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Julian Hurtado Date

**Understanding the Functional Contribution of Heme to the Progression of Atherosclerosis in Beta Thalassemia**

By

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Graduate Division of Biological and Biomedical Sciences
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# Understanding the Functional Contribution of Heme to the Progression of Atherosclerosis in Beta Thalassemia

By

**Julian Hurtado**
**B.S., Johns Hopkins University 2017**

Advisor: W. Robert Taylor M.D., Ph.D.

An abstract of
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## Abstract

### Understanding the Functional Contribution of Heme to the Progression of Atherosclerosis in Beta Thalassemia

By

Julian Hurtado

***Background***

Children with Beta Thalassemia (BT) present with an increase in carotid intima-medial thickness, an early sign suggestive of premature atherosclerosis. However, it is unknown if there is a direct relationship between BT and atherosclerotic disease. BT is a hemolytic anemia which has increased free heme in the vasculature. We hypothesize that heme-mediated oxidative stress creates a proatherogenic environment.

**Methods**

Both male and female, WT (littermates) and BT (Hbbth3/+) mice were placed on a 3-month high fat diet with LDL receptor suppression via proprotein convertase subtilisin/kexin type 9 (PCSK9) gain-of-function mutation (D377Y). In addition, the effect of hemopexin (HPX) on the progression of atherosclerosis was evaluated by overexpressing HPX with an adeno-associated virus. Atherosclerosis was evaluated in the descending aorta via *en face* analysis and histology of the aortic roots. In parallel, we evaluated atherosclerotic plaque accumulation in a drug-induced model of hemolysis using phenylhydrazine (PHZ). We also evaluated the effect of defiriprone (DFP)-mediated iron chelation in the progression of atherosclerosis in BT mice.

**Results**

Aortic *en face* analysis revealed elevated plaque accumulation in both male and female BT mice compared to WT mice (WT female: 10.6 ± 2.9 % vs. BT female: 23.3 ± 1.5 % and WT male: 28.1 ± 2.4 % vs. BT male: 45.5 ± 2.4 %, total percentage plaque area relative to luminal area, mean ± SEM). Hemopexin therapy was able to decrease plaque accumulation in BT mice measured via aortic en face (BT: 45.5 ±. 2.4 % vs. BT + HPX: 31.7 ± 2.5 %) and aortic root lesion area analysis (BT: 444.7 ± 38.1 $\mu m^2 \times 10^3$ vs BT + HPX: 318.8 ± 28.6 $\mu m^2 \times 10^3$).

**Conclusion**

Our data demonstrate for the first time that the underlying pathophysiology of BT leads to accelerated atherosclerosis and shows that heme contributes to atherosclerotic plaque development in BT.

# Understanding the Functional Contribution of Heme to the Progression of Atherosclerosis in Beta Thalassemia

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**List of abbreviations**

Adeno-associated virus (AAV)

Beta Thalassemia (BT)

Bone marrow transplants (BMT)

Divalent metal transporter 1 (DMT-1)

Ferroprotein (FPN)

Hematopoietic stem cell transplantation (HSCT)

Hematoxylin and eosin (H&E)

Heme Oxygenease-1 (HO-1)

Hemoglobin (Hb)

Hemopexin (HPX)

Hereditary haemochromatosis (HH)

High density lipoprotein (HDL)

human leukocyte antigens (HLA)

Interleukin-6 (IL-6)

Lactate dehydrogenase (LDH)

Low density lipoprotein (LDL)

Nicotinamide adenine dinucleotide phosphate (NADPH)

Nitric Oxide (NO)

Nitric oxide synthase (NOS)

Non-transfusion dependent thalassemia (NTDT)

Nuclear factor kappa-light-chain-enhancer of activated B (nF-kB)

Phenylhydrazine (PHZ)

Proprotein convertase subtilisin/kexin type 9 (PCSK-9)

Protein quality control (PQC)

Reactive oxygen species (ROS)

Sickle cell disease (SCD)

Signal transducer and activator of transcription (STAT)

Smooth muscle cells (SMC)

Toll-like receptor 4 (TLR4)

Transferrin (Tf)

Transferrin Saturation (TS)

Transfusion dependent thalassemia (TDT)

Tumor necrosis factor (TNF)

# Chapter 1: Introduction

## 1.1 Introduction

Beta Thalassemia (BT) is an autosomal recessive hemoglobinopathy with a global prevalence of 80 million carriers (Galanello and Origa, 2010). Previously, patients with BT had shortened life spans due to severe anemia and iron toxicity, but patients are now living longer due to regular blood transfusions and improvements in iron chelation (Ansari-Moghaddam *et al.*, 2018; Dhanya *et al.*, 2020). Consequently, a growing body of literature has highlighted the importance of vascular complications related to the underlying pathophysiology of hemoglobinopathies (Morris, 2008a; Kato *et al.*, 2009; Potoka and Gladwin, 2015; Tantawy *et al.*, 2015). Here, we will provide conclusive evidence for accelerated atherogenesis in BT and elucidate the contribution of heme to this phenotype. Furthermore, we will investigate adeno-associated virus (AAV) hemopexin therapy as a potential therapeutic strategy in this disease paradigm.

### 1.1.1 Underlying Pathophysiology in Beta Thalassemia

BT is primarily characterized as a disease of aberrant hemoglobin synthesis in the beta globin gene whose severity is determined by inherited genes, co-mutations, and disease management.

#### *1.1.1.1 Aberrant Hemoglobin Synthesis*

BT is an autosomal recessive disease defined by a mutation in the beta globin gene, HBB, found on chromosome 11 which leads to an absence ($B^0$) or reduced ($B^+$) formation of the beta globin protein (Nienhuis and Nathan, 2012; Needs *et al.*, 2022). BT is a molecularly heterogenous disease characterized by over 200 mutations that impair beta globin production (Cao and Galanello, 2010). These mutations are most commonly point mutations, such as IVS1-nt1 G→A mutation (Galanello and Origa, 2010). The

hemoglobin tetramer is comprised of two pairs of polypeptide subunits: two α-globin like (α, ζ) chains and two β-globin like (β, ε, γ, δ) chains. An adult hemoglobin molecule (HbA [$\alpha_{1.2}$ $\beta_{1,2}$]) is a tetramer comprised of two alpha chains ($\alpha_1$, $\alpha_2$) and two beta chains ($\beta_1$, $\beta_2$) that have a heme (a porphyrin ring bound to an iron) group as seen in figure 1.1 (Sheffield S, 2022). Typically, humans gradually transition from fetal hemoglobin (HbF [$\alpha_{1,2}$ $\gamma_{1,2}$]) to HbA around 12 weeks of gestation, and by 6 months of age, they are using greater than 95% HbA with the remainder in $HbA_2$ [$\alpha_{1.2}\delta_{1,2}$] and HbF (Wang and Thein, 2018). Ultimately, adult hemoglobin synthesis is decreased in BT patients which causes anemia and a compensatory increase in HbF and $HbA_2$ to meet physiologic demands.

Due to decreased beta globin gene production, excess alpha-globin aggregates, called hemichromes, form large inclusion bodies that lead to instability and premature erythrocyte destruction (Galanello and Origa, 2010). Hemichromes generate intracellular reactive oxygen species (ROS) and clustering of cytoplasmic Band 3 anion transport protein (BAND-3) (Morris, 2008). Band-3 is a glycolytic enzyme that mediates the flux of chloride and bicarbonate, links with ankyrin and protein 4.2 for membrane stability, and helps promote NADPH generation (a necessary cofactor for antioxidant enzymes) by stabilizing enzymes in the membrane for the pentose phosphate pathway. BAND-3 clusters lead to membrane blebbing and binding of anti-BAND-3 antibodies that can lead to complement activation and subsequent macrophage clearance. As a result, the hallmark of BT is ineffective erythropoiesis: premature destruction of erythrocytes caused by aggregates of excess, unpaired alpha hemichromes and pools of immature erythroblasts (Needs *et al.*, 2022). As a result, the body compensates through

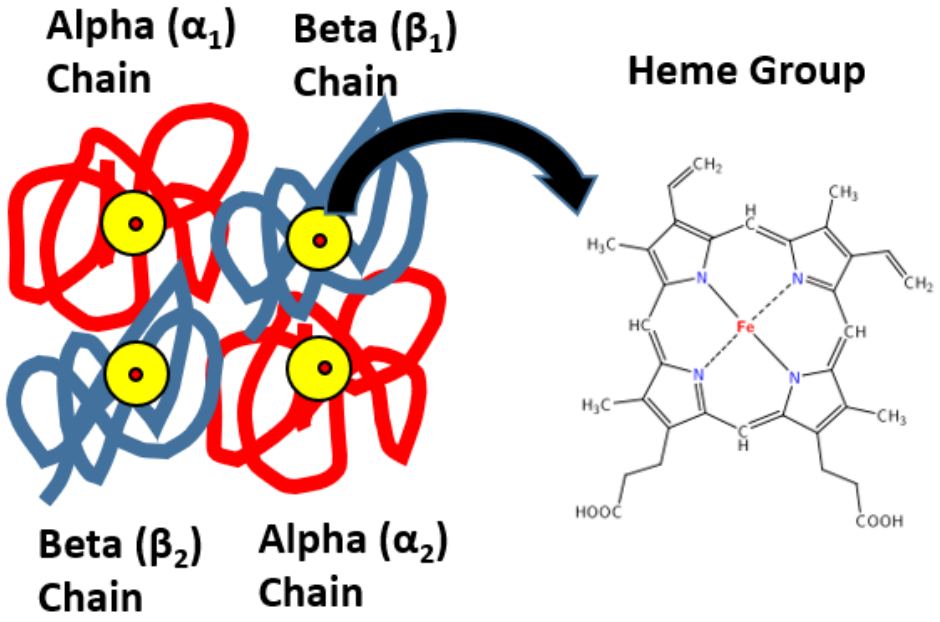


**Figure 1.1 Adult Hemoglobin.** Hemoglobin is a protein molecule consisting of four subunits arranged in a quaternary structure. Each subunit is made up of a heme group and a globin chain. The heme group is a porphyrin ring that contains an iron ion in its center, which binds to oxygen molecules. The globin chain, on the other hand, provides the structural framework for the protein and is made up of alpha and beta chains, depending on the type of hemoglobin. When fully assembled, the four subunits come together to form a spherical shape with a central cavity where the heme groups are located. The iron ions in each heme group can bind to oxygen, and when all four heme groups are bound to oxygen, the protein is in its oxygenated state, known as oxyhemoglobin.

extramedullary hematopoiesis, increased gastrointestinal iron absorption, upregulation of erythropoietin, and decreased hepcidin expression (Cao and Galanello, 2010; Needs *et al.*, 2022). Erythropoietin (EPO) is a kidney-secreted glycolytic hormone whose production is stimulated by decreased $pO_2$ and binds to the surface of CD34+ hematopoietic stem cells via the EPO receptor (Schoener and Borger, 2022). EPO mediates increases in production of red blood cells by activating the Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Hodges *et al.*, 2007). Nisli et al. and many other have found that EPO levels were significantly higher in BT (215.1 ± 144.5 mU/mL) versus control (9.3 ± 4.6. mU/mL) (Nişli *et al.*, 1997). Hepcidin is a peptide liver hormone that is the key regulator of iron metabolism by triggering the degradation of ferroportin (FPN), the only known iron exporter. In BT, hepcidin is decreased due to anemia, and consequently, (FPN) located in enterocytes and tissue/macrophages leads to increase iron absorption and tissue accumulation of iron, respectively (Nemeth, 2010). BT pathophysiology leads to premature destruction of erythrocytes caused by excess/unpaired alpha globin chain aggregates, anemia, and ineffective compensatory mechanisms.

