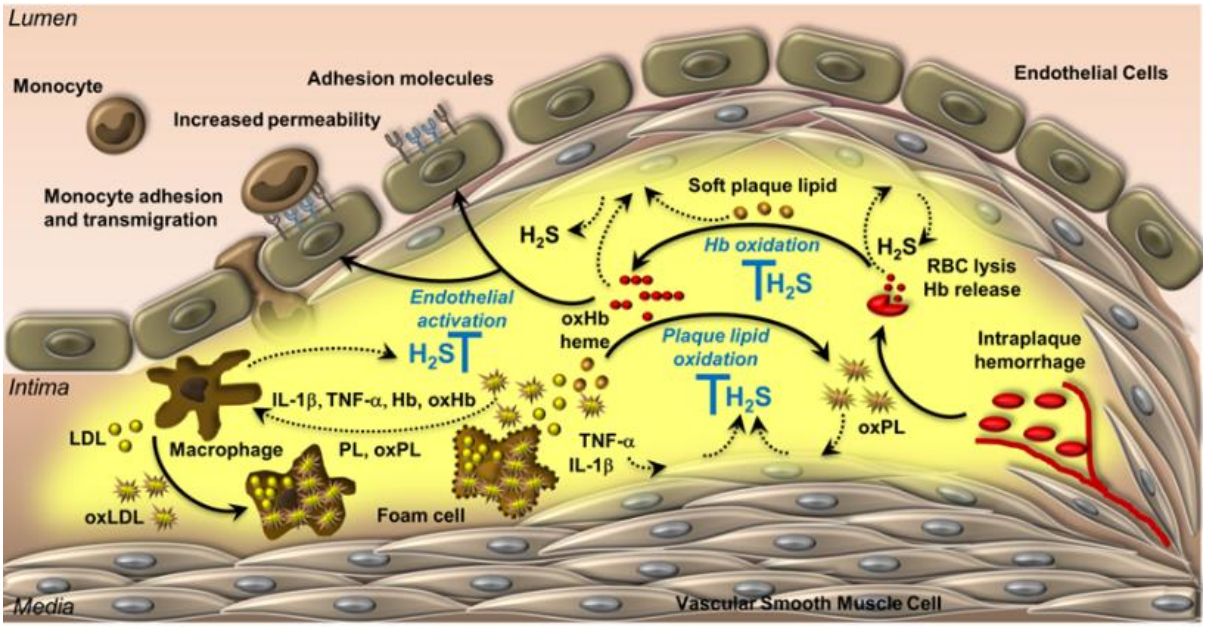


Figure 3. Proposed protective actions of H_2S in the atherosclerotic plaque. Upon infiltration of RBCs into the atherosclerotic lesion RBCs are lysed and oxidation of extracellular Hb occurs followed by heme release. Oxidized Hb forms and the released heme trigger further lipid peroxidation. FerrylHb exhibits proinflammatory property provoking endothelial cell activation characterized by intercellular gap formation and increased adhesion molecule expression. Endothelial activation facilitates monocyte adhesion and transendothelial migration. In the reactions between Hb and plaque lipids, different oxidized Hb derivatives are formed including metHb and ferrylHb species. Macrophages, foam cells, and smooth muscle cell-derived myofibroblasts respond to such an insult (ferrylHb, heme, plaque lipids, and the proinflammatory cytokines IL-1β and TNF-α) by upregulating CSE expression. The increased production of H^2S inhibits (i) oxidation of Hb preventing the formation of ferrylHb derivatives (a novel function as a heme-redox-intermediate-scavenging antioxidant), (ii) oxidation of plaque lipids, and subsequently (iii) activation of endothelium.

This work has been published: Potor L, Nagy P, Méhes G, Hendrik Z, Jeney V. et al. Hydrogen Sulfide Abrogates Hemoglobin-Lipid Interaction in Atherosclerotic Lesion. ***Oxid Med Cell Longev.*** 2018 Jan 21;2018:3812568. doi: 10.1155/2018/3812568. (IF: 4.936).

