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Neutrophil and Macrophage Interactions with Ferryl Hemoglobin Mediate Oxidative Inflammation and Arterial Wall Remodeling in Abdominal Aortic Aneurysm

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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, the 19th of May 2026 2:00 PM.

1 Literature Review

1.1 Overview of abdominal aortic aneurysm

Abdominal aortic aneurysm (AAA) is a large-vessel disease characterized by localized or diffuse dilation of the abdominal aorta caused by structural degeneration of the aortic wall. Progressive enlargement of the aneurysm may ultimately result in rupture, which is associated with a high risk of mortality. The development of AAA is typically a chronic, irreversible, and largely asymptomatic process, and rupture often represents the first clinical manifestation.

At present, no effective pharmacological therapies are available to prevent AAA formation or progression. Surgical repair and endovascular aortic repair remain the primary treatment options; however, both strategies are associated with substantial perioperative risk and long-term morbidity. Consequently, a deeper understanding of the epidemiological characteristics and disease progression of AAA is essential for the development of targeted therapeutic strategies.

Since the early 20th century, the reported incidence of AAA has shown a continuous upward trend. This increase is closely related to improvements in diagnostic techniques, widespread screening programs, and long-term cohort studies. In Western countries, population aging, lifestyle changes, and dietary patterns are considered major contributors to the growing disease burden. With the ongoing demographic shift toward an older population, the global incidence of AAA is expected to continue rising.

The prevalence of AAA exhibits marked geographic variability. Epidemiological studies consistently demonstrate a significantly higher prevalence in Europe, North America, and Australia compared with Asian populations. Large-scale screening programs in Western countries report prevalence rates generally ranging from 1% to over 7%, particularly among older male populations. In contrast, screening studies conducted in East Asian regions,

including Japan, South Korea, and Hong Kong, reveal substantially lower prevalence rates, typically below 0.5%.

Comparative analyses across continents indicate that AAA is considerably less common in Asian populations than in Caucasian populations from Europe, the Americas, and Australia. These differences are likely influenced by genetic susceptibility, environmental factors, lifestyle behaviors, and cardiovascular risk profiles.

AAA predominantly affects older individuals and is rare in those under 50 years of age. The majority of patients are diagnosed after the age of 65, making advanced age one of the most important risk factors. In Western countries, the average age at diagnosis is approximately 70 years. AAA rupture before the age of 65 is uncommon, and the disease accounts for a notable proportion of mortality in elderly men.

A pronounced gender disparity exists in AAA prevalence. Men are significantly more likely to develop AAA than women, with reported male-to-female ratios of approximately 5:1. Women typically present with AAA at an older age than men, often with a delay of about 10 years. Despite this difference in prevalence and age at onset, aneurysm growth rates and rupture risk relative to aneurysm size appear comparable between sexes.

1.2 Etiology and pathological basis of abdominal aortic aneurysm

AAA is a multifactorial vascular disease resulting from the interaction of genetic susceptibility, environmental influences, and biochemical abnormalities. Risk factors such as family history, altered collagen and elastin metabolism, increased proteolytic activity, and impaired inhibitory mechanisms contribute to AAA development; however, its precise pathogenesis remains incompletely defined.

Although alterations in individual AAA-related genes have been reported, evidence increasingly indicates that AAA is driven by dysregulation of multiple gene networks rather than single-gene mutations. The aortic wall is rich in type I and type III collagen, and structural

or metabolic abnormalities in these components compromise vascular integrity. Several collagen-related genetic variants have been associated with increased AAA susceptibility, but their roles in aneurysm progression and rupture remain controversial.

High-throughput gene expression analyses suggest that genes involved in apoptosis, receptor signaling, cytoskeletal organization, and protein synthesis are differentially expressed during AAA formation. These gene clusters are closely linked to vascular smooth muscle cell (SMC) phenotypic modulation and extracellular matrix (ECM) remodeling, highlighting the complexity of AAA-related genetic regulation.

AAA progression is characterized by a chronic inflammatory cascade accompanied by profound structural remodeling of the aortic wall. Hallmark pathological features include inflammatory cell infiltration, vascular SMC senescence and apoptosis, endothelial dysfunction, and progressive ECM degradation.

AAA is widely regarded as an inflammation-driven vascular disorder. Inflammatory cells infiltrating the aortic wall secrete matrix metalloproteinases and other proteolytic enzymes that promote elastin degradation and collagen disorganization. Endothelial cells (ECs), vascular SMCs, and fibroblasts further amplify this process through sustained protease production. The interaction between inflammation and ECM degradation constitutes the central pathological basis of aneurysmal dilation.

1.3 Current clinical management of abdominal aortic aneurysm

Surgical repair remains the primary treatment for advanced AAA to prevent rupture. Endovascular aneurysm repair is widely used due to its minimally invasive nature, but perioperative mortality and postoperative complications remain significant. Common complications include endoleaks, graft migration, occlusion, and infection, with larger aneurysm diameter being a major predictor of adverse outcomes.

Currently, no pharmacological therapies have been shown to effectively halt AAA progression in clinical practice. Nonetheless, increasing insight into AAA pathophysiology has identified potential therapeutic targets. Experimental studies suggest that inhibiting inflammation, suppressing vascular SMC phenotypic switching, reducing protease activity and oxidative stress, and stabilizing ECM structure may attenuate aneurysm progression, providing a basis for future drug development.

1.4 The role of neutrophils and neutrophil extracellular traps in abdominal aortic aneurysm

Neutrophils are among the earliest immune cells recruited to sites of vascular injury and play a critical role in the initiation and progression of AAA. Beyond their classical inflammatory functions, neutrophils contribute to aneurysmal degeneration through the formation of neutrophil extracellular traps (NETs), a specialized process characterized by the extracellular release of chromatin fibers decorated with histones and granular enzymes.

Neutrophil-derived proteases, such as neutrophil elastase (NE), promote ECM degradation and facilitate NET formation, thereby amplifying local inflammatory responses within the aortic wall. NETs serve as a structural scaffold that concentrates inflammatory mediators and proteolytic enzymes, accelerating elastin breakdown and collagen disorganization, which are central features of AAA pathology.