*1.1.1.2 Disease Severity*

The severity of the anemia in BT is dictated by the imbalance of alpha (α) and gamma (γ) globin chains. Mutations that decreases γ chain synthesis and/or increase α chain synthesis lead to a greater imbalance in the two globin chains needed for HbF formation which worsens anemia in BT patients (Srinoun *et al.*, 2009; Needs *et al.*, 2022). Conversely, an increase γ chain and/or decreases in α chain synthesis would improve anemia. The severity of BT is clinically categorized as major, intermedia, or minor. This

is determined by the inherited HBB mutation ($B^0$ or $B^+$) and coinheritance of other genetic factors (Galanello and Origa, 2010). The reduced production of the α-globin gene leads to fetal hemoglobin synthesis of over 95% for BT major, 30-90% for BT intermedia, and up to 30% for BT minor (Bajwa and Basit, 2022). In BT patients, there are several mutations and coinherited hemoglobinopathies that affect disease severity by creating an imbalance in the alpha and gamma globin chains. For example, α-hemoglobin stabilizing protein (AHSP) is a molecular chaperone protein for alpha globin chains that facilitates folding, stabilizes the protein before hemoglobin incorporation, and prevents alpha chains aggregates if its binding capacity is not exceeded. Thus, mutations that decrease the level of AHSP expression will worsen disease severity by increasing unpaired alpha chains. Likewise, mutations decreasing the protein quality control (PQC) pathway, ubiquitin–proteasome system (UPS), and lysosome-autophagy impair the cells ability to clear unpaired alpha chains, which can worsen disease severity (Nienhuis and Nathan, 2012). On the other hand, genome-wide association studies have identified mutations leading to decreased B-cell lymphoma/ leukemia 11A (BCL11A) expression (a gene that suppresses γ production with mRNA transcription regulator Lin-28 Homolog B (LIN28B) causing increased HbF production, decreased α/γ globin imbalance all of which effectively decreases the severity of BT (Khosravi *et al.*, 2019). In addition, coinherited hemoglobinopathies such as α-thalassemia can decrease the severity of BT because of the reduced production of alpha chains (Needs *et al.*, 2022). In BT patients, it is crucial to understand beta globin production, alpha/gamma globin chain production/protein stabilization/clearance, and other coinherited hemoglobinopathies.

## 1.2 Clinical Manifestation and Disease Management in Beta Thalassemia

### 1.2.1 Clinical Manifestation

Despite BT being a disease that only affects beta globin gene production, aberrant hemoglobin synthesis leads to several pathophysiological complications. Traditionally, clinical characteristics in untreated BT patients are jaundice, growth retardation, hepatosplenomegaly, leg ulcers, extramedullary hematopoiesis, and skeletal changes (e.g. “hair on end appearance”) that result from expansion of the bone marrow (Needs *et al.*, 2022). BT patients have abnormal hematologic parameters indicating conditions such as anemia and iron overload. For example, *Sherief et al.*, in a population of 65 BT major children age 5-18 compared to 50 age and sex match children found the following: Elevated WBC (BT: 11.3 ± 4.6 vs. Control: 7.3 ± 1.8,units=$10^3/mm^3$), decreased hemoglobin (BT: 6.8 ± 0.9 vs. Control: 12.6 ± 0.7, units=gm/dl), increased platelets (BT: 478.4 ± 279.4 vs. Control: 276 ± 77.6, units=$10^3/mm^3$), increased bilirubin (BT: 1.4 ± 0.6 vs. Control: 0.6 ±0.2, units =mg/dl), increased serum ferritin ( BT: 2490 ± 1579 vs. Control: 83 ± 32, units =ng/ml), and C-Reactive Protein ( BT: 5.9 ± 5.9 vs. Control: 0.9 ± 0.9, units =ng/dl) (Sherief *et al.*, 2017). Due to the excess hemolysis in BT that causes splenomegaly, patients undergo a splenectomy which reduces the transfusion requirement. BT major and intermedia/minor with mutations that lead to greater α/γ imbalance require a transfusion and iron chelation regiment. As a result, transfusion dependent status has emerged as the guiding phenotypic classification because it better reflects disease severity as opposed to major, intermedia, and minor classification which have huge variations in clinical manifestation within each classification (Cao and Galanello, 2010).

*1.2.2 Disease management*

*1.2.2.1 Anemia and Iron*

BT is categorized into transfusion-dependent thalassemia (TDT) or non-transfusion-dependent thalassemia (NTDT). TDT patients require regular, lifelong blood transfusions every two to five weeks targeting hemoglobin levels of around >9-10 g/dl (Cappellini *et al.*, 2008). In addition to anemia and subsequent blood transfusion management, iron overload is the most important clinical manifestation to monitor in BT via serum ferritin and T2* weighted MRI. Both TDT and NTDT patients have iron overload because of constant blood transfusion and increased iron absorption (Origa, 2017). Clinically, myocardial siderosis, iron accumulation in the heart, is the leading cause of death in BT (Yang *et al.*, 2014). In fact, cardiac complications were responsible for 71% (Borgna-Pignatti *et al.*, 2005) of the causes of premature death in BT major patients, but survival rates in recent years have improved due to iron chelation therapy (Ansari-Moghaddam *et al.*, 2018). Serum ferritin levels over 2500 ng/ml and below 1000 ng/ml are associated with increased risk of heart disease/death and prolonged survival respectively (Krittayaphong *et al.*, 2018). NTDT patients also have increased iron accumulation via decreased levels of the hepatic hormone hepcidin by inhibiting the negative regulation of FPN which leads to increased iron accumulation (Cappellini and Motta, 2017). Three iron chelators used alone and in combination are the mainstays in BT: deferoxamine (subcutaneous), deferiprone (oral), and deferasirox (oral) (Hoffbrand *et al.*, 2012). In addition, there are several iron restricting drugs in preclinical development as seen in table 1. Disease management in BT focuses on managing anemia through blood transfusion and iron overload through chelation therapy.

| Drug | Mechanism | Material | Reference |
|---|---|---|---|
| Minihepcidins | Hepcidin upregulation | Th3/+ mice | (Casu *et al.*, 2016) |
| TMPRSS6-LRx | TMPRSS6 disruption | Th3/+ mice, monkeys | (Nai *et al.*, 2016) |
| Exogenous Transferrin | TfR1 downregulation | Th3/+ mice | (Li *et al.*, 2010b) |
| ERFE downregulation | Hepcidin upregulation | Th3/+ mice | (Nai *et al.*, 2016) |
| IOX1 | Selective silencing of α-globin | Erythroid cultures | (Mettananda *et al.*, 2017) |

**Table 1.1 Preclinical Iron Restricting Agents in BT.** There are several different iron modulating pharmacological agents currently in preclinical development for BT.

*1.2.2.2 Bone marrow transplantation and other therapies*

*1.2.2.2.1 Bone marrow transplantation*

Advancement in our use and understanding of bone marrow transplants and other pharmacological/gene therapies for BT have improved patient disease management. In BT allogeneic hematopoietic stem cell transplantation (HSCT) is the only established cure. HSCT uses a human leukocyte antigen (HLA)-identical donor, but limited family donors, transplant-related mortality, and limited resource availability in developing nations leads to limited use. In adult BT patients with risk factor such as poor iron chelation history, the disease free survival (DFS) is 65% and the mortality in risk is 7% (Lucarelli *et al.*, 2012). In addition, widespread use of bone marrow transplants for BT has been affected due to socio-economic factors; BT prevalence is largely regionalized to the Mediterranean region, southeast Asia, and Africa which have several barriers for treatment. For example, in India, the use of bone marrow transplants is lower because 71% of health spending is out of pocket and there is a lower living wage (John *et al.*, 2018). The alternative therapy (transfusion-chelation) is cheaper at $629-$2300 (John *et al.*, 2018) yearly compared to BMT $36,000-$88,000 (Khera *et al.*, 2012). Despite being curative, bone marrow transplant availability is affected by limited donors, BMT rejection/mortality exacerbated by poor disease management, and socio-economic factors.

*1.2.2.2.2 Other therapies*

Several other therapies targeting new pathways are currently under investigation. Pharmacologic induction of gamma chain via hydroxyurea, a drug used for decades in sickle cell disease (SCD), has improved hematological parameters in NTDT by reducing

spleen size and improving anemia in a few clinical studies (Yasara *et al.*, 2021). Hydroxyurea is an antimetabolite S-phase-specific drug that reversibly inhibits ribonucleoside diphosphate reductase enzyme, an enzyme used in DNA synthesis that converts ribonucleosides into deoxyribonucleosides. This ultimately provides selective advantage to the expansion of the HbF cell population over the rapidly dividing HbA population producing erythroid progenitors. The full mechanism of hydroxyurea upregulating HbF is poorly understood (Agrawal *et al.*, 2014; Yasara *et al.*, 2021). There are no conclusive, large clinical studies on hydroxyurea that have provided clinical effectiveness. Studies have indicated large non-responder groups that could be caused by several confounding factors such as chelation and splenectomy Algiraigri *et al.*, 2017).

Pharmacological targets improving ineffective erythropoiesis is an ongoing area of clinical investigation with the three drugs: Luspatercept (ACE-536), Sotatercept (ACE-011), and Ruxolitinib (Cappellini and Motta, 2017; Needs *et al.*, 2022). Both, Luspatercept and Sotatercept inhibit SMAD2/3 and downstream growth differentiation factor 11 (GDF11) signaling by binding to activin receptors which are negative regulators of late-stage erythropoiesis (Cappellini and Motta, 2017). Luspatercept and Sotatercept are both recombinant fusion proteins with a Fc domain of human IgG1, but different extracellular domains of activin receptor type IIB and type IIA, respectively (Piga *et al.*, 2019). Both, compounds have shown improvements in transfusion burden by improving hematocrit (Piga *et al.*, 2019; Humbert *et al.*, 2021). Ruxolitinib Is a topical, competitive inhibitor of the ATP-binding catalytic site on Janus Kinase 1/2, key proteins in the signaling pathway used by erythropoietin to stimulate RBC production. Ruxolitinib has shown promising results to decrease splenomegaly in TDT and reduce transfusion burden (Taher *et al.*,

2018). In addition, there are several preclinical proposed approaches to improve gamma production that are under investigation: knocking down B-cell lymphoma/leukemia 11A, a strong silencer of the γ-globin gene, using RNA interference, forced chromatin looping using LIM domain-binding protein 1, and Forkhead-box-class-O3 inducers such as metformin (Yasara *et al.*, 2021).There are several exciting pharmacological agents being studied that aim to improve transfusion burden in BT by inhibiting ineffective erythropoiesis pathways.

### 1.3 Clinical observations suggest that Beta Thalassemia is associated with Accelerated Atherosclerosis

Children with BT have increased carotid artery intimal-medial thickness (CAIMT), suggestive of the early stages of atherosclerosis (Tantawy *et al.*, 2009; Gursel *et al.*, 2012a; Sherief *et al.*, 2017; Ahmad Ibrahim *et al.*, 2021a). Atherosclerosis, described in more detail in Section 2.1, is a slowly progressive disease with a long subclinical lag phase characterized by gradual thickening of the intima (Hansson, 2005; Manios *et al.*, 2009). *Sherief et al.* noted both right and left CAIMT as assessed by ultrasonography was significantly increased in BT major patients compared to control subjects (Rt 0.62 ± 0.2 mm vs. 0.29 ± 0.07 mm, p = 0.001 & Lt 0.66 ± 0.17 mm vs 0.29 ± 0.05 mm, p = 0.001). Several studies have found similar increases in CAIMT compared to baseline with slightly different raw numbers due to baseline patient demographics (intermedia vs. major, appropriate chelation/blood chelation, age, location, etc.). BT patients also have dyslipidemia characterized by elevated triglycerides (128 ± 20 vs 101 ± 7 mg/dl, p = 0.009) and low HDL levels resulting in a high atherogenic index (0.45 ± 0.12 vs 0.22 ± 0.04, p = 0.001, calculated as log (TG/HDL-C)) (Sherief *et al.*, 2017). In addition, BT patients

show elevated lipid peroxidation and reduced antioxidant proteins (Tantawy *et al.*, 2009). Despite parodically having documented lower total cholesterol and LDL, patients with BT have a clear proatherogenic environment suggesting premature atherosclerosis. Several biomarkers such as Osteoprotegerin and Cystatin C correlate with CAIMT in BT (Sherief *et al.*, 2017). The true incidence of atherosclerosis in BT is hard to delineate due to varying degrees of iron chelation therapy compliance, small sample sizes in clinical studies, and until recently, premature death. A list of atherosclerosis studies association studies in BT is found in table 2. Globally, BT patients have increased CAIMT in both TDT and NTDT. In addition, BT patients have endothelial dysfunction as assessed by impaired flow-mediated vasodilation (Stoyanova *et al.*, 2012). Taken together, these clinical studies suggest that a proatherogenic environment could be present in BT.