2.4. Non-immune protection against malaria

Malaria, the disease caused by Plasmodium spp. infection, remains a major global cause of morbidity and mortality. Host resistance to malaria relies on immune-driven mechanisms exerting a negative impact on different stages of Plasmodium infection. This defence strategy however, is not sufficient per se to avoid the onset of severe and eventually lethal forms of malaria. This is accomplished instead via the establishment of disease tolerance, a defence strategy that operates irrespectively of immune-driven resistance mechanisms targeting Plasmodium.

The blood stage of Plasmodium spp. infection is characterized by the invasion of host red blood cells (RBC), in which this protozoan parasite proliferates extensively, consuming up to 60–80% of the RBC Hb content. Plasmodium spp. do not express a heme oxygenase-1 ortholog gene and cannot catalyze the extraction of Fe from heme, acquiring Fe via heme auto-oxidation while also polymerizing labile heme into redox-inert hemozoin and avoiding its cytolytic effects. Once the physical integrity of infected RBC becomes compromised, the remaining RBC Hb content is released into plasma, where extracellular α2β2 Hb tetramers disassemble into αβ dimers that undergo auto-oxidation, eventually releasing their non-covalently bound heme. As it accumulates in plasma, labile heme is loosely bound to plasma acceptor proteins, macromolecules, or low-molecular-weight ligands that fail, however, to

control its redox activity. A fraction of the labile heme in plasma becomes bioavailable, acting in a pathogenic manner and compromising the establishment of disease tolerance to malaria.

Heme accumulation in plasma and urine of malaria patients is associated with the development of acute kidney injury (AKI), a clinical hallmark of severe malaria. Similarly, heme accumulation in plasma, as a consequence of rhabdomyolysis, is also associated with the development of AKI. While heme partakes in the pathogenesis of AKI associated with rhabdomyolysis, whether this is the case for severe malaria has not been established. We have previously shown that heme detoxification by the stress-responsive enzyme heme oxygenase-1 (HO-1) is a limiting factor in the establishment of disease tolerance to malaria. In a similar manner, heme detoxification by HO-1 also prevents the development of AKI following rhabdomyolysis. This protective effect requires that the Fe extracted from heme is neutralized by the ferroxidase active ferritin H (FTH) component of the ferritin complex, establishing disease tolerance to malaria and preventing development of AKI following rhabdomyolysis.

Under this project we investigated whether heme catabolism by HO-1 and Fe sequestration by FTH act locally in the kidney to prevent the development of AKI and establish disease tolerance to malaria.

We demonstrated that disease tolerance to malaria relies on the capacity of renal proximal tubule epithelial cells to detoxify labile iron (Fe)-containing protoporphyrin (heme), which accumulates in plasma and urine during the blood stage of Plasmodium infection. This protective mechanism operates via the induction by renal proximal tubular epithelial cells of the heme catabolizing enzyme heme oxygenase-1 and the ferroxidase active ferritin H chain component of the Fe-sequestering protein complex ferritin. Induction of this heme/Fe-detoxifying system is controlled by the transcription factor nuclear factor E2-related factor-2, which prevents the development of acute kidney injury, a clinical hallmark of severe malaria. Targeting this non-immune defence mechanism should provide a unique therapeutic window to limit malaria severity, without exerting a selective pressure on Plasmodium.

This work has been published: Ramos S, Carlos AR, Sundaram B, Jeney V, Ribeiro A, Gozzelino R, Bank C, Gjini E, Braza F, Martins R, Ademolue TW, Blankenhaus B, Gouveia Z, Faísca P, Trujillo D, Cardoso S, Rebelo S, Del Barrio L, Zarjou A, Bolisetty S, Agarwal A, Soares MP. Renal control of disease tolerance to malaria. ***Proc Natl Acad Sci U S A.*** 2019 Mar 19;116(12):5681-5686. doi: 10.1073/pnas.1822024116 (IF: 9.504).