In addition, NETs exert direct cytotoxic effects on ECs and vascular SMCs, inducing apoptosis or necrosis and compromising aortic wall integrity. Histones released during NET formation exhibit potent cytotoxicity, further aggravating vascular injury and promoting sustained inflammatory cell infiltration.

Experimental studies demonstrate that inhibition of NET formation reduces inflammatory cell accumulation and attenuates aneurysm development. Genetic or pharmacological targeting of

key mediators of NETosis, such as peptidyl arginine deiminase 4 (PAD4), has been shown to suppress aneurysm progression, highlighting NETs as a potential therapeutic target.

Furthermore, NETs interact with platelets and the coagulation system, contributing to intraluminal thrombus formation within aneurysmal lesions. Thrombus-associated hypoxia enhances protease release and oxidative stress, establishing a vicious cycle of NET formation, persistent inflammation, and aortic wall degeneration. Collectively, these findings underscore the pivotal role of neutrophils and NETs in driving the inflammatory and structural remodeling processes underlying AAA progression.

1.5 The role of macrophages in abdominal aortic aneurysm

AAA is a chronic inflammatory disease characterized by progressive ECM degradation, vascular smooth muscle cell loss, and structural weakening of the aortic wall. Among the proteolytic enzymes involved, matrix metalloproteinases, particularly MMP-9, play a central role in aneurysmal degeneration. MMP-9 is highly expressed in aneurysmal tissue, especially in regions of elastin disruption and rupture, and is predominantly produced by infiltrating macrophages, underscoring their key contribution to ECM destruction and aortic wall destabilization.

AAA is often described as an inflammatory aneurysm due to extensive immune cell infiltration within the aortic wall, among which macrophages represent a dominant and functionally critical population. Activated macrophages release pro-inflammatory cytokines, chemokines, proteases, and reactive oxygen species, collectively promoting ECM degradation, smooth muscle cell apoptosis, and pathological vascular remodeling.

Macrophages exhibit pronounced phenotypic plasticity, with both pro-inflammatory and tissue-remodeling subsets present in aneurysmal lesions. A shift toward pro-inflammatory macrophage dominance sustains chronic inflammation and accelerates aneurysm progression. Given their involvement throughout multiple stages of AAA development, macrophages are

considered a central cellular driver of disease pathogenesis and an attractive target for therapeutic intervention. Strategies aimed at modulating macrophage recruitment, activation, and polarization hold promise for limiting aneurysm growth and reducing rupture risk.

1.6 Hemoglobin oxidation in abdominal aortic aneurysm

Intramural hemorrhage is a key pathological feature of AAA, with the accumulation of erythrocytes and hemoglobin (Hb) in the aortic wall exacerbating oxidative stress and inflammation, thereby promoting ECM degradation, smooth muscle cell apoptosis, and aneurysm expansion. Neutrophils and macrophages catalyze Hb oxidation, converting metHb (Fe^{3+}) into highly reactive ferryl (Fe^{4+}) heme species and protein-centered radicals. These rapid redox reactions create a strongly pro-oxidative microenvironment that accelerates vascular injury.

Oxidized Hb species, particularly ferrylHb, mediate lipid peroxidation, endothelial dysfunction, and cell death, while enhancing adhesion molecule expression to recruit leukocytes and amplify local inflammation. Additionally, oxidized Hb modulates the immunogenicity of Hb-derived peptides, potentially triggering autoimmune responses that further degrade vascular structural proteins. Intraluminal thrombus enriched with erythrocytes and Hb serves as a continuous source of oxidative and proteolytic mediators, sustaining the pathological remodeling of the aneurysmal wall.

Overall, Hb oxidation links intramural hemorrhage to oxidative stress, inflammation, and vascular remodeling in AAA. Targeting Hb redox pathways and neutralizing ferrylHb represent promising strategies to mitigate oxidative damage, limit inflammation, and reduce aneurysm progression and rupture risk.

1.7 Endothelial and smooth muscle cell dysfunction in abdominal aortic aneurysm

Vascular ECs and SMCs are essential for maintaining arterial homeostasis. ECs regulate vascular permeability, leukocyte adhesion, coagulation, and inflammatory signaling, while

SMCs sustain mechanical integrity and ECM remodeling. Both cell types exhibit phenotypic plasticity, with SMCs switching from a contractile to a synthetic or pro-inflammatory state under stress.

Endothelial dysfunction is an early event in AAA, promoting leukocyte infiltration and local inflammation. This pro-inflammatory milieu induces SMC apoptosis and phenotypic modulation, disrupts ECM homeostasis, and weakens the arterial wall. The combined dysfunction of ECs and SMCs drives progressive vascular degeneration, positioning these cells at the center of AAA pathogenesis.

1.8 Heme Oxygenase-1 in Abdominal Aortic Aneurysm: Mechanistic and Therapeutic Insights

Heme oxygenase-1 (HO-1) exerts protective effects against AAA by regulating oxidative stress, inflammation, and ECM remodeling. HO-1 deficiency accelerates aneurysm formation and rupture in experimental models, and human genetic variants associated with reduced HO-1 inducibility correlate with higher AAA prevalence.

Intramural hemorrhage releases hemoglobin and heme, which undergo oxidation to generate reactive oxygen species, further exacerbated by neutrophils, macrophages, and oxidized lipids. Free heme amplifies oxidative stress, triggers endothelial dysfunction, and promotes inflammatory signaling, creating a vicious cycle that drives aneurysm progression.

Pharmacological induction of HO-1, for example using Normosang (heme arginate), can mitigate vascular inflammation, suppress matrix metalloproteinase activity, and reduce oxidative damage without pro-oxidant effects. These properties highlight HO-1 as a promising therapeutic target for limiting AAA growth and preventing rupture.