**1.4 Intravascular hemolysis in Beta Thalassemia**

BT is a hemoglobinopathy that is characterized in by oxidative stress, elevated hemolysis, ineffective erythropoiesis, and endothelial dysfunction (Cao and Galanello, 2010; Galanello and Origa, 2010). Unpaired alpha hemichromes cause erythrocyte instability which increase hemolysis in the bone marrow and vascular. Hemolysis exposes the vasculature to elevated levels of free hemoglobin and subsequently free heme. Intravascular hemolysis is a prominent, underappreciated feature of BT whose contribution to vascular disease in BT is poorly understood (Morris, 2008).

| **Name (Author Year)** | **Patient Population (BT Sample size)** | **Findings (cIMT- BT vs WT)** | **Reference** |
|---|---|---|---|
| Premature Atherosclerosis in Non-Transfusion-Dependent β-Thalassemia Intermedia | BT-Intermedia (20) | cIMT: 0.51 ± 0.09 vs. 0.46 ± 0.07 mm; p = 0.049 | (Hahalis *et al.*, 2011) |
| Premature atherosclerosis in children with beta-thalassemia major: New diagnostic marker | BT-Major (65) | cIMT: (Right 0.62 ± 0.2 vs. 0.29 ± 0.07mm, p = 0.001 & Left 0.66 ± 0.17 vs 0.29 ± 0.05 mm, p = 0.001 | (Sherief *et al.*, 2017) |
| Subclinical atherosclerotic predictive value of inflammatory markers in thalassemia intermedia patients | BT-Intermedia (45) | cIMT: 0.06 ± 0.01 vs 0.05 ± 0.01 (cm) | (Ahmad Ibrahim *et al.*, 2021a) |
| The evaluation of carotid intima-media thickness in children with beta-thalassemia major | BT major (33) | cIMT: 0.87 vs. 0.74, with a p-value less than 0.005 | (Dogan and Citak, 2012) |
| Premature atherosclerosis in children with β-thalassemia major | BT major (31) | Significant Increase in BT cIMT and | (Gursel *et al.*, 2012a) |
| Premature Atherosclerosis in Children With Transfusion-Dependent Thalassemia: A Twin-Center Cross-Sectional Study | BT major (49) | cIMT: controls:0.27(0.07) mm, 0.39 (0.03) mm, and 0.46 (0.05) mm vs. BT: 0.43 (0.08) mm, 0.55 (0.07) mm and 0.63 (0.08) mm in 2 to 5 years, 6 to 10 years, and 11 to 15 years age groups respectively, as against (P<0.001). | (Kumaravel *et al.*, 2022) |
| Subclinical Atherosclerosis In Young β-thalassemia Major Patients | BT major (30) | cIMT: 0.73 and 0.63 mm) CIMT was positively correlated with age, Hb F, ferritin and cholesterol levels. | (Tantawy *et al.*, 2009) |
| Evaluation of carotid artery dynamics & correlation with cardiac & hepatic iron in β-thalassaemia patients | BT transfusion dependent ( 53) | cIMT: 0.48 ± 0.04 and 0.44±0.02 mm, respectively and these were significantly different (*P*<0.001) | (Merchant *et al.*, 2016) |

| | | | |
|---|---|---|---|
| Coronary atherosclerosis burden is not advanced in patients with β-thalassemia despite premature extracardiac atherosclerosis: a coronary artery calcium score and carotid intima-media thickness study | BT intermedia and major (37) | Median intima-media thickness was higher in β-thalassemia patients compared to control group [0.45 (0.06–0.65) vs. 0.062 (0.054–0.086); P = 0.04]. | (Hahalis *et al.*, 2016) |
| Surrogate Markers of Subclinical Atherosclerosis and Its Associated Factors in Patients with β-Thalassemia Major | BT major (60) | significant cIMT and correlation with age, cholesterol, HDL-c, and insulin | (Soltani *et al.*, 2021) |
| GDF-15 is associated with atherosclerosis in adults with transfusion-dependent beta-thalassemia | BT transfusion dependent (60) | (median of 0.08 cm) than in the controls (median of 0.04). | (Efat *et al.*, 2022) |

**Table 1.2: Clinical studies suggesting increased atherosclerosis in BT.** Several clinical studies have shown an increased in carotid intima medial thickness in BT patients.

1.4.1 Pathophysiology of Hemolysis

Hemolysis is the destruction of red blood cells (RBC) in the body. Hemolysis can vary temporally (acute or chronic), pathologically (congenital or acquired), and by mechanism of occurrence (induced by mechanical factors, genetic abnormalities, or toxins) (Baldwin *et al.*, 2022). Hemolysis is categorized into extra- and intra-vascular hemolysis. Intravascular hemolysis exposes the vasculature to the contents of RBC. In extravascular hemolysis, macrophages in the reticuloendothelial system target defective red blood cells for destruction largely in the spleen and liver (Morris, 2008). As a result, BT patients have enlarged spleens due to the increase in damaged RBC (Needs *et al.*, 2022). In recent years, intravascular hemolysis has been shown to contribute to the vasculopathy found in hemoglobinopathies (Morris, 2008b; Kato *et al.*, 2009).

1.4.2 Contribution intravascular hemolysis to oxidative stress

In BT and other hemolytic anemias, RBC contents expose the vasculature to high levels of hemoglobin that is oxidized with subsequent release of free heme into the vasculature (Cao and Galanello, 2010). Under normal hemolysis rates, scavenging proteins haptoglobin and hemopexin can bind to hemoglobin and free heme respectively to prevent toxicity and ROS generation as discussed in 1.4.2.1-1.4.4.2 Furthermore, NO bioavailability is limited via arginase release upon RBC lysis as discussed in 1.4.2.3. A visual summary of hemolysis mediated oxidate stress is seen in figure 1.2.

*1.4.2.1 Hemoglobin-mediated oxidative stress*

Hemoglobin is the most abundant protein in RBC. Upon full maturation, erythroblasts and reticulocytes lose nuclei and organelles respectively to accommodate

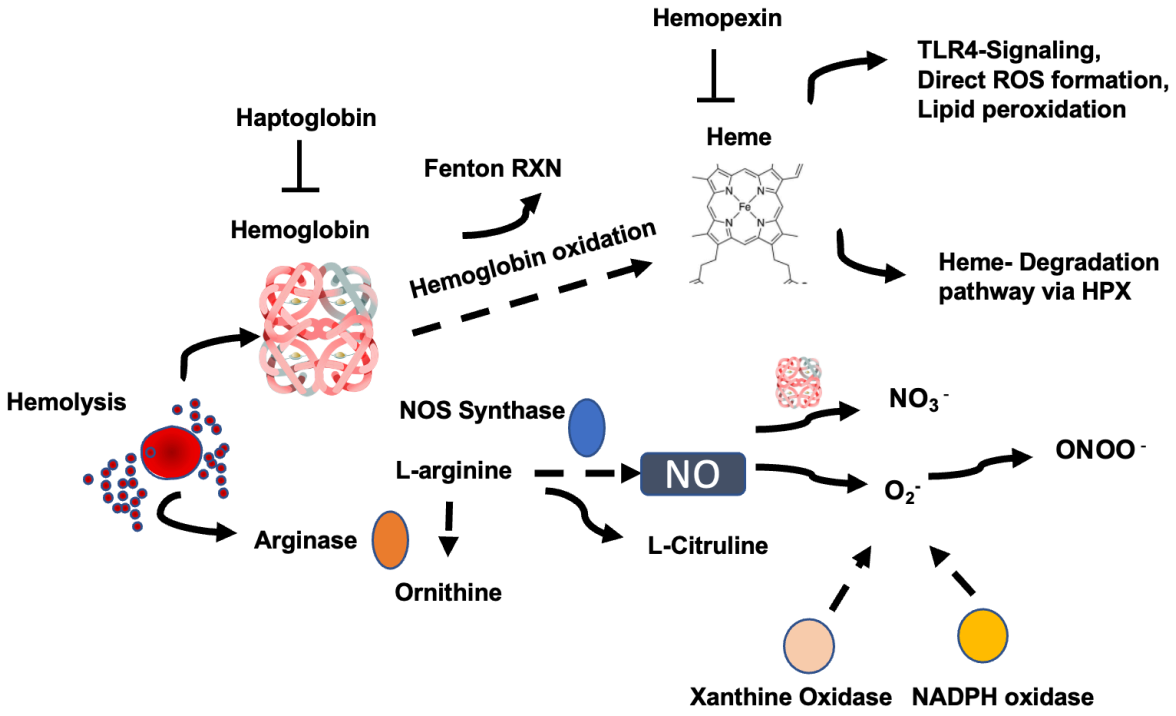


**Figure 1.2 Hemolysis signaling pathways.** Upon hemolysis, hemoglobin is released and subsequently oxidize to heme that can lead to oxidative stress. In addition, arginase is released upon hemolysis which limits NO by degrading L-arginine. Nitric oxide can react with superoxide to form peroxynitrate. Nitric oxide can also be directly oxidized by free hemoglobin.

260 million hemoglobin molecules which comprise 95% of the volume of RBC (Yoshida *et al.*, 2019). Hemoglobin, as previously described, is a 4-globin subunit that can be autoxidized at a rate of 0.5-3% per day and at higher rates in high oxidative stress environments (Voskou *et al.*, 2015). As seen in Equation 1, hemoglobin can autoxidize to generate superoxide ion.

$$Equation\ 1: HbFe(II)O_2 \rightarrow HbFe(III) + O_2^{\cdot -}$$

Superoxide is unstable and converted to hydrogen peroxide ($H_2O_2$) both spontaneously and via superoxide dismutase as seen in Equation 2.

$$Equation\ 2: O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2$$

Hydrogen peroxide can react with oxygenated hemoglobin (HbFe(II) $O_2$) or methemoglobin (HbFe(III)) to generate ferryl hemoglobin ($HbFe(IV) = O$ ) and oxyferryl hemoglobin ($HbFe(IV) = O^-$) as seen in equation 3 and 4 respectively.

$$Equation\ 3: HbFe(II)O_2 + H_2O_2 \rightarrow HbFe(IV) = O + H_2O + O_2$$

$$Equation\ 4: HbFe(III) + H_2O_2 \rightarrow HbFe(IV) = O + H_2O$$

In addition, Ferryl hemoglobin and oxyferryl hemoglobin can generate methemoglobin as seen in Equation 5 and 6 respectively.

$$Equation\ 5: HbFe(IV) = O + H_2O_2 \rightarrow HbFe(III) + O_2^{\cdot -} + H_2O$$

$$Equation\ 6: HbFe(II)O_2 + H_2O_2 \rightarrow HbFe(III) + H_2O + O_2$$

These oxygenated hemoglobin moieties have less affinity for heme which results in increased heme dissociation from hemoglobin. In addition, cell-free hemoglobin can react with nitric oxide to form methemoglobin and nitrate (Yeo *et al.*, 2009). Cell-free hemoglobin leads to increased ROS through autoxidation and increased vasoconstriction through consumption of NO.