2.5. The role of hypoxia in vascular calcification.

Neovascularization of atheroma is triggered by tissue hypoxia. Vascular calcification is present in advanced atherosclerotic lesions characterized by neovascularization and intra-plaque hemorrhage. Vascular calcification is associated with high risk of cardiovascular events and mortality. Osteochondrogenic differentiation of vascular smooth muscle cells (VSMCs) is the major cellular mechanism underlying vascular calcification.

Because tissue hypoxia is a common denominator in vascular calcification, our aim in this project was to investigate whether hypoxia per se triggers osteochondrogenic differentiation of VSMCs. We studied osteochondrogenic differentiation of human aorta VSMCs cultured under normoxic (21% O2) and hypoxic (5% O2) conditions. Hypoxia increased protein expression of HIF (hypoxia-inducible factor)-1α and its target genes GLUT1 (glucose transporter 1) and VEGFA (vascular endothelial growth factor A) and induced mRNA and protein expressions of osteochondrogenic markers, that is, RUNX2 (runt-related transcription factor 2), SOX9 (Sry-related HMG box-9), OCN (osteocalcin) and ALP (alkaline phosphatase), and induced a time-dependent calcification of the extracellular matrix of VSMCs. HIF-1 inhibition by chetomin abrogated the effect of hypoxia on osteochondrogenic markers and abolished extracellular matrix calcification. Hypoxia triggered the production of reactive oxygen species, which was inhibited by chetomin. Scavenging reactive oxygen species by N-acetyl cysteine attenuated hypoxia-mediated upregulation of HIF-1α, RUNX2, and OCN protein expressions and inhibited extracellular matrix calcification, which effect was mimicked by a specific hydrogen peroxide scavenger sodium pyruvate and a mitochondrial reactive oxygen species inhibitor rotenone. Ex vivo

culture of mice aorta under hypoxic conditions triggered calcification which was inhibited by chetomin and N-acetyl cysteine. In vivo hypoxia exposure (10% O2) increased RUNX2 mRNA levels in mice lung and the aorta.

We have concluded that hypoxia contributes to vascular calcification through the induction of osteochondrogenic differentiation of VSMCs in an HIF-1-dependent and mitochondria-derived reactive oxygen species-dependent manner.

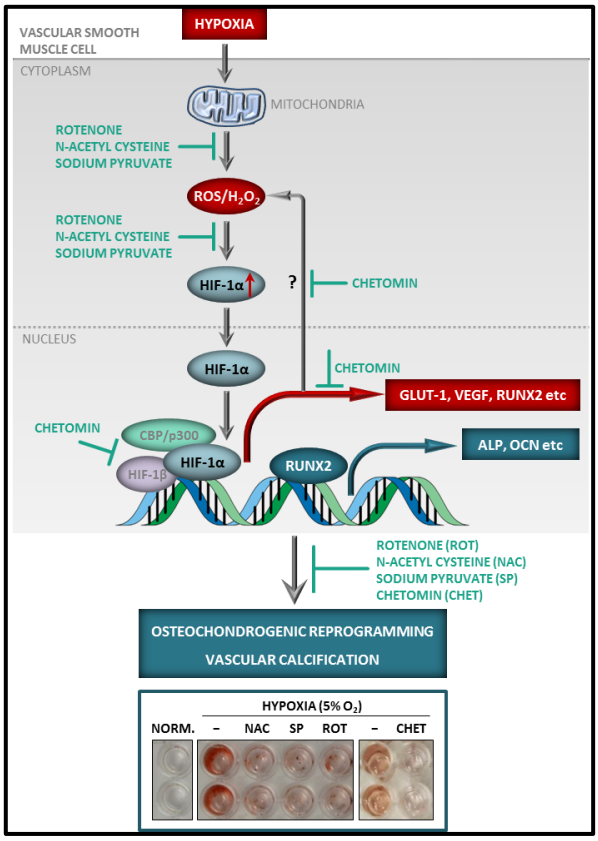


Figure 4. Proposed mechanism of hypoxia-induced osteochondrogenic transdifferentiation of VSMCs. Hypoxia induces unfettered generation of mitochondria-derived reactive oxygen species (ROS) in VSMCs. ROS is involved in the stabilization of HIF-1α. HIF-1α translocates into the nucleus and induces the transcription of genes (RUNX2, osteocalcin (OCN), alkaline phosphatase (ALP)) involved in the osteochondrogenic differentiation program of VSMCs that leads eventually to vascular calcification. Inhibition of mitochondrial ROS production or HIF-1α transcriptional activity inhibit hypoxia-induced osteochondrogenic transdifferentiation and extracellular matrix mineralization of VSMCs.