2 Aim of the Study

Develop translational tools to detect ferrylHb in circulation, and assess whether circulating ferrylHb is associated with ruptured AAA, supporting its potential as a rupture-related biomarker.

Determine the causal effects of Hb and ferrylHb on aneurysm progression, with emphasis on neutrophil and macrophage activation, cell injury, and NET formation within the aortic wall.

Characterize Hb oxidation-associated signatures in AAA, including accumulation of oxidized Hb species, heme loading, and inflammatory immune cell markers in human and experimental mice aneurysmal tissues.

Transcriptomic signatures associated with inflammation and neutrophil activation in human and mouse hemorrhaged AAA, and to determine whether ferrylHb drives similar transcriptomic changes in human macrophages.

How ferrylHb influences neutrophil behavior, including gene programs involved in macrophage recruitment, activation, elastase release with potential elastin degradation, and PAD4-dependent NET formation, thereby linking Hb oxidation to immune mediated vascular wall remodeling.

Heme overload elucidates its impact on ECs and SMCs activation, including induction of IL1β, ICAM1, and NLRP3-dependent inflammatory pathways.

Evaluate the therapeutic relevance of the heme-HO-1 axis, testing whether pharmacological HO-1 induction mitigates aneurysm formation and intramural hemorrhage, while HO-1 inhibition exacerbates disease severity.

3 Methods

3.1 Ferryl hemoglobin level quantification in serum

Based on the development of a novel monoclonal antibody specifically recognizing oxidized ferrylHb, we established an in-house sandwich enzyme-linked immunosorbent assay (ELISA) for the quantification of ferrylHb levels in serum or plasma. The antibody targets an epitope within amino acids 32-41 (LLVVYPWTQR) of the Hb β-chain, which becomes exposed only upon oxidative modification of Hb.

High-binding 96-well ELISA plates were coated overnight at 4 °C with capture antibody (10 µg/ml) diluted in 0.2 M sodium bicarbonate buffer (pH 9.6). After blocking with buffer containing bovine serum albumin, sodium chloride, and Tween-20, pre-diluted serum or plasma samples (1:2500) or ferrylHb standards were added and incubated for 1 hour at room temperature. Bound ferrylHb was detected using an HRP-conjugated anti-ferrylHb monoclonal antibody, followed by color development with TMB substrate. The reaction was stopped with sulfuric acid, and absorbance was measured at 450 nm.

Washing steps were performed three times between all incubation stages using PBS with Tween-20. Standard curves were generated using ferrylHb concentrations ranging from 2.5 to 100 µg/l. The assay demonstrated good reproducibility, with intra-assay coefficients of variation below 8% across a range of control concentrations.

3.2 Establishment of a murine abdominal aortic aneurysm model

Male apolipoprotein E deficient ($ApoE^{-/-}$) mice aged 8-10 weeks were randomly assigned to receive either angiotensin II (AngII) or saline infusion. Only male mice were used to avoid hormonal influences on AngII responsiveness and due to the higher incidence of aortic hematoma observed in males.

All animals were maintained on either a high-fat diet (HFD) or standard chow (STD) for 12 weeks. During the final 4 weeks, osmotic minipumps were subcutaneously implanted to deliver

AngII at a dose of 1000 ng/min/kg or saline as a control. At the end of the experimental period, mice were anesthetized and euthanized, and the thoracic and abdominal aorta were carefully harvested following saline perfusion.

Excised aortas were cleaned of surrounding tissue, rinsed to remove intraluminal blood, and sectioned. Abdominal segments were used for aneurysm evaluation and histological analysis, while additional samples were snap-frozen for protein studies. Gross morphology of the arterial specimens was documented using digital photography.

3.3 Ultrasonographic evaluation, hemodynamic measurements, and aneurysm grading

In vivo assessment of abdominal aortic morphology was performed using a high-resolution ultrasound imaging system equipped with a 30 MHz linear transducer. Mice were anesthetized with isoflurane, positioned supine on a temperature-controlled platform, and the abdominal region was prepared for imaging. Longitudinal scans of the suprarenal abdominal aorta were obtained at mid-systole, and maximal luminal diameters were measured. All measurements were independently conducted by two blinded investigators to ensure reproducibility.

Systolic blood pressure and heart rate were measured noninvasively using a tail-cuff system before and after osmotic minipump implantation. Animals were placed in restrainers on a heated platform to maintain peripheral circulation, and multiple measurement cycles were performed per session. Valid readings were automatically recorded and averaged for subsequent analysis.

Aneurysm severity was classified according to a modified grading system based on morphological characteristics. Intact aortas without dilation were defined as Stage I, dilated aneurysms without hemorrhage as Stage II, aneurysms with hemorrhage as Stage III, and ruptured aneurysms as Stage IV. Grading was performed independently by three blinded investigators, with discrepancies resolved by consensus.

3.4 Histological, immunohistochemical, and immunofluorescent analyses

Formalin-fixed, paraffin-embedded human and murine tissue sections (4 μm) were prepared for histological and immunostaining analyses. Conventional histological staining included hematoxylin-eosin (HE), Naphthol AS-D (NASD) chloroacetate esterase, Verhoeff-Van Gieson (EVG), and Masson's trichrome. For immunohistochemistry (IHC) tissue sections were deparaffinized, subjected to antigen retrieval, and incubated with primary antibodies against myeloperoxidase (MPO), Hb, ferrylHb, and CD163, followed by HRP-based signal detection with DAB and hematoxylin counterstaining. Whole-slide images were acquired using a digital slide scanner.

For immunofluorescence analysis, cultured cells were fixed, permeabilized, and incubated with primary antibodies targeting ferrylHb, MPO, or CD163, followed by fluorophore-conjugated secondary antibodies. Nuclei were counterstained with Hoechst dye. Paraffin-embedded human and murine tissue sections were similarly processed for immunofluorescence labeling of ferrylHb in combination with macrophage- or neutrophil-associated markers, including CD163, MPO, or carboxypeptidase M (CPM). Fluorescent signals were visualized using appropriate Alexa Fluor-conjugated secondary antibodies.