*1.4.2.2 Heme-mediated oxidative stress*

Heme is a porphyrin ring shaped molecule that consists of 4 pyrroles, (pentagon shaped molecules made up of 4 carbons and a nitrogen) and a central iron atom (Fortes *et al.*, 2012). Functionally, heme has a multitude of functions including oxygen transport, electron transfer, and enzymatic catalysis. Heme-mediated oxidative stress occurs when free heme is released from hemoproteins such as hemoglobin, myoglobin, and cytochromes due to oxidative or proteolytic damage (Immenschuh *et al.*, 2017). Free heme can directly interact with various biomolecules such as lipids, proteins, and DNA, leading to oxidative damage, inflammation, and cell death. Free heme and iron are oxidizing agents that can act as a Fenton reagent in the Haber-Weiss cycle to generate hydroxyl/hydroxyl radicals as seen in equation 7 and hydroxyperoxyl as seen in equation 8.

$$Equation\ 7: H_2O_2 + Fe(II) \rightarrow OH^{\cdot} + OH^{-} + Fe(III)$$

$$Equation\ 8: Fe(III) + H_2O_2 \rightarrow Fe(II) + HOO^{\cdot} + H^{+}$$

Heme has been shown to activate neutrophils through protein kinase C (PKC) activation with NADPH oxidase, G-protein-coupled receptor, and macrophage-derived leukotriene B4 production (Dutra and Bozza, 2014). Heme also acts as potent ligand to Toll-like death receptor 4 (TLR4) to generate TNFa and IL6 in endothelium and macrophages (Dutra and Bozza, 2014). In SCD mice studies, heme activation of endothelial TLR4 has been shown to induce vascular occlusion through NF-κB activation, weibel-Palade body degranulation, and adhesion molecule expression (Belcher *et al.*, 2014). Heme also causes early macrophage death via TLR4/MyD88-dependent TNF production (Lin *et al.*,

2012). Heme-mediated stress is caused by directly intercalating with molecules, ROS generation via Fenton reaction, and as a TLR4 agonist.

Heme-mediated oxidative stress has been implicated in many pathological conditions such as hemolytic diseases, including sickle cell disease, thalassemia, and malaria (Immenschuh *et al.*, 2017). In these diseases, the release of heme from damaged red blood cells can lead to oxidative stress and inflammation, contributing to the pathogenesis of the disease. Heme-mediated oxidative stress is also involved in the pathogenesis of atherosclerosis, a chronic inflammatory disease that affects the arterial wall. Heme can accumulate in atherosclerotic lesions and promote the formation of foam cells, which are the hallmark of the disease (Libby, 2021b). Heme can also promote the production of pro-inflammatory cytokines and chemokines, further contributing to the progression of the disease (Lin *et al.*, 2012). Moreover, heme-mediated oxidative stress is implicated in the development of cancer (Fiorito *et al.*, 2020). Heme can promote the proliferation and survival of cancer cells by activating various signaling pathways such as NF-κB and MAPKs (Fiorito *et al.*, 2020). Heme can also induce the expression of genes that promote angiogenesis and invasion, leading to the metastasis of cancer cells (Gamage *et al.*, 2021). Elevated levels of free heme have led to increase oxidative stress in many different disease paradigms.

*1.4.2.2.1 Heme degradation pathway*

To counteract heme-mediated oxidative stress, cells have developed various defense mechanisms. One of the main defense mechanisms is the production of heme oxygenase, the rate-limiting enzyme in heme degradation pathway that degrades heme into biliverdin, iron, and carbon monoxide. Heme oxygenase is an enzyme that has three

distinct isoforms (HO-1, HO-2, HO-3) (Immenschuh *et al.*, 2017). HO-1 (32kda protein) is a highly inducible enzyme that is upregulated via stimuli such as high heme levels, heat shock, oxidative stress, and LPS (Araujo *et al.*, 2012). HO-2 (36kda) is constitutively expressed in most cell types and helps maintain regulatory physiological process (Araujo *et al.*, 2012). HO-3 (33kda) is also constitutively expressed but has poor heme-degrading capacity (Araujo *et al.*, 2012). Heme oxygenases use electron transfer from NADPH to cleave the α-meso carbon bridge of the heme molecule into its components: CO (a vasodilatory gas that has anti-inflammatory properties), biliverdin (antioxidant), and iron (that can be sequestered by ferritin) (Campbell *et al.*, 2021). Ferritin is an acute phase protein that stores iron in tissues, Ferritin is elevated in iron overload, but is also elevated in inflammation, infection, and liver disease (Araujo *et al.*, 1995). In iron overload, there are high levels of both ferritin and transferrin, while in other disease such as in fatty liver disease have high ferritin and low transferrin levels (Krittayaphong *et al.*, 2018; McDowell *et al.*, 2023). High levels of ferritin (>1000 ng/ml) have correlated with increased likelihood of liver damage (McDowell *et al.*, 2023). Biliverdin is metabolized to bilirubin, a bile pigment, via biliverdin reductase. Heme is not directly used for the formation for new hemoglobin despite being able to renature in-vivo because ferric free heme must be reduced to ferrous free heme in a non-spontaneous manner and free heme would have to travel to the bone marrow to form new hemoglobin molecules (Araujo *et al.*, 2012; Chau, 2015).

HO-1 has been a broadly studied protein in many disease paradigms due to its ability to breakdown heme and the transcriptional regulation of HMOX1 gene, (the gene encoding HO-1) in various disease states (Kato *et al.*, 2009). Clinically, HO-1 deficiency

leads to a chronic inflammation and accumulation of lymphocytes and macrophages in the spleen (Radhakrishnan *et al.*, 2011). However, the compensatory role heme-oxygenase plays in a chronic hemolytic anemia such as BT is complicated. In BT mice, Garcia-Santos et al. showed that pharmacological inhibition of heme-oxygenase via tin protoporphyrin IX improves anemia and ineffective erythropoiesis (Garcia-Santos *et al.*, 2018). In Ldlr $^{-/-}$ mice, *Ishikawa et al.* showed that pharmacologically inhibition of heme-oxygenase mice via Sn-protoporphyrin IX lead to increased atherosclerotic plaque accumulation (Kazunobu Ishikawa *et al.*, 2001). Free heme generated from phenylhydrazine in rabbits has been shown to increase atherosclerosis (Fernandez *et al.*, 2001a).

*1.4.2.3 Decreased Nitric Oxide NO bioavailability.*

In addition to the above effects, hemolysis decreases nitric oxide (NO) and subsequent vasodilation by limiting the availability of the NO synthase (NOS) substrate, arginine.

*1.4.2.3.1 Nitric oxide overview*

NO is a lipid-soluble, signaling molecule that is one nitrogen atom covalently bonded to an oxygen atom with one free radical electron. Traditionally, NO was first recognized as a endothelium-derived relaxing factor (EDRF) that promoted blood vessel relaxation and regulation of vascular tone (El-Hady *et al.*, 2012). NO is a short-lived molecule that penetrates the cell membrane and is produced by endothelial cells (Thomas *et al.*, 2001). The formation of NO is catalyzed by NOS, a dimeric flavoprotein containing tetrahydrobioprotein, with oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as cofactors. The three forms of NOS are neuronal NOS (nNOS), inducible

NOS (iNOS), and endothelial NOS (eNOS) with most current nomenclature being NOS I, II, and III respectively (Moskaleva *et al.*, 2021). NOS I and NOS III are , for the most part, constitutively expressed and require calcium, while NOS II is inducible and calcium-insensitive (Ghimire *et al.*, 2017). Classically, NO acts as a vasodilator by diffusing across endothelial cell membranes and activating the enzyme guanylate cyclase, which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in smooth muscle cell (Thomas *et al.*, 2001). cGMP then activates various downstream signaling pathways that regulate physiological processes, such as vasodilation, platelet aggregation, and neuronal transmission. Therefore, the role of NO in inflammation is complex and depends on a balance between its pro-inflammatory and anti-inflammatory effects (Ghimire *et al.*, 2017).

Superoxide anion is a reactive oxygen species produced by cellular oxidases such as NADPH oxidase, xanthine oxidoreductase enzyme system, and uncoupled NOS (described in more detail in 1.4.2.3.2) (Lubos *et al.*, 2008). Superoxide can react rapidly with nitric oxide (NO), producing peroxynitrite. The reaction between NO and superoxide occurs at an almost diffusion-limited rate, 6 times greater than the removal of superoxide by copper-zinc superoxide dismutase (Cu/Zn SOD) (Wang *et al.*, 2018). The interaction between NO and superoxide depletes NO bioactivity and is functionally important because NO is a pivotal mediator of key vascular functions, including regulation of smooth muscle tone and blood pressure, platelet activation, and vascular cell signaling (Ghimire *et al.*, 2017). Consequently, the loss of NO bioactivity associated with increased vascular superoxide plays a potentially important role in the pathogenesis of endothelial dysfunction associated with atherosclerosis (Lubos *et al.*, 2008).

*1.4.2.3.2 Nitric oxide in the context of hemolytic anemia*

Chronic intravascular hemolysis leads to the release of free hemoglobin, arginase, and altered NOS co-factors. Arginase, an enzyme that facilitates conversion of L-arginine to L-ornithine and the formation of polyamines and L-proline, is released upon hemolysis (Morris, 2008b; El-Hady *et al.*, 2012). There are two forms of arginases: type1: a cytosolic enzyme found in liver and RBCs and type 2: a mitochondrial enzyme found in the kidney, prostate, testis, and small intestine. In mice, there is only one form of arginase, arginase-1, which is mostly expressed in cells other than erythrocytes, and consequently, this limits mouse studies to understand the functional contribution of erythrocyte arginase (Yang *et al.*, 2013). In BT major patients, NO levels are lower and free arginase-1 levels are higher compared to age-matched controls (NO: BT-12.1 ± 5.1 vs WT-33 ± 8.9 mmol/L; Arginase-1: BT- 47.1 ± 5.6 vs WT- 21.9 ± 9.2 ng/ml) (El-Hady *et al.*, 2012). Under chronic hemolysis and oxidative stress, NOSs are inhibited by increased levels of dimethylarginine (ADMA) and degradation of the important co-factor tetrahydrobiopterin (BH4). As a result of low arginine concentration and eNOS inhibition via ADMA/BH4 alteration, eNOS becomes uncoupled and produces superoxide instead of NO (Ghimire *et al.*, 2017). In BT and other hemolytic anemia, pulmonary hypertension is likely in part due to limited NO bioavailability caused by chronic hemolysis (Morris *et al.*, 2007). BT patients have decreased NO bioavailability caused by several factors including increased free arginase and NOS decoupling caused by diminished cofactors leading to increased superoxide formation.

*1.4.2.4 Antioxidant systems*

Hemolysis degradation pathway is summarized in figure 1.3.

*1.4.2.4.1 Haptoglobin*

Haptoglobin binds to cell-free hemoglobin to prevent its autoxidation, pro-oxidant effects, and intravascular free heme release. Haptoglobin dimers bind to hemoglobin dimers with high affinity $K_d$=1.5X10$^{-6}$ M (Nagel and Gibson, 1971). The Haptoglobin: Hemoglobin complex is removed via binding to CD163 receptors in macrophages and subsequently degraded and removed by heme oxygenase. The clearance time of haptoglobin is slow with a half -life ($t_{1/2}$) of 12 hours (Ragab *et al.*, 2015). The haptoglobin protein consists of two alpha and two beta subunits that are connected by disulfide bonds. There are three major types of haptoglobin in humans: Hp1-1, Hp2-1, and Hp2-2 (Nagel and Gibson, 1971). These differ in their ability to bind to hemoglobin and their susceptibility to proteolysis. Elevated haptoglobin levels can be a sign of an acute-phase response, which is the body's reaction to infection, inflammation, or injury (Graw *et al.*, 2022). Furthermore, haptoglobin binding prevents renal filtration of hemoglobin and subsequent kidney damage. In sumary, haptoglobin is a protein that binds to free hemoglobin and prevents hemoglobin-mediated oxidative stress.