This work has been published: Balogh E, Tóth A, Méhes G, Trencsényi G, Paragh G, Jeney V. Hypoxia Triggers Osteochondrogenic Differentiation of Vascular Smooth Muscle Cells in an HIF-1 (Hypoxia-Inducible Factor 1)-Dependent and Reactive Oxygen Species-Dependent Manner. ***Arterioscler Thromb Vasc Biol.*** 2019 Jun;39(6):1088-1099. doi: 10.1161/ATVBAHA.119.312509 (IF: 6.618).

3. Major results not yet published

The following works are closely associated with the original proposal and the manuscripts will likely be published within a year, therefore we kindly ask the judges for the re-evaluation of the outcome of our grant next September.

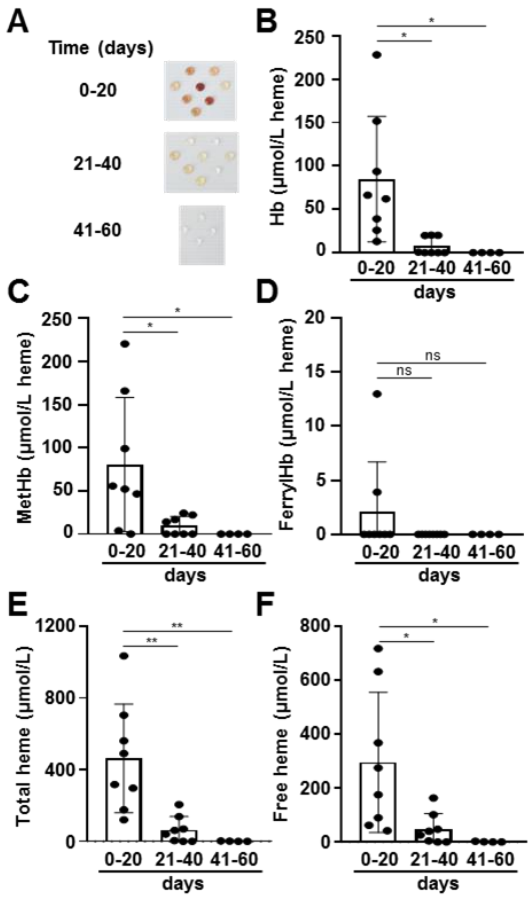
3.1. Oxidized Hb forms drives the pathogenesis of intraventricular hemorrhage (IVH)

Intraventricular hemorrhage (IVH) is a frequent complication of prematurity, occurring in about 15% to 20% of very low birth-weight (<1500 g) preterm infants, and its incidence is even higher (~45%) in extremely low birth-weight infants (500-750 g). IVH is associated with high neonatal mortality (20-50%), and increases the risk of neurodevelopmental impairment in the surviving infants beyond the risk associated with prematurity alone.

IVH in preterm infants leads to systemic inflammation, characterized by elevation of pro-inflammatory cytokines e.g. tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8), IL-1β; chemokines such as monocyte adhesion molecule-1, and increased levels of soluble adhesion molecules i.e. vascular cell adhesion molecule-1 (Vcam-1), intercellular adhesion molecule-1 (Icam-1). As a sign of local inflammatory response the levels of soluble adhesion molecules E-selectin, Vcam-1, Icam-1 and L-selectin were found to be elevated in the cerebrospinal fluid (CSF) of patients after subarachnoid hemorrhage. Rupture of the microvasculature of germinal matrix cause extravasation of RBCs in the

CSF followed by lysis of RBCs. Outside of the RBCs Hb is prone to oxidation, giving a rise to the formation of different Hb oxidation products and subsequent release of heme.