3.5 Western blot analysis

Protein extracts were prepared from human arterial tissues, murine aneurysm samples, and cultured immune cells using standard lysis procedures supplemented with protease inhibitors. Tissue samples were homogenized under cold conditions, while cell lysates were obtained following experimental treatments with Hb, ferrylHb, phorbol 12-myristate 13-acetate (PMA), or GSK484. Protein concentrations were determined, and equal amounts of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes.

Membranes were incubated overnight with primary antibodies against Hb, ferrylHb, MPO, or CD163, followed by incubation with appropriate HRP-conjugated secondary antibodies where

applicable. Immunoreactive bands were visualized using a chemiluminescence detection system. Western blot analyses were performed on human control and aneurysmal tissues, murine aortic samples, and treated neutrophils or macrophages, as specified in the respective experiments.

3.6 Proteomic analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Proteins from murine and human aneurysmal aortic tissues were extracted, denatured, reduced, alkylated, and digested with trypsin. Resulting peptides were desalted, dried, and reconstituted in formic acid for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Peptides were separated on a C18 analytical column using a gradient of increasing acetonitrile concentration and analyzed on a high-resolution Orbitrap mass spectrometer operated in data-dependent acquisition mode, with dynamic exclusion to prevent repeated fragmentation of identical precursor ions.

Protein identification was performed using MaxQuant software against the respective human or mouse SwissProt databases, allowing common variable modifications and up to two missed cleavages. Identifications were validated in Scaffold software, with proteins required to have at least two unique peptides, a false discovery rate of 1%, and a minimum peptide probability of 95%. Structural visualization of hemoglobin was conducted using CAVER Analyst based on available PDB entries for human and murine hemoglobin.

3.7 Hemoglobin preparation and redox analysis

OxyHb (Fe^{2+}) was isolated from fresh human blood via DEAE ion-exchange chromatography, yielding 98-99% purity. Aliquots were snap-frozen in liquid nitrogen and stored at -70 °C. Purity was confirmed by silver staining, and concentrations were determined based on heme content.

Human and murine aortic tissues, as well as treated neutrophils, were homogenized and centrifuged, and the supernatants were analyzed spectrophotometrically to quantify hemoglobin in distinct redox states (oxyHb, metHb, ferrylHb) using established absorbance formulas. Total heme content in healthy and aneurysmal aortic tissues was measured according to standard protocols.

3.8 Isolation and *in vitro* treatment of neutrophils

Peripheral blood neutrophils were isolated from healthy donors using density-gradient centrifugation, followed by brief red blood cell lysis and washing steps. Purified cells were resuspended in culture medium and seeded into multiwell plates for experimental treatments. Neutrophils were exposed to Hb or ferrylHb at defined concentrations and time points, with some experiments including PMA activation or GSK484 co-treatment. Cellular responses and Hb oxidation were assessed at pre-specified intervals using microscopy and spectrophotometric analysis.

3.9 Polymorphonuclear leukocyte elastase measurement

Polymorphonuclear (PMN) elastase levels were quantified using a commercial ELISA kit. Test samples were incubated with detection reagents, followed by substrate addition and colorimetric development. Absorbance was measured at 450 nm, and concentrations were calculated in ng/ml according to the manufacturer's instructions.

3.10 RNA sequencing (RNA-seq) and transcriptomic analysis

Total RNA was extracted from human neutrophils, patient-derived aortic tissues, and murine aneurysm samples. Ribosomal RNA was depleted, and sequencing libraries were prepared using standard protocols for Illumina platforms. Libraries were sequenced to generate single end reads, which were aligned to the respective human or mouse reference genomes.

Raw data were normalized and analyzed for differential gene expression using DESeq2. Differentially expressed genes were identified between treatment groups or disease versus control samples, with statistical significance assessed via analysis of variance (ANOVA) or post hoc tests as appropriate. Gene Ontology (GO) enrichment analyses were performed to interpret functional significance, and results were visualized using heatmaps, dot plots, and bar graphs.

3.11 Cell culture, siRNA (small interfering RNA) transfection, and qRT-PCR (quantitative reverse transcription-polymerase chain reaction)

Human aortic endothelial cells (HAoECs) and smooth muscle cells (HAoSMCs) were maintained in CM199 or DMEM supplemented with 10% FBS, penicillin, streptomycin, and amphotericin B. Cells were expanded to -90% confluence and used between passages 4-6. For heme treatments, cells were washed with HBSS+ and incubated in serum- and antibiotic-free medium with defined concentrations of hemin for 2 hours. Following treatment, cells were washed and cultured in complete medium for 3, 6, or 16 hours to assess both early and delayed transcriptional responses.

HO-1 expression was selectively silenced in HAoECs using siRNA transfection with a non-targeting control for comparison. Transfections were performed according to standard protocols at a final siRNA concentration of 10 nM.

Total RNA was extracted from cultured cells, and cDNA was synthesized from 1 μg RNA. Quantitative PCR was performed to determine transcript levels of HO-1, IL1β, ICAM1, and NLRP3, with GAPDH as the endogenous control. Relative gene expression was calculated using the ΔΔCt method, enabling standardized assessment of heme-induced or HO-1-dependent transcriptional changes.

3.12 Statistical analysis

All statistical computations were carried out using GraphPad Prism 10 software (GraphPad Software Inc., La Jolla, CA, USA). Experimental data are presented as mean ± standard error of the mean (SEM), with the exception of measurements for mouse blood pressure, heart rate, and survival rates. The Shapiro-Wilk test was employed to verify the normality of data distribution, while Levene's test was utilized to assess the homogeneity of variances across groups. For datasets that met the assumptions of normality and equal variance, group comparisons were performed using Student's t-test or one-way ANOVA, followed by Sidak's post hoc testing as detailed in the corresponding figure legends. Survival curves were analyzed using the Log-rank test and Gehan-Breslow-Wilcoxon test. Pearson's correlation analysis was applied to explore the association between ferrylHb levels and heme content. A *P* value less than 0.05 was defined as statistically significant.