Haptoglobin therapy is an experimental approach to treating conditions characterized by excessive hemolysis, or the destruction of red blood cells. The goal of haptoglobin therapy is to provide patients with exogenous haptoglobin, which can bind to and neutralize free hemoglobin in the bloodstream, preventing the potential harm caused by its accumulation (Santiago *et al.*, 2018). Haptoglobin therapy is still in the experimental stage and has not yet been approved for widespread use. However,

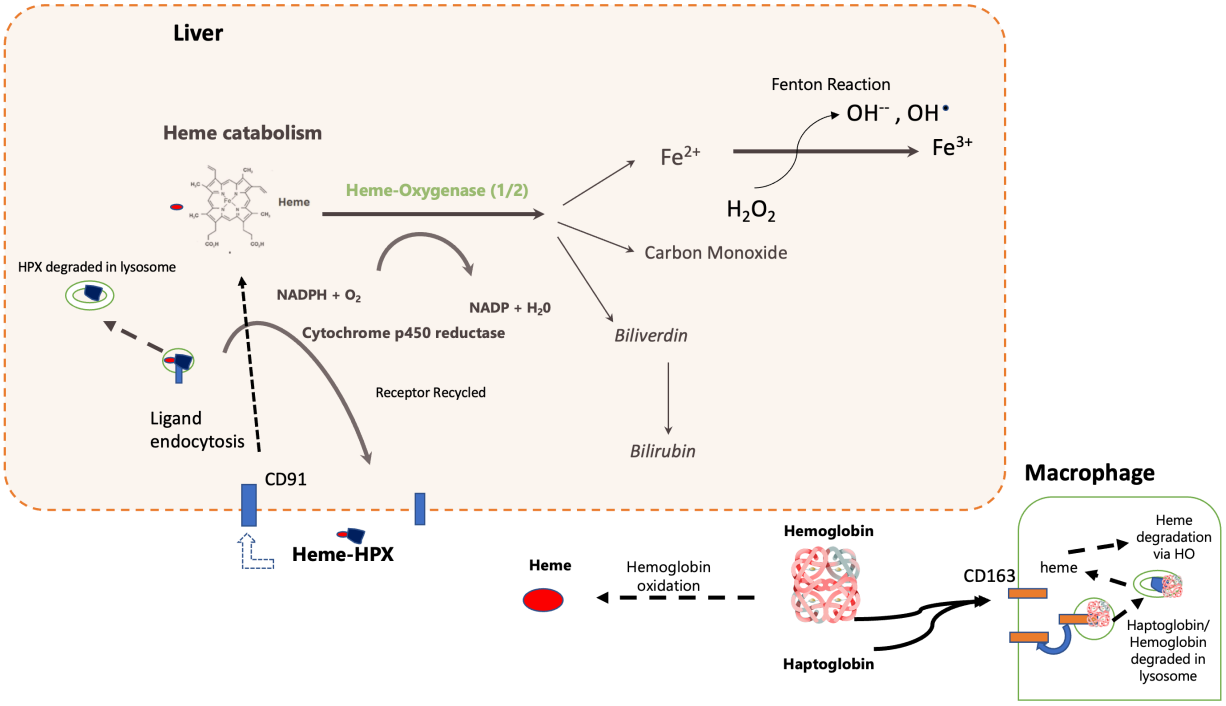


**Figure 1.3. Heme/Hemoglobin degradation pathway.** Hemoglobin is scavenged by haptoglobin via CD163 primarily in macrophages. Heme is scavenged by Hemopexin via CD163 in various cell types but most prominently in the liver. Both receptors are recycled, but hemopexin and haptoglobin are degraded in the lysosome. Heme is broken down by heme oxygenase to iron, CO, and biliverdin.

preclinical and clinical studies have shown promising results in animal models and in humans with various conditions characterized by hemolysis, including sickle cell disease and hemolytic anemia (Ragab *et al.*, 2015; Santiago *et al.*, 2018; Buehler *et al.*, 2020). One approach is to administer recombinant haptoglobin directly to patients (Buehler *et al.*, 2020). This involves the production of large quantities of haptoglobin in the laboratory, which can then be purified and administered intravenously. Haptoglobin therapy is not widely used but is in preclinical development.

*1.4.2.4.2 Hemopexin (HPX)*

Hemopexin is a glycoprotein that is found in the blood of many animals, including humans. Its primary function is to bind to heme, a toxic molecule that is released when red blood cells are broken down. During hemolysis, haptoglobin levels are decreased and rapidly consumed; initially, hemopexin levels remain consistent, but in severe chronic hemolysis, such as BT, HPX levels are exhausted (Deuel *et al.*, 2015). Once the binding capacity of haptoglobin saturates, hemoglobin is oxidized and releases free heme. Free heme can be scavenged by HPX, albumin, and α-micro-globulin to prevent its pro-oxidant effects. HPX is an acute phase, glycoprotein that is synthesized in the liver and has the highest binding affinity for plasma heme by binding to its heme binding pocket ($K_d$ =10-14). The HPX:heme complex is recognized by the macrophages receptor, CD91 and heme is broken down to its components by HO.

Originally, HPX and CD91 receptor were believed to act like the transferrin receptor which is recycled and does not degrade ligand, but HPX is degraded in the lysosome while only the receptor is recycled (Hvidberg et al., 2005). Furthermore, HPX levels are depleted in diseases with extensive hemolysis (Vinchi, Vercellotti, *et al.*, 2016).

Both HPX and/or Hp gene knockout mice develop a normal phenotype in non-challenged conditions while exhibiting severe renal/ hepatic damage when subjected to experimental hemolysis such as hemin or phenylhydrazine administration (Tolosano et al., 1999; Tolosano et al., 2002; Vinchi et al., 2008). *Vinchi et al.* showed that 700 µg purified human HPX intraperitoneally twice a week for 1 month improved cardiovascular function by normalizing blood pressure in SCD mice and decreased vascular adhesion molecules both BT and SCD mice (Vinchi et al., 2013). Furthermore, in SCD, HPX therapy (4 mg/kg HPX intraperitoneally once a week, for 3 weeks) reverted heme-induced switching of macrophages from proinflammatory phenotype M1 as evidence by decreased expression of M1 markers (CD86, iNOS, and MHC II) (Vinchi et al., 2016). In lipopolysaccharide (LPS)-activated macrophages, HPX down-regulates pro-inflammatory cytokines [such as TNFa, interleukin-6 (IL-6) and IL-1β] production and acts as a negative regulator of Th17 response in EAE. (Rolla et al., 2013) Clinically, CSL889, a plasma-derived hemopexin, is in a phase-1 clinical trial (www.clinicaltrials.gov identifier NCT04285827) based on pre-clinical evidence showing improved endothelial activation and heme-mediated vaso-occlusion in SC mice. Globally, HPX therapies have shown preclinical efficacy as a promising therapeutic target in numerous different clinical settings including hemolytic anemias and acute kidney injury (Vinchi, Vercellotti, *et al.*, 2016; Ghosh *et al.*, 2019; Gentinetta *et al.*, 2022a). A comprehensive list of HPX therapy is shown in table 3.

1.4.3 Evidence of Intravascular hemolysis in Beta Thalassemia

Intravascular hemolysis is present in BT as seen in RBC survival studies, spectrometric analysis of serum, and clinical evidence. There are two main RBC

| ***Disease model*** | ***Animals*** | ***Used to dose of HPX*** | ***Effect of HPX*** | **Ref.** |
|---|---|---|---|---|
| Rat liver model of cold storage and reperfusion and tested the potential anti-oxidant effects of HPX | Rat Sprague-Dawley | Reperfused with 5μm HPX | Decreasing oxyradical production in a model of cold storage/reperfusion | (Brass *et al.*, 1998) |
| Implanted intracranially with 50,000 U87 glioma cells | Nude and BALB/C Mice | Intra cerebral delivery to PEX (recombinant HPX) 0.25-1 with minipumps mg/kg/day (29 days) | Local intracerebral delivery of endogenous inhibitors decreased of tumors growth | (Giussani *et al.*, 2003) |
| Mesenchymal stem cells-PEX (hMSC-HPX) injected adjacent to glioblastoma tumors | Nude mice | No dose reported | Mice treated with hMSC-PEX reduction tumor volume and weight measurements decrease 22 days | (Goren *et al.*, 2010) |
| SCD and B-thalassemic model | Hbs SCD mice and B-thalassemic mice | I.P. To 700μg injection purified human HPX | HPX to treat vasculopathy in hemolytic disorders. Decrease cardiac output, aortic valve peak pressure in different mice model | (Vinchi *et al.*, 2013) |
| SCD mouse models | NYDD and Townes SCD mice | I.V. 0.4 or 1.6 μmol/kg | Hemoglobin-induced vaso-occlusion was blocked by the heme-binding protein HPX | (Belcher *et al.*, 2014) |
| Hemorrhagic schock (HS) and resuscitated with either FRBCs or SRBCs | C57BL/6 mice | HS and resuscitated with FRBCs/SRBCs to simultaneous infusion of 7.5 mg HPX | Increase the survival rate and reduced the early proinflammatory response after HS resuscitation with stored blood | (Graw *et al.*, 2016) |
| Atherosclerosis | HPX and HPX/ApoE KO mice | I.P. human HPX to HPXApoE KO during 24 h | HPX significantly reduced serum heme levels. Increased in the expression of LXR-α and ABCA1 genes. Reduction in expression of CCR-2, and a significant increase in expression of Arg-1 | (Mehta *et al.*, 2016) |
| SCD model | Townes SCD model | I.P human HPX (4 mg) | Administration of HPX is beneficial to counteract heme-driven macrophage-mediated inflammation and its pathophysiologic consequences in sickle cell disease | (Vinchi, Vercellotti, *et al.*, 2016) |
| HPX KO and B-thalassemic model | C57BL/6 $HPX^{-/-}$ and $Hbb^{th3/+}$ mice | 160 mg/kg HPX | HPX rescued contraction defects of heme-treated cardiomyocytes and preserved cardiac function in hemolytic mice | (Ingoglia *et al.*, 2017) |
| Spinal Cord Injury (SCI) | HPX KO mice | I.P. 0.5-50ng/mL HPX | Acute-phase plasma glycoprotein, in the regulation of microglia polarization HPX in alleviating the secondary injury and improving functional repair after SCI | (Han *et al.*, 2018) |

| | | | | |
|---|---|---|---|---|
| Intravascular hemolysis induced by PHZ and Heme injection | C57BL/6 and C3 $^{-/-}$ mice | I.P. injection of 40µmol/kg of human HPX 1 h before heme or PHZ injection | Decreased Kidney Complement deposition | (Merle *et al.*, 2018) |
| Intravascular hemolysis induced by PHZ and Heme injection | C57BL/6 mice | I.P. injection of 40µmol/kg of human HPX 1 h before heme or PHZ injection | No effect on renal NGAL, Kim1, and HO-1 genes expression | (Merle *et al.*, 2018) |
| Cerebral Ischemia reperfusion injury (CIRI) | Rat Sprague-Dawley | Insert beneath the dural surface to inject rat HPX (10µL, 1.86 g/L HPX) | HPX can alleviate cognitive dysfunction after focal CIRI through HO-1 pathway and preventing the impairment of the blood-brain barrier in rats | (Dong *et al.*, 2019) |
| SCD Model | HPX KO and littermate SCD mice HPX KO(SS HPX$^{-/-}$ and SS HPX $^{+/+}$) | | HPX deficiency promotes AKI development in SCD, and we provide proof-of-principle for HPX replacement therapy to treat AKI in SCD | (Ghosh *et al.*, 2019) |
| Intravascular hemolysis induced by PHZ and heme injection | C57BL/6, C3$^{-/-}$, and TLR4$^{-/-}$ mice | I.P. injection of 40µmol/kg of human HPX 1 h before heme or PHZ injection | Decreased NGAL gene expression, decreased liver complement deposition | (Merle *et al.*, 2018) |

**Table 1.3: List of preclinical HPX therapies.** Hemopexin therapy has been used to rescue cardiovascular function, acute kidney injury, cerebral ischemia reperfusion, and drug-induced intravascular injury. Hemopexin therapy has been used to in preclinical development in hemoglobinopathies such as sickle cell disease and BT mice. Adapted from (Poillerat *et al.*, 2020).

survival assays: Chromium-51 and Biotin-labeled studies. In Chromium-51 studies, BT patients have decreased RBC survival more than two-fold compared to age and sex matched controls (Smith *et al.*, 1955; Dimitriou *et al.*, 1991). Biotin labeled studies are the current preferred method to determine erythrocyte lifespan because it nonisotopic, safe and equivalently effective compared to $^{51}$C. Biotin-labeled studies have shown similar results in that BT intermedia patients have decreased RBC survival of 36 ± 15 days compared to controls that typically have survival of over 100 days (Singer *et al.*, 2004; Thiagarajan *et al.*, 2021).