The goal of the present study was to perform a qualitative and quantitative analysis of Hb content of CSF samples obtained from premature infants following IVH, with a special interest for the presence of ferrylHb/covalently crosslinked Hb species. We also aimed to investigate whether there is a correlation between the levels of Hb oxidation products and the concentration of pro-inflammatory adhesion molecules in CSF.



We have evaluated Hb levels in CSF samples obtained from 20 premature infants suffered from IVH grade III. We grouped the samples based on the time lapse between IVH and CSF sampling and found that Hb, metHb, ferrylHb, total heme and free heme levels were markedly elevated in CSF samples obtained between day 0-20 following IVH. The levels of Hb-derived species were significantly less in IVH samples obtained at later time points between day 21-40 post-IVH and were absent in samples taken at day 41-60 post-IVH (Fig. 4).

Figure 5. Evaluation of Hb, metHb, ferrylHb, total heme and labile heme levels in CSF samples post-IVH. (A-D) Concentrations of Hb, metHb, and ferrylHb were quantified spectrophotometrically in CSF obtained from premature infants suffering from IVH (n=20). (E-F) Total heme was determined by heme assay kit and labile heme was calculated.

Hb levels correlated strongly to the levels of oxidized Hb forms, as well as to total heme levels, suggesting that Hb is the origin of these species. Oxidized Hb forms readily release their heme prosthetic group. In accordance of this notion, we found that labile heme levels correlated most strongly with oxidized Hb levels in post-IVH CSF samples.

Brain endothelial cells play a critical role in post-IVH neuroinflammatory responses. Previously we have shown that ferrylHb induces the expression of adhesion molecules including Vcam-1 in human umbilical vein endothelial cells. Here we tested the effect of Hb forms and free heme on Vcam-1 expression in brain microvascular endothelial cells (BMVECs). Among the Hb forms ferrylHb and free heme were the most potent to induce Vcam-1 expression in BMVECs (Fig. 5).

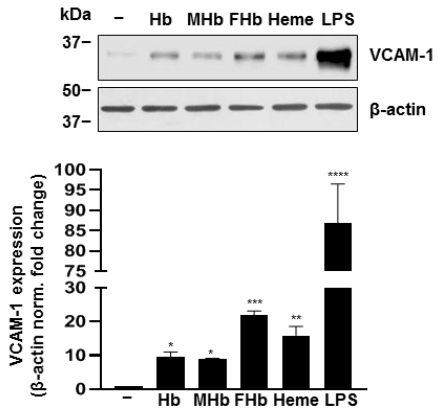


Figure 6. Evaluation of Vcam-1 expression in BMVECs. Confluent BMVECs were exposed to vehicle, Hb, metHb, ferrylHb, heme or LPS for 12 hours. Protein expressions of Vcam-1 was evaluated in whole cell lysates by Western blot. Membranes were re-probed for β-actin. Representative Western blots from three independent experiments are shown.

We have evaluated Vcam-1 levels in post-IVH CSF samples and found that Vcam-1 levels were high in early post-IVH samples and that the level gradually decreased by the time, reaching a significant reduction in CSF samples obtained between day 41-60 post-IVH (Fig. 7A). We found a moderate correlation between total heme levels and Vcam-1 levels in post-IVH CSF samples (Fig. 7B).