4 Results

4.1 Ferryl hemoglobin in the circulation of patients with ruptured abdominal aortic aneurysms

We developed a monoclonal antibody-based ELISA to quantitatively measure ferrylHb in serum. Blood samples were collected from 40 patients with ruptured AAA and 26 age- and sex-matched healthy controls. Serum ferrylHb concentrations were significantly higher in AAA patients (320.12 ± 339.26 μg/ml) compared to controls (122.36 ± 106.53 μg/ml), representing an approximately 2.6-fold increase ($P < 0.01$). These results indicate a strong association between elevated circulating ferrylHb levels and AAA rupture, suggesting a potential mechanistic role in disease progression.

4.2 Ferryl hemoglobin accumulates in human abdominal aortic aneurysms and is internalized by neutrophils and macrophages

Aneurysmal tissue specimens from ten AAA patients and healthy aortic tissues from five donors were analyzed to assess local Hb oxidation. Histopathology revealed intramural hemorrhage and mural thrombus formation in AAA tissues, in contrast to the intact structure of healthy aortas. Immunohistochemistry demonstrated pronounced ferrylHb accumulation in AAA walls, both extracellularly and within infiltrating cells, while healthy tissues lacked ferrylHb staining. Infiltrating cells exhibited granulocyte-like nuclear morphology and stained positively for NASD esterase and MPO, indicating neutrophil presence and activation, predominantly in adventitial and periadventitial hemorrhagic regions. CD163-positive macrophages were markedly increased in AAA tissues, suggesting enhanced recruitment or activation. Double immunostaining showed ferrylHb colocalization with MPO-positive neutrophils and CPM- or CD163-positive macrophages, indicating active uptake of oxidized Hb by these immune cells. Quantitative analysis revealed colocalization rates of 72.37% ± 3.88% with neutrophils and

78.92% ± 5.89% with CD163-positive macrophages, confirming that both cell types participate in ferrylHb internalization within AAA lesions.

4.3 Hemorrhagic abdominal aortic aneurysm is associated with ferryl hemoglobin formation, globin oxidation, and heme release

Western blot analysis of AAA tissue extracts revealed bands corresponding to Hb dimers and tetramers, indicating covalent crosslinking of globin chains characteristic of ferrylHb formation, whereas healthy aortic tissues showed minimal reactivity. Immunoprecipitation with anti-ferrylHb antibodies confirmed ferrylHb presence in AAA samples. Spectral analysis further demonstrated loss of the characteristic Hb peaks at 542 and 577 nm, consistent with oxidation, and quantitative assays confirmed markedly elevated ferrylHb levels in AAA tissues compared to undetectable levels in controls.

Total heme measurements showed significantly higher concentrations in AAA tissues (78.29 ± 23.27 μmol/mg protein) versus healthy controls (7.67 ± 5.86 μmol/mg protein), indicating heme release from oxidized Hb. High-resolution mass spectrometry identified oxidative and trioxidative modifications at βCys93, as well as additional modifications at βCys112 and αCys104, demonstrating that multiple cysteine residues undergo oxidative alterations. These findings provide biochemical evidence that hemorrhagic AAA promotes ferrylHb formation, globin oxidation, and heme liberation, contributing to local oxidative stress and inflammation.

4.4 Ferryl hemoglobin formation in angiotensin II -induced abdominal aortic aneurysm in apolipoprotein E knockout mice

ApoE$^{-/-}$ mice fed STD or HFD for 12 weeks were infused with AngII or saline to induce AAA. AngII caused progressive aortic dilation, more pronounced in HFD-fed mice, while blood pressure remained similar across groups. Control mice without AngII did not develop aneurysms. AAA severity was graded I-IV, with hemorrhage and rupture more frequent in HFD + AngII mice (17/19 and 8/19) than in STD + AngII mice (9/19 and 4/19). Spectral analysis

confirmed elevated metHb and ferrylHb in hemorrhagic tissues. Western blot showed Hb dimers and covalent globin cross-links, indicating ferrylHb formation.

Mass spectrometry revealed predominant βCys93 oxidation in mice, while human AAA also showed βCys112 and αCys104 modifications. FerrylHb accumulation correlated with increased heme levels ($R = 0.717$, $P = 0.030$).

These data indicate that AngII-induced AAA, especially with HFD, mimics the oxidative environment of human AAA, featuring ferrylHb formation, globin cross-linking, heme release, and cysteine oxidation, supporting this model for mechanistic studies of Hb oxidation in aneurysm progression.

4.5 Hemoglobin internalization by neutrophils and macrophages in murine hemorrhagic abdominal aortic aneurysm

To assess whether Hb uptake by immune cells occurs in murine AAA, aortic tissues from AngII-infused $ApoE^{-/-}$ mice fed STD or HFD were analyzed. Saline-infused controls maintained intact aortic architecture with preserved elastin. AngII treatment induced aneurysm formation with extensive elastic fiber degradation and increased cellular infiltration, which was more severe in HFD-fed mice.

IHC revealed extracellular Hb accumulation in regions of elastin disruption and adventitial hemorrhage. High-resolution imaging showed active internalization of Hb by neutrophils and macrophages within these areas, mirroring patterns observed in human AAA. This suggests a conserved mechanism whereby immune cells interact with oxidized Hb, promoting local oxidative stress and inflammation.

Extended AngII infusion (15 weeks) led to the formation of NETs in advanced aneurysmal lesions, evidenced by extracellular DNA decorated with MPO and NE. The emergence of NETs indicates an additional layer of neutrophil-mediated inflammation that may interact with extracellular Hb to amplify oxidative and proteolytic activity.

Overall, these results demonstrate that in AngII-induced hemorrhagic AAAs, extracellular Hb is internalized by neutrophils and macrophages, and NET formation contributes to the inflammatory and oxidative milieu, recapitulating mechanisms observed in human AAA tissues.