Clinically, the exact degree of hemolysis in hemolytic anemia is complex due various degrees of disease severity, blood transfusion, splenectomy, chelation therapy, age, and natural variation in hemolytic markers. The contribution of heme to the pathophysiology in SCD is widely accepted and is receiving increasing attention in BT (Morris, 2008; Kato *et al.*, 2009; Kato and Taylor, 2010). For example, Kiger and colleagues collected blood from 77 SCD and 23 BT intermedia patients and used light absorption spectrophotometry of plasma samples to determine hemolysis markers present in these hemolytic anemias. Table 1.22 provides a summary of findings with literature baseline values (Kiger *et al.*, 2019a). Serum free hemoglobin and Lactate Dehydrogenase (LDH) are commonly used as the most obvious markers of intravascular hemolysis. Lactate dehydrogenase is an enzyme that catalyzes the conversion of lactate into pyruvic acid and NADH to NAD+ and is widely used as a biomarker of hemolysis that correlates with cell-free hemoglobin (Farhana and Lappin, 2022). Intravascular hemolysis is more extensive in SCD compared to BT intermedia (SCD: plasma Hb (μmol/L) of 6.3 [3.4-11] / LDH (IU/L) 395 ± 120 vs BT: plasma Hb 2.6 [1-5.4] (μmol/L) / LDH 209 ± 81 209

± 81 LDH (IU/L)). When compared to control values for adults (Plasma Hb is typically under 2μmol/L) /LDH 140-280(IU/L), SCD has a clear intravascular hemolysis profile, while BT patients have slightly elevated LDH levels and serum free hemoglobin. However as previously described, disease severity varies drastically between BT major/TDT and the BT intermedia patient population that is largely NTDT. For instance, in BT major, LDH levels were found to be 524.48 ± 167.44 [IU/L] which is clearly higher than the LDH levels found in controls 140-280(IU/L) (Gunawan *et al.*, 2021). In SCD despite chronic intravascular hemolysis, hemolysis markers can fluctuate depending on sickle crises that cause increased RBC lysis, while levels in BT are relatively more stable (Origa, 2017).

Related to this thesis, levels of hemopexin in BT are extremely depleted which is associated with elevated levels of total and free heme. Both Vinchi et al. and Kiger et al. reported more than a 10-fold decreases in serum hemopexin levels (Vinchi, Vercellotti, *et al.*, 2016). Hemopexin levels in BT have been reported to be lower than in SCD (BT: 0.08 g/dl [0.05-.55] vs SCD: 0.28 g/dl [0.13-0.35]) and lower than controls (0.4-1.5 g/dl) (Smith and McCulloh, 2015; Santiago *et al.*, 2018; Kiger *et al.*, 2019a). Furthermore, plasma free heme was reported to be higher in BT than in SCD (BT: 10.5 [3.5-24] vs SCD: 1.05 [0.05-3] mM) (Kiger *et al.*, 2019a). Total heme, although not compared in the same study, appears to be similar in BT intermedia and SCD with levels of ~80μM in serum compared to ~ 40μM in controls (Vinchi, Vercellotti, *et al.*, 2016; Santiago *et al.*, 2018).

| | **SCD** **(Kiger *et al.*, 2019a)** | **BT** **(Kiger *et al.*, 2019a)** | **Control (Literature)** |
|---|---|---|---|
| HB(g/dl) | 8.9 ± 1.5 | 9.6 ± 1.7 | 12-16 (Female) 14-18 (Male) (Billett, 1990) |
| LDH (ui/L) | 395 ± 120 | 209 ± 81 | 140-280 (Farhana and Lappin, 2022) |
| Total Bilirubin (umol/L) | 42 ± 29 | 41 ± 22 | 1.71-20.5 (Chaudhary *et al.*, 2013) |
| Plasma Hb (µmol/L) | 6.3 [3.4-11] | 2.6 [1-5.4] | Up to 3 (Smith and McCulloh, 2015) |
| Plasma heme (µmol/L) | 1.05 [0.05-3] | 10.5 [3.5-24] | Not available |
| Hemopexin(g/L) | 0.28 [0.13-0.35] | 0.08 [0.05-.55] | ~0.4-1.5 (Smith and McCulloh, 2015) |

**Table 1.4 Serum levels indicate intravascular hemolysis is present in SCD and BT mice.** BT and SCD both have elevated levels of heme and hemoglobin. They also have exhausted levels of HPX.

## 1.5. Summary and Objectives

### 1.5.1. Summary

BT is a disease that leads to reduced production of the beta-globin gene. Two-alpha and two beta-globin chains are needed to form an adult hemoglobin, the main oxygen carrying protein found inside red blood cells. Due to the reduced beta-globin gene production, BT patients rely on the fetal hemoglobin system and have unpaired alpha-chains called hemichromes that aggregate to the surface of red blood cell precursors and lead to instability. As a result, BT patients have a large pools of immature blood cells, anemia, and enhanced erythropoiesis compensatory mechanism collectively called “ineffective erythropoiesis”. A prominent feature of BT is intravascular hemolysis which exposes the blood vessels to high levels of hemoglobin and subsequent heme. Typically, heme is scavenged by hemopexin, but with high levels of free heme, hemopexin is exhausted. High levels of free heme in the vasculature can lead to increased ROS formation and a proatherogenic environment as seen in figure 1.4.

### 1.5.2 Objectives

1. Does BT underlying pathophysiology lead to atherosclerosis?
2. What is the functional contribution of heme to the progression of atherosclerosis in BT?
3. Does iron chelation provide a synergistic effect with hemopexin therapy to reduce atherosclerosis in BT?

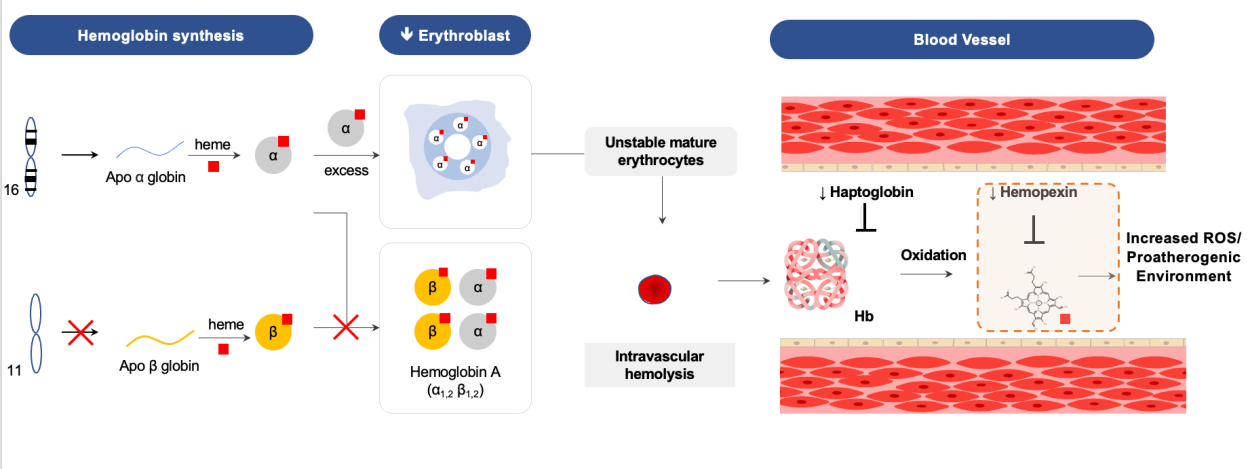


**Figure 1.4. Summary of BT pathophysiology.** In BT, there is reduced beta-globin gene production that leads to excess pairs of alpha-chains. These alpha-chains aggregate to the surface of red blood cell and leads to instability. This instability leads to increased intravascular hemolysis that exposes heme to the vasculature.

# Chapter 2: Accelerated Atherosclerosis in Beta Thalassemia

## 2.1 Introduction to Atherosclerosis

Atherosclerosis is a complex inflammatory disease that is characterized by the deposition of fatty material, cholesterol, immune cells, and necrotic tissue in arteries that leads to multiple cardiovascular diseases including coronary artery disease, peripheral arterial disease, stroke, and myocardial infarction (Jebari-Benslaiman *et al.*, 2022). Atherosclerosis is driven by multiple genetic (e.g. genes affecting lipid metabolism) and environmental risk factors (e.g. smoking, diabetes mellitus, hypertension, obesity, physical inactivity) (Fruchart *et al.*, 2004).

### 2.1.1 Blood Vessel Structure

Atherosclerosis is a slowly progressive disease in which atherosclerotic plaque forms in medium to large arteries (e.g. aorta and carotid arteries) and in areas of disturbed flow (e.g. bifurcations and branching points) (Björkegren and Lusis, 2022). Arteries have a larger muscle layer (more elastic) compared to veins, and can accumulate lipoprotein/macrophage/necrotic cells that lead to plaque formation (Mercadante and Raja, 2022). Blood vessels consist of 3 layers that are called tunica intima, tunica media, and tunica externa (Mercadante and Raja, 2022). The tunica intima is comprised of endothelial cells (ECs), a monolayer of squamous cells lining the most inner layer of blood vessels, at the blood interface that is supported by an internal elastic lamina layer (Milutinović *et al.*, 2020). ECs are not merely a passive barrier in human pathophysiology. When EC are “activated” such in the context of oxidative stress and atherosclerosis, ECs have the following core changes that can mediate numerous pathological effects: loss of vascular integrity, expression of leucocyte adhesion molecules, change in phenotype from antithrombotic to prothrombotic, cytokine production, and upregulation of HLA

molecules (Hunt and Jurd, 1998). The tunica media is comprised of smooth muscle cells (SMC), collagen, and an outer elastic lamina layer (Milutinović *et al.*, 2020). SMCs are the major determinant of arterial wall mechanical properties and can contract or constrict in response to multiple stimuli (Mercadante and Raja, 2022). SMCs play a critical role in atherosclerosis including migrating from the media to the intima, proliferating, and synthesizing extracellular matrix and inflammatory proteins. In more developed plaques, SMC and macrophage death leads to the formation of the necrotic core. The tunic externa/adventitia is a layer of connective collagenous and elastic tissue that supports and shapes the blood vessel (Harman and Jørgensen, 2019). Fibroblasts are the main component of the adventitia along with progenitor cells for other cell types. The adventitia becomes more complex over atherosclerotic plaques (Milutinović *et al.*, 2020). A growing body of literature indicates the importance of the adventitia to inflammation and atherosclerosis. The "Outside-in" is a hypothesis that states that vascular inflammation is initiated from the adventitia which includes the accumulation of inflammatory cells in the adventitia, the phenotypic switch of adventitial fibroblasts into migratory myofibroblasts, enhanced collagen and matrix protein deposition, and chemokine and growth factor responses to foam and antigen presenting cells (Maiellaro and Taylor, 2007; Pagano, 2007).