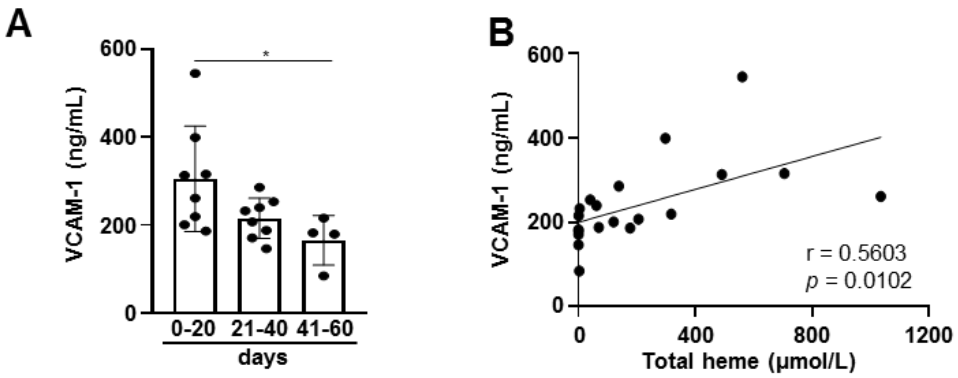


Figure 7. Soluble adhesion molecule Vcam-1 levels in post-IVH CSF samples and its correlation with the level of total heme. Soluble Vcam-1 levels were determined by AlphaLISA in post-IVH CSF samples (n=20) in triplicates. Total heme levels were determined by heme assay kit. The correlation between soluble Vcam-1 and total heme was were determined by linear regression analysis. R represents Pearson's correlation coefficient.

Based on mainly these findings a manuscript is currently under construction. We wish to submit it in the next couple of weeks to the journal of BBA Molecular Basis of Disease.

3.2. Formation and pro-inflammatory actions of Hb-derived DAMPs (invited review)

Hemoglobin, the highly specialized molecule for transporting oxygen, is a major component of RBCs. RBCs are equipped with a highly efficient antioxidant system to protect Hb from oxidation. In contrast, Hb outside of the protective environment of RBCs is prone to oxidation, in which process different Hb oxidation products are formed. Oxidized Hb forms release their heme prosthetic groups that increase the amount of labile heme.

Hemolytic diseases are often associated with sterile inflammation which is driven, at least partially, by Hb-related damage associated molecular patterns (DAMPs) which are being produced under hemolytic conditions. Hb-related DAMPs includes metHb, ferrylHb and covalently crosslinked oxidized Hb forms as well as labile heme. Hb-related DAMPs act as chemo-attractants, activate endothelial cells, target different cells of the innate immune system including macrophages, microglia, neutrophils, and activate the complement system.

The review, we are currently working on, aims to summarize our current knowledge about the formation and pro-inflammatory actions of Hb-derived DAMPs.

4. Results not strictly associated with the proposal

Besides of the above mentioned articles and the ongoing manuscripts we have published several articles in the time-frame of the proposal which topics were not closely associated with the original proposal. The OTKA grant has been acknowledged in them, because – besides other grants – we used resources (human and/or infrastructural) supported by this grant to accomplish those projects. We would appreciate if these publications would be considered as a result of this grant.

4.1. Balogh E, Tolnai E, Nagy B Jr, Nagy B, Balla G, Balla J, Jeney V. Iron overload inhibits osteogenic commitment and differentiation of mesenchymal stem cells via the induction of ferritin. ***Biochim Biophys Acta.*** 2016 Sep;1862(9):1640-9. doi: 10.1016/j.bbadis.2016.06.003. (IF: 5.158)

4.2. Balogh E, Tóth A, Tolnai E, Bodó T, Bányai E, Szabó DJ, Petrovski G, Jeney V. Osteogenic differentiation of human lens epithelial cells might contribute to lens calcification. ***Biochim Biophys Acta.*** 2016 Sep;1862(9):1724-31. doi: 10.1016/j.bbadis.2016.06.012. (IF: 5.158)

4.3. Jeney V. Clinical Impact and Cellular Mechanisms of Iron Overload-Associated Bone Loss. ***Front Pharmacol.*** 2017 Feb 21;8:77. doi: 10.3389/fphar.2017.00077. (IF: 3.831)

4.4. Balogh E, Paragh G, Jeney V. Influence of Iron on Bone Homeostasis. ***Pharmaceuticals*** (Basel). 2018 Oct 18;11(4). pii: E107. doi: 10.3390/ph11040107. (IF: 4.2)

4.5. Váradi J, Hermenean A, Gesztelyi R, Jeney V. et al. Pharmacokinetic Properties of Fluorescently Labelled Hydroxypropyl-Beta-Cyclodextrin. ***Biomolecules.*** 2019 Sep 20;9(10). pii: E509. doi: 10.3390/biom9100509. (IF: 4.694)