4.6 Ferryl hemoglobin uptake by neutrophils is mediated via CD163

CD163, a scavenger receptor known for clearing Hb-haptoglobin complexes and free Hb, was found to mediate ferrylHb internalization in neutrophils within aneurysmal tissues. In AngII- and HFD-induced AAA in ApoE$^{-/-}$ mice, ferrylHb strongly colocalized with CD163 on both neutrophils and macrophages, whereas healthy control tissues lacked such colocalization. Functional blockade of CD163 in isolated neutrophils significantly reduced ferrylHb uptake, confirming the receptor's key role in this process. These results indicate that CD163 on neutrophils actively mediates ferrylHb clearance, likely mitigating local oxidative stress and inflammation, and highlight a previously underappreciated protective function of neutrophil-expressed CD163 in hemorrhagic AAA.

4.7 Transcriptomic signature of human hemorrhaged abdominal aortic aneurysms

Bulk RNA sequencing (RNA-seq) of hemorrhaged AAA tissues compared with healthy aortic controls revealed distinct transcriptomic profiles, with principal component analysis showing clear separation between groups. A total of 4,327 differentially expressed genes (DEGs) were identified, including 2,473 upregulated and 1,854 downregulated genes in AAA. Upregulated genes were enriched in pathways related to neutrophil activation, inflammatory signaling, oxidative stress, and iron metabolism, highlighting the central role of neutrophil-driven inflammation and iron dysregulation in AAA pathogenesis.

Targeted analysis of neutrophil-associated genes showed marked upregulation of S100A8, ADAM8, DOCK8, and SELL, consistent with enhanced neutrophil recruitment, motility, and adhesion within aneurysmal lesions. Key inflammatory cytokines, including IL1β and IL6, were also elevated, supporting a pro-inflammatory microenvironment. Genes involved in iron

metabolism, such as HBB, HMOX1, and LTF, exhibited altered expression, reflecting extracellular Hb accumulation, heme catabolism, and protective iron sequestration responses. Collectively, these data indicate that ferrylHb accumulation, neutrophil activation, and iron homeostasis dysregulation act in concert to drive inflammation and oxidative stress in hemorrhagic AAA, providing molecular evidence for potential therapeutic targets to mitigate aneurysm progression and rupture.

4.8 Ferryl hemoglobin drives macrophage transcriptomic reprogramming overlapping with abdominal aortic aneurysms

Comparative transcriptomic analysis of human AAA tissues and primary macrophages exposed to ferrylHb for 8 hours revealed a substantial overlap of 884 DEGs, including 685 upregulated and 199 downregulated genes, indicating that ferrylHb is a major driver of the transcriptional changes observed in aneurysmal tissue. Overlapping genes included oxidative stress-related enzymes (HMOX1, HBA1), pro-inflammatory mediators (IL6, CCL8, CCL9), and multiple MMP family members, linking ferrylHb exposure to redox imbalance, inflammation, and ECM remodeling. Functional enrichment further highlighted processes such as inflammatory responses, vascular remodeling, angiogenesis, and blood vessel development. Disease enrichment analysis corroborated associations with AAA, vascular inflammation, and arteriosclerotic lesions. Collectively, these data demonstrate that ferrylHb acts as an upstream regulator in macrophages, orchestrating oxidative stress, inflammatory signaling, and vascular remodeling pathways that recapitulate the pathological transcriptomic signature of human AAA.

4.9 Murine abdominal aortic aneurysms transcriptomes recapitulate human hemorrhagic signatures

Bulk RNA-seq of abdominal aortic tissues from $ApoE^{-/-}$ mice revealed that AngII-induced AAA, particularly under HFD conditions, produced robust and reproducible transcriptomic remodeling. Comparisons identified thousands of DEGs, with upregulated genes linked to

neutrophil functions (S100A8, S100A9), inflammatory cytokines (IL6, IL1β, CCL7, IL1R2), and hemoglobin family members (HBA, HBB), mirroring patterns observed in human hemorrhagic AAA. GO enrichment highlighted pathways including NET formation, inflammatory responses, iron homeostasis, and oxidative stress, reflecting key pathological mechanisms. Cellular deconvolution indicated neutrophils as the predominant population in aneurysmal tissue, emphasizing their central role in driving inflammation and oxidative damage. Overall, HFD synergized with AngII to exacerbate neutrophil activation, inflammation, iron dysregulation, and oxidative stress, recapitulating the molecular and cellular landscape of human hemorrhagic aneurysms and highlighting factors that promote rupture risk.

4.10 Ferryl hemoglobin activates neutrophil transcription and promotes macrophage recruitment

Bulk RNA-seq of primary human neutrophils exposed to ferrylHb revealed that, contrary to their conventional view as transcriptionally quiescent cells, neutrophils exhibit robust transcriptional activity. FerrylHb stimulation upregulated pathways linked to inflammatory signaling, oxidative stress responses, and iron homeostasis, recapitulating signatures observed in human and murine aneurysmal tissues. Notably, genes and pathways associated with macrophage chemotaxis, migration, and phagocytosis, such as CCL4, CCR7, IL18R1, and IL6, were significantly induced, indicating that ferrylHb-activated neutrophils may actively recruit and activate macrophages within the aneurysmal microenvironment. These findings suggest a mechanistic cascade in which ferrylHb drives neutrophil transcriptional responses that, in turn, facilitate macrophage-mediated inflammation and tissue remodeling, thereby amplifying oxidative stress and contributing to AAA progression and rupture.

4.11 Neutrophil activation by ferryl hemoglobin

FerrylHb potently activates neutrophils, as evidenced by robust secretion of MPO and NE following 24-hour exposure, with levels significantly higher than those induced by native Hb.

Untreated neutrophils showed no detectable release, confirming the specificity of this activation. Moreover, co-incubation with neutrophils promoted the oxidation of Hb, with ferrylHb constituting a notable fraction of the total hemoglobin pool. These results indicate a feed-forward mechanism in which ferrylHb triggers neutrophil degranulation while maintaining its oxidized state, thereby sustaining a pro-inflammatory and oxidative environment within aneurysmal lesions.