### 2.1.2 Pathogenesis of Atherosclerosis

Atherosclerosis is a multifactorial disease that is characterized by vascular inflammation, dyslipidemia, endothelial dysfunction, foam cell generation, vascular smooth muscle activation, and thrombosis (Jebari-Benslaiman *et al.*, 2022). The pathogenesis of atherosclerosis has been previously understood from numerous different

hypotheses: response to injury hypothesis, response to retention hypothesis, low-density lipoprotein (LDL)-oxidation hypothesis, lipid hypothesis, and inflammation hypothesis (Libby *et al.*, 2019; Björkegren and Lusis, 2022). The common motif that arises is an initiation phase followed by disease progression with potential complications.

The lipid hypothesis suggests that both the initiation and progression of atherosclerosis is primarily due to hyperlipidemia (Steinberg, 2013). Experimentally, in the first half of the twentieth century, several scientists described atherosclerotic lesions in rabbits who were fed high-cholesterol and high-fat diets (Fan *et al.*, 2015). Several studies have shown the association between hypercholesterolemia, specifically of high levels of LDL (low-density lipoprotein) cholesterol, and the severity of atherosclerotic lesions (Holvoet *et al.*, 1998). For instance, humans with loss-of function proprotein convertase subtilisin/kexin type 9 (PCSK9) mutations have life-long low LDL cholesterol concentrations and show a great reduction in coronary events (Bayona *et al.*, 2020).

Over the past several decades, the widespread use of statins have shown that reducing cholesterol biosynthesis through inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), the rate limiting step in the cholesterol biosynthesis pathway, results in decreased cardiovascular morbidity and mortality (Pinal-Fernandez *et al.*, 2018) . High-density lipoprotein cholesterol (HDL-C) levels are inversely related to the risk of atherosclerotic events, but numerous therapies that raise HDL-C have failed to improve cardiovascular outcomes (Fogelman, 2010; Bohm and Werner, 2008.), and HDL seem to have a U-shape of risk in which both extreme high and low concentrations are associated with worse cardiovascular outcomes (Madsen *et al.*, 2017; Singh and Rohatgi, 2018). Triglyceride-rich lipoproteins have been to show a clear causal

role in atherosclerosis (Talayero and Sacks, 2011). Ultimately, the lipid hypothesis states that lipids are important to the development of atherosclerosis, but this hypothesis does not completely explain the pathogenesis of atherosclerosis as it does not provide insights into the contributions of other cell types.

The LDL oxidation hypothesis proposes that oxidatively LDL modified by reactive oxygen species (ROS) is the key driver to the pathogenesis in atherosclerosis (Steinberg, 2009). According to the hypothesis, when LDL cholesterol becomes oxidized, it can trigger an inflammatory response in the arterial wall, which leads to the accumulation of immune cells and the formation of atherosclerotic plaques (Westhuyzen, 1997). The process of LDL oxidation can be caused by a variety of factors, including smoking, high blood pressure, diabetes, and inflammation. While the LDL oxidation hypothesis has been a popular theory in the field of cardiovascular disease for several decades, there is still some debate about its validity due to failed clinical trials with antioxidants such as vitamin E or β-carotene (Steinberg, 2009). Some studies have suggested that other factors, such as inflammation and insulin resistance, may play a more significant role in the development of atherosclerosis rather than LDL oxidation alone (Libby *et al.*, 2019; Milutinović *et al.*, 2020). Monocyte/macrophages that are recruited to the endothelium and move to the sub endothelium engulf oxidized LDL to form foam cells/fatty streaks (Björkegren and Lusis, 2022). Several mouse studies have corroborated this hypothesis such as inhibiting oxidized LDL through anti-oxidant treatment (probucol and vitamin E) and studies using the 12/15-lipoxygenase knockout mice showing diminished atherosclerosis in apo E–deficient mice (Cyrus *et al.*, 1999). However, there is not conclusive evidence that oxidized LDL particles initiate human atherosclerosis despite its

clear role in plaque development. For example, in the Aggressive Reduction of Inflammation Stops Events (ARISE) trial, succinobucol, a lipid-soluble antioxidant that blocks LDL oxidation, did not reduce cardiovascular events (Ridker and Lüscher, 2014). Overall, while the LDL oxidation hypothesis remains a topic of ongoing research and discussion, atherosclerosis is a complex condition with multiple contributing factors. LDL oxidation likely contributes to the pathogenesis, but a comprehensive approach to prevention and treatment is likely to involve addressing additional mechanisms and a range of risk factors.

In 1977 Ross et al., proposed a more contemporary view of atherosclerosis called the response-to-injury hypothesis which stated that atherosclerosis forms subsequent to injury to the endothelium (Ross *et al.*, 1977). Endothelial dysfunction caused by factors such as altered shear stress from disturbed flow can lead to increased permeability to cholesterol-containing LDL particles, monocytes, and lymphocytes. Ultimately, adhesion molecule recruit monocytes and platelets at the sites of “focal injury” with resultant endothelial cell activation and expression of surface adhesion molecules such as VCAM-1, ICAM-1, selectins, and integrins. As a result of endothelial cell activation, there is a cascade of events including monocyte rolling /infiltration to the subendothelium, smooth muscle proliferation, deposition of lipids, and decreased NO bioavailability. The response-to injury hypothesis laid the groundwork for understanding the relationship between endothelium, lipids, and monocytes to the progression of atherosclerosis.

In 1995, Williams et al proposed the response-to-retention hypothesis that proposes that the retention of cholesterol-rich lipoprotein within the sub-endothelium is the initiating driver in atherosclerosis (Williams and Tabas, 1995). This advancement

indicates that endothelial cells are intact (and thus an insufficient driver of atherosclerosis) through lipoprotein retention and fatty streak formation (Delgado-Roche, 2014). Plasma LDL accumulate in the endothelium, and the ROS from macrophages and damaged EC generates oxidized LDL (oxLDL) (Libby *et al.*, 2019). oxLDL promotes inflammation, foam cell formation, smooth muscle cell proliferation, monocyte/T-cell recruitment, and activates EC. OxLDL is engulfed by macrophages through SR-A, CD36, and Lox1 receptors to form foam cells (Björkegren and Lusis, 2022). The response-to-retention hypothesis states the primary cause for atherogenesis is lipid accumulation in the subendothelial space independent of endothelium injury.

The inflammation hypothesis broadens our understanding of atherosclerosis from a disease of lipid accumulation associated with hypercholesteremia to the crucial involvement from innate/adaptive immune systems through immune cells (e.g.monocytes, macrophages, dendritic cells, and T cells) that generate an inflammatory cascade (Lusis, 2000; Hansson, 2005; Saaoud, 2017; Libby, 2021a). Endothelial cell activation increases adhesion molecules (e.g. VCAM-1, ICAM-1) and chemoattracts (e.g. Monocyte Chemoattractant Protein-1/MCP-1) that promote diapedesis (entry of leukocytes into blood vessel wall). Macrophage colony-stimulating factor (M-CSF) promotes monocyte differentiation of receptors SR-A and CD36 on the surface of macrophages to help uptake oxLDL (Ley *et al.*, 2011). Activated macrophages also secrete MCP-1, interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) that recruit more macrophages. Furthermore, inflammation mediated by several risk conditions such as hypertension, tobacco use, and insulin resistance can alter inflammatory cell function. Human atherosclerotic lesions contain T lymphocytes in which some subtypes such as

type 1 T helper (TH1) cells can promote atherosclerosis whereas others such as regulatory T (Treg) cells can decrease atherosclerosis (Saigusa *et al.*, 2020). The clinical meaningfulness of therapy for the inflammation pathways in atherosclerosis is complex. Recent, clinical trials have shown that targeting inflammation can reduce cardiovascular events for certain patient populations in both the 'Canakinumab, Anti-inflammatory Thrombosis Outcomes Study' (CANTOS) which tested an antibody that neutralizes the proinflammatory cytokine IL-1β and the 'Colchicine Cardiovascular Outcomes Trial' (COLCOT). However, other anti-inflammatory therapies such as methotrexate did not improve cardiovascular outcomes (Hassan, 2018). Inflammation is a key driver in the pathophysiology of atherosclerosis and the inflammation hypothesis provides a unifying theory for the development and progression of atherosclerosis.

During the progression of atherosclerosis, atherosclerotic plaques continue to accumulate lipids and lipid-engorged cells that form fatty-streaks. In the sub-endothelium, macrophages are engorged with lipids to form lipid-laden foam cells and smooth muscle cells migrate from the media to the intima while changing to a proliferative phenotype that exacerbates the forming atheroma and promotes deposition of collagen fibers. Macrophage and smooth muscle cells can undergo programmed cell death and impaired clearance of dead cells leading to the formation of a necrotic atherosclerotic plaque core. In atherosclerosis, monocytes can differentiate into the M1 and M2 state which promote inflammation and stimulate resolution of inflammation respectively. As macrophages engulf oxLDL they undergo apoptosis, a programmed cell-death, that results in cellular debris that can be cleared by M2 macrophages, a process called efferocytosis. However, as atherosclerosis progress, efferocytosis is impaired promoting a necrotic core. In

addition, an accumulation of inflammatory cells/smooth muscle cells releases matrix metalloproteinases (MMPs), proteolytic enzymes that degrade ECM, resulting in the thinning and degradation of the fibrous cap. After forming an advanced lesion, complications arise from events such as plaque erosion/rupture. Atherosclerotic plaques rupture, most commonly seen in plaques with large lipid cores with <60uM fibrous cap, is the most common trigger of acute thrombosis of coronary arteries that leads to myocardial infarction. Plaque erosion refers to erosion in atherosclerotic plaque that have a rich extracellular matrix without a thin, friable fibrous cap, few leukocytes, and little lipid. In addition, during advance stages of plaque development, calcification, a process of accumulation calcium phosphate hydroxyapatite crystals into the extracellular matrix of the growing atheroma, is found in complex lesions. Atherosclerosis progression is summarized 2.1.

Taken together, atherosclerosis is an exciting field that, as Libby et al states, is a moving target (Libby, 2021). There are numerous recent advancements in our understanding of the pathophysiology of atherosclerosis that have increased our knowledge of this complex disease. For example, smooth muscle cells can differentiate into foam-like cells similar to macrophages, a relationship between clonal hematopoiesis (clones of mutant myeloid cells) and cardiovascular disease has been established, and utilization of single-cell RNA sequencing to understand specific cell type contributions (Saigusa *et al.*, 2020; Libby, 2021c; Björkegren and Lusis, 2022). Atherosclerosis is a complex a multifactorial disease that is an interplay between lipids and inflammation in blood vessel.

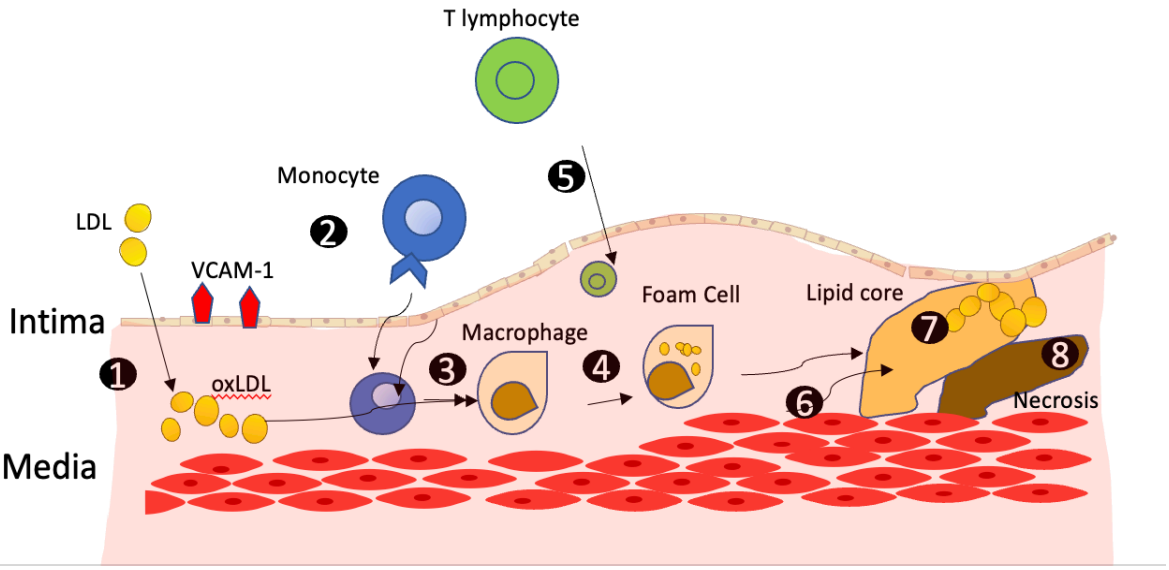


**Figure 2.1 Progression of atherosclerosis pathophysiology.** Atherosclerosis is a multifactorial disease that leads from cholesterol accumulation in the sub endothelium, foam cell generation, and eventually necrosis. 1. Cholesterol accumulates in the sub-endothelium, and it is oxidized, 2. Activated endothelium attracts Monocyte to sub-endothelium, 3. Monocyte differentiating into macrophage, 4. Macrophages filled with cholesterol to form foam cells 5. Immune cell-infiltration, 6. Smooth muscle cell migration, 7. Lipid core formation 8. Necrosis. Adapted from (Linton *et al.*, 2000)

2.1.3 Models of Atherosclerosis

Historically, rabbits and hamsters fed a high fat diet were the primary animal models used for atherosclerosis studies in the first half of the twentieth century (Fan *et al.*, 2015). Contemporarily, mice became the most used model of atherosclerosis due to their ease of breeding, established inbred strains, ease of genetic mutation, and low cost (Lusis, 2000; Saaoud, 2017). However, mice have a few drawbacks such as they are mostly a suitable model of atherogenesis (not plaque instability), do not spontaneous form lesions, provide smaller tissue samples, and have higher endogenous HDL levels (Santiago-Raber *et al.*, 2020a). Apolipoprotein-E knockout ($apoE^{-/-}$) and LDL receptor knockout ($LDLR^{-/-}$) genetic modifications in conjunction with a high fat diet are the most common mouse models of atherosclerosis (Getz and Reardon, 2006). In addition, large animal models such as pigs have been commonly used because of human similarity, size of vessels facilitates experimental inquiry, and as a secondary confirmatory model before clinical trials (Fan *et al.*, 2015).

Apolipoprotein-E is a plasma lipoprotein secreted from liver that facilitates binding, uptake, and clearance of triglyceride-rich lipoproteins such as very low-density lipoprotein (VLDL) and low density lipoprotein (Yachie, 2021). In $apoE^{-/-}$ mice, atherosclerotic lesions form throughout the entire of the aorta. In addition, these animals maintain high levels of plasma cholesterol and can form lesions without a high fat diet (although commonly incorporated) (Emini Veseli *et al.*, 2017). LDLR is a cell surface receptor, mostly expressed in the liver, that facilitates the endocytosis of LDL from the circulation. In $LDLR^{-/-}$ mice, LDL is not cleared which leads to accumulation of LDL in the circulation. These

animals typically are used with a high fat diet in order to generate atherosclerotic lesions (Fan *et al.*, 2015).

Recently, overexpressing proprotein convertase subtilisin/kexin type 9-(PCSK9) via an adeno-associated virus (AAV) vector has been used because it avoids the need of breading genetically engineered mice onto a hyperlipidemic background (Bayona *et al.*, 2020). PCSK9 was discovered in 2003 by a team of researchers led by Dr. Nabil Seidah and Dr. Catherine Boileau (Abifadel *et al.*, 2003). The researchers were studying the genetic causes of a rare disorder called familial hypercholesterolemia (FH), which is characterized by very high levels of LDL cholesterol in the blood and a high risk of premature heart disease. In their research, they identified a gene located on chromosome 1 that was associated with FH, but the gene's function was unknown. They found that this gene encoded a protein that they named PCSK9, which is involved in the regulation of cholesterol levels in the blood. The researchers discovered that PCSK9 binds to the LDL receptor, which is responsible for removing LDL cholesterol from the bloodstream and causes it to be degraded instead of being recycled back into the liver. This discovery provided a new understanding of how cholesterol is regulated in the body and opened new avenues for the development of cholesterol-lowering drugs. In fact, the discovery of PCSK9 led to the development of a new class of cholesterol-lowering drugs called PCSK9 inhibitors, which have been shown to be highly effective at reducing LDL cholesterol levels in patients with hypercholesterolemia who are at high risk for heart disease.

After a single injection with murine D377Y gain-of-function mutant PCSK9, mice develop an increase in total cholesterol (double baseline at 30 days) that remains even after 1-year post infection (Lu *et al.*, 2016) . These mice do not show adverse side effects

or liver damage. Cholesterol levels in mice at baseline range from ~ 128 mg/dl to 206mg/dl depending on strain (Getz and Reardon, 2006; Emini Veseli *et al.*, 2017). Mice with AAV- PSCK-9 gain of function mutation on chow alone can generate approximately 300 mg/dl of total cholesterol, and on a high fat diet (e.g. western diet), mice on this model (Lu *et al.*, 2016) can generate cholesterol levels greater than 1000 mg/dl which are similar values to those seen in apoE$^{-/-}$ and LDLR$^{-/-}$ mice (Getz and Reardon, 2006).

As seen in table 2.1, there are several diets used in mouse models of atherosclerosis disease that vary in composition. The goal of mouse diets is to increase atherosclerosis in genetically modified mouse models by increasing cholesterol and fat intake. There are several pros and cons for different diets. For example, western diet, the most widely used diet, leads to higher level of insulin resistance which confounds the result in atherogenesis studies in mice (Getz and Reardon, 2006). On the other hand, the Paigen diet can lead to hepatic toxicity in mice after three months because cholate alters cholesterol absorption (Fan *et al.*, 2015). Ultimately, mimicking atherosclerosis is complex and nuanced, but the advancement in genetically engineered mice and diets helps enhance our understanding of the pathophysiology of atherosclerosis.

| Diet | Composition | Model | Comments |
|---|---|---|---|
| **Paigen diet** | 1.25% Cholesterol, 0.5% Cholic acid, 15% Cocoa Butter 1% Corn Oil | C57BL/6 and C3H | Evaluated content of fat, cholesterol, and cholate on plasma lipids, and atherosclerosis |
| **Paigen diet without cholate** | 15.8% fat, 1.25% cholesterol, no cholate | LDLR $^{-/-}$ and various strains | contains cocoa butter or anhydrous milk fat as source of saturated fatty acids |
| **High-fat semisynthetic diet (AIN-76a)** | 17.4% cocoa butter, 2.8% soy oil, 0-1.25% cholesterol, 0-0.5% cholate | LDLR $^{-/-}$ | Contrasted diets with low/high-cholesterol levels in the presence or absence of cholate |
| **Low-fat semisynthetic diet** | 1.9% cocoa butter, 2.4% soy oil, 0%-0.5% cholesterol | LDLR $^{-/-}$ | Contrasted LDLR$^{-/-}$ mice in 2 genetic backgrounds for response to dietary cholesterol |
| **Western type diet** | 21% milk fat, 0.2% cholesterol (0.15% added, 0.05% from milk fat) | LDLR$^{-/-}$ and apoE$^{-/-}$ (other models also) | Most widely used diet. Commercial diets differ in carbohydrate source and presence of 1% corn oil |
| **Modified western type diet** | 18% milk fat, 0.25% cholesterol | LDLR$^{-/-}$ | Compared Western type and modified Western type diet |
| **Modified western type diet without cholesterol** | 16% milk fat, 5% lard, 0% added cholesterol | LDLR$^{-/-}$ | Increased insulin resistance and atherosclerosis compared to fructose/lard diet |
| **Semisynthetic diet with alternative sources of fat** | 18.5% fat from various plant sources, 0.2% cholesterol | LDLR$^{-/-}$ and apoE$^{-/-}$ | Atherosclerosis in LDLR$^{-/-}$ mice but not apoE$^{-/-}$ mice was influenced by type of dietary fat |
| **Semisynthetic diet with alternative protein sources** | 10% olive oil, 0%--1% cholesterol, 20% casein or soy protein, 0% - 0.25% cholate | apoE$^{-/-}$and LDLR$^{-/-}$ | Compared effect of protein source and isoflavones in soy protein extracts on atherosclerosis |
| **Palm oil diet** | 10% palm oil, 0.1% cholesterol | LDLR$^{-/-}$, apoE$^{-/-}$ | Equal amounts of saturated and monounsaturated fatty acids |

**Table 2.1. List of High Fat Diets used in Animal studies.** There are several mice diets used in atherosclerosis research which vary in composition, fat source and cholesterol percentage. Adapted from (Getz and Reardon, 2006).

## 2.2 Methods

### 2.2.1 Rationale

Patients with BT have been shown to have several severe vascular complications including thromboembolism, stroke, and impaired flow-mediated dilation of brachial arteries (Needs *et al.*, 2022). Recently, there is growing body of literature that shows that children with BT have increased, carotid intimal media thickness, a sign of premature atherosclerosis (Manios *et al.*, 2009; Hahalis *et al.*, 2011, 2016; Dogan and Citak, 2012; Merchant *et al.*, 2016; Sherief *et al.*, 2017; Ibrahim *et al.*, 2020; Efat *et al.*, 2022). However, it is not known if there is a direct relationship between BT and atherosclerotic disease. BT patients have increased leukocyte aggregation, high triglycerides levels, endothelial dysfunction, anemia, and iron overload that could contribute to a proatherogenic environment (Cao and Galanello, 2010; Hahalis *et al.*, 2016; Sherief *et al.*, 2017; Needs *et al.*, 2022). Our central hypothesis is that there is a proatherogenic environment in BT that leads to advanced atherosclerotic lesion formation.

$Hbb^{th3/+}$ is a widely acceptable heterozygous mouse model that recapitulates BT by displaying ineffective erythropoiesis, intravascular hemolysis, anemia, and decreased beta chain synthesis similar to what is found in human patients (Kumfu *et al.*, 2017). By using an adeno-associated virus vector (AAV) gain of function mutation (D377Y) of PCSK9, we can study atherosclerosis and induce hyperlipidemia in a mouse model without extensive and time-consuming breeding protocols (Lu *et al.*, 2016). PCSK9 directly binds to the LDL receptor and targets it for lysosomal degradation which results in elevated serum cholesterol levels (Lu *et al.*, 2016). The Paigen diet was selected because we selected both a 1- and 3-month model of atherosclerosis which could

generate more atherosclerosis than a western diet, due to the higher cholesterol percentage, while avoiding the long-term effects of cholate that arise after 3 months.

2.2.2 Methods

*2.2.2.1 Animals*

$Hbb^{th3/+}$ mice (Jax No.000996) on a C57BL/6 background were used as a model of BT-intermedia. C57BL/6 mice wildtype (WT) littermates were used as normal controls. Both male and female WT and BT mice ($Hbb^{th3/+}$) between 8 and 12 weeks old were used in this study to determine if accelerated atherosclerosis is present in BT. Mouse colonies were established, maintained, and bred in-house at the Emory University Department of Animal Resources.

*2.2.2.2 Models of Atherosclerosis*

Susceptibility to atherosclerosis was induced by feeding mice a high fat diet (HFD, Research Diets, CAT # D12336) and inducing low-density lipoprotein receptor suppression by administering gain-of-function proprotein convertase subtilisin/kexin type 9 (Vector Biolabs, AAV8-D377Y-mPCSK9) (Lu *et al.*, 2016). Mice were retro-orbitally injected with 1E11 genomic copies of AAV8-D377Y-