4.12 Neutrophil-released elastase promotes aortic elastin degradation

Supernatants from ferrylHb-stimulated neutrophils were applied to human aortic tissue sections, revealing extensive fragmentation of elastin fibers compared to intact fibers in untreated controls or tissue exposed to supernatants from unstimulated neutrophils. Quantitative analysis confirmed significantly enhanced elastin degradation, comparable to the effect of purified elastase. These results demonstrate that neutrophil activation by ferrylHb releases elastase capable of degrading vascular elastin, highlighting a key mechanism by which ferrylHb contributes to aortic wall weakening in AAA.

4.13 Hemoglobin induces NETosis in neutrophils

Prolonged exposure of neutrophils to ferrylHb or Hb (16-24 hours) triggered NET formation, with extracellular DNA, MPO, and ferrylHb co-localizing within NET structures. FerrylHb was detected in neutrophils treated with Hb alone, indicating that neutrophils promote Hb oxidation during NETosis. Spectrophotometric and Western blot analyses confirmed the generation of ferrylHb by neutrophils, demonstrating that Hb-driven NETosis contributes to oxidative and inflammatory amplification within aneurysmal lesions.

4.14 PAD4 (peptidylarginine deiminase 4) mediates ferryl hemoglobin-induced NETosis

FerrylHb-triggered NET formation is dependent on PAD4, which facilitates histone citrullination and chromatin decondensation. Inhibition of PAD4 with GSK484 markedly

reduced extracellular MPO release and NET extrusion in neutrophils, while intracellular activation markers, including CD163 and MPO, remained detectable. Quantitative analysis showed strong colocalization of ferrylHb with CD163, indicating that PAD4 inhibition selectively blocks NET release without abolishing neutrophil activation. Similarly, ferrylHb-induced CD163 upregulation in macrophages was reversed by GSK484. These results demonstrate that PAD4 is a key mediator of ferrylHb-driven NETosis, and its pharmacological inhibition may mitigate NET-associated vascular damage while preserving essential immune functions in hemorrhagic AAA.

4.15 Heme-induced inflammatory activation in vascular cells is modulated by heme oxygenase-1

Heme exposure triggered inflammatory responses in both vascular ECs and SMCs, but these responses were differentially regulated by HO-1. In ECs, heme induced robust HO-1 expression, which effectively suppressed IL1β and ICAM1 upregulation; silencing HO-1 unmasked strong pro-inflammatory activation. In contrast, SMCs responded to heme with IL1β and ICAM1 induction independently of HO-1, while NLRP3 inflammasome expression was further amplified upon HO-1 knockdown. These findings indicate that HO-1 provides cell type-specific anti-inflammatory protection in the vascular wall, mitigating IL1β-ICAM1 signaling in ECs and modulating inflammasome-driven responses in SMCs, highlighting its critical role in limiting heme-driven vascular inflammation during AAA pathogenesis.

4.16 Normosang (heme arginate) attenuates abdominal aortic aneurysm progression via heme oxygenase-1 induction

To evaluate the protective role of HO-1 in AAA, $ApoE^{-/-}$ mice on HFD receiving AngII infusion were treated with Normosang, a pharmacological HO-1 inducer, with or without the HO-1 inhibitor tin protoporphyrin IX (SnPP). Normosang markedly reduced aneurysm formation, luminal expansion, and the incidence of intramural hemorrhage, whereas co-treatment with

SnPP abolished these protective effects. Biochemical and histological analyses confirmed that Normosang robustly upregulated HO-1 and H-ferritin expression in the vascular wall, whereas SnPP blunted this induction. Correspondingly, Normosang treatment suppressed pro-inflammatory cytokines IL1β and TNFα, an effect reversed by HO-1 inhibition. These findings demonstrate that pharmacological induction of HO-1 confers structural and anti-inflammatory protection against AAA, while its enzymatic activity is essential for these benefits.

5 Discussion

Hb undergoes continuous auto-oxidation, generating metHb and reactive oxygen species, a process intensified in inflammatory microenvironments by neutrophil- and macrophage-derived ROS. Here, we demonstrate for the first time the presence of ferrylHb in the circulation of patients with ruptured AAA, indicating substantial Hb oxidation during hemorrhagic transformation. FerrylHb accumulation was confirmed in human AAA tissues, characterized by Hb dimers, tetramers, and oxidative modifications at βCys93, βCys112, and αCys104, supporting its role in amplifying oxidative stress within aneurysmal walls. The AngII-induced AAA model in $ApoE^{-/-}$ mice recapitulated key pathological features, including Hb oxidation, intramural hemorrhage, and neutrophil/macrophage infiltration, validating its use for mechanistic studies.

FerrylHb localizes both extracellularly in hematomas and intracellularly within neutrophils and macrophages, where it is internalized via CD163-mediated endocytosis independent of haptoglobin. Internalized ferrylHb triggers neutrophil transcriptional activation, degranulation, and NETosis, promoting elastin degradation and amplifying local inflammation. PAD4 inhibition selectively blocked NET formation while preserving intracellular activation, highlighting a potential therapeutic strategy to limit tissue damage without impairing essential immune functions. Similarly, ferrylHb induced PAD4-dependent CD163 upregulation in macrophages, underscoring its role in regulating immune responses in AAA.

Extracellular heme released from oxidized Hb activates vascular endothelial cells and smooth muscle cells, upregulating IL1β, ICAM1, and NLRP3 and driving inflammation. HO-1 counteracts heme-mediated cytotoxicity, degrading heme and sequestering iron via ferritin. Pharmacological induction of HO-1 with Normosang enhanced these protective mechanisms, reduced aneurysm formation and hemorrhage, and suppressed local cytokine expression, whereas HO-1 inhibition worsened vascular damage and inflammation. Collectively, our

findings reveal a coordinated interplay between ferrylHb, neutrophils, macrophages, and heme-HO-1 signaling in AAA pathogenesis, positioning ferrylHb as both a mechanistic driver of lesion progression and a potential biomarker for rupture risk, while highlighting PAD4 and HO-1 as therapeutic targets.

6 Summary

Abdominal aortic aneurysm (AAA) compromises vascular integrity through inflammation-driven tissue remodeling and intramural hemorrhage, elevating rupture risk. In this study, oxidized hemoglobin (Hb), particularly ferrylHb, was detected in the circulation of patients with ruptured AAA, indicating systemic dissemination of oxidative stress products. FerrylHb formation was confirmed in hemorrhagic AAA tissues, involving oxidative modifications at βCys93, βCys112, and αCys104, alongside the formation of covalent Hb dimers, tetramers, and higher order multimers. The angiotensin II -induced AAA model in apolipoprotein E deficient mice recapitulated these pathological features, including vascular hemorrhage and ferrylHb accumulation. Immunohistochemistry revealed that extracellular ferrylHb is internalized by neutrophils via CD163-mediated endocytosis, activating granulocytes within the aneurysmal wall.

Following uptake, neutrophils exhibited transcriptional reprogramming toward a proinflammatory state, with upregulation of genes related to chemotaxis, cytokine production, and macrophage recruitment. Prolonged ferrylHb exposure triggered degranulation, releasing elastase and myeloperoxidase, which accelerated elastin degradation and weakened the aortic wall. FerrylHb also promoted neutrophil extracellular traps formation, enhancing local inflammation and thrombogenic potential. RNA-seq profiling of human AAA tissues identified 4,327 differentially expressed genes, enriched for neutrophil activation, inflammatory signaling, iron metabolism, and vascular remodeling. Notably, 43% of these DEGs overlapped with genes upregulated in ferrylHb-treated macrophages *in vitro*, highlighting ferrylHb as a key driver of inflammatory and tissue remodeling programs.

Mechanistic studies in endothelial cells and vascular smooth muscle cells demonstrated that heme induces IL1β, ICAM1, and NLRP3 expression, while HO-1 counteracts this proinflammatory response. Pharmacological induction of HO-1 with Normosang mitigated

aneurysm progression and inflammation, whereas HO-1 inhibition exacerbated vascular injury, supporting the heme-HO-1-H-ferritin axis as a central regulator of AAA pathogenesis. Collectively, these findings reveal a coordinated interplay between ferrylHb, neutrophils, macrophages, and vascular cells in driving oxidative stress, immune activation, and extracellular matrix degradation. Detection of ferrylHb in circulation may serve as a biomarker for disease severity and rupture risk, and targeting the Hb- neutrophils or macrophages axis offers a promising strategy for therapeutic intervention in AAA.

7 Novel findings

1. Circulating ferryl hemoglobin is elevated in patients with ruptured abdominal aortic aneurysms and accumulates within hemorrhaged aneurysmal tissue.
2. Hemoglobin oxidation to the ferryl state in abdominal aortic aneurysm is accompanied by globin crosslinking and heme release.
3. Oxidative modifications of hemoglobin in abdominal aortic aneurysm involve specific cysteine residues.
4. Ferryl hemoglobin accumulation and heme release are main features in angiotensin II-induced abdominal aortic aneurysms in ApoE$^{-/-}$ mice.
5. Ferryl hemoglobin and free heme levels are strongly correlated across human and mouse aneurysms.
6. CD163 mediates ferryl hemoglobin uptake in neutrophils as well as macrophages.
7. Ferryl hemoglobin induces transcriptomic changes in macrophages that overlap with those observed in aneurysmal tissue.
8. Ferryl hemoglobin-stimulated neutrophil products promote elastin degradation in human aortic tissue.
9. Ferryl hemoglobin induced NETosis and CD163 upregulation are dependent on PAD4 signaling.
10. Heme induces inflammatory responses in endothelial and smooth muscle cells that are restrained by heme oxygenase-1.
11. Pharmacological induction of heme oxygenase-1 attenuates abdominal aortic aneurysm formation and intramural hemorrhage.
12. Heme oxygenase-1 induction promotes H-ferritin expression and suppresses inflammatory cytokine production in aneurysmal tissue.

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List of Publications

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List of publications related to the dissertation

1. **Ding, Y.**, Potor, L., Sótonyi, P., Szappanos, Á., Gyurok, G. P., Póliska, S., Patsalos, A., Méhes, G., Beke, L., Sikura, K. É., Zavaczki, E., Gáll, T., Pethő, D., Fintha, A., Nagy, B., Juhász, B., Nagy, L., Balla, G., Balla, J.: Heme as a Pro-Inflammatory Stimulus in Abdominal Aortic Aneurysm.
   Antioxidants. 15 (2), 1-21, 2026.
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   IF: 6.6 (2024)
2. **Ding, Y.**, Potor, L., Katona, É., Sótonyi, P., Szappanos, Á., Gyurok, G. P., Méhes, G., Hendrik, Z., Fintha, A., Gergely, P., Benyó, Z., Combi, Z., Sikura, K. É., Beke, L., Németh, N., Szabó, B., Fürtös, I., Kalló, G., Csősz, É., Póliska, S., Patsalos, A., Debus, E. S., Nagy, L., Balla, G., Balla, J.: Oxidation of hemoglobin to ferryl hemoglobin contributes to remodeling of the artery wall in abdominal aortic aneurysm.
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3. Combi, Z., Potor, L., Nagy, P., Sikura, K. É., Ditrói, T., Jurányi, E. P., Galambos, K., Szerafin, T., Gergely, P., Whiteman, M., Torregrossa, R., **Ding, Y.**, Beke, L., Hendrik, Z., Méhes, G., Balla, G., Balla, J.: Hydrogen sulfide as an anti-calcification stratagem in human aortic valve: altered biogenesis and mitochondrial metabolism of H2S lead to H2S deficiency in calcific aortic valve disease.
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6. Jiang, B., Zhang, X., **Ding, Y.**, Zhai, Z.: Medicine for treating artery related diseases and application thereof. 2021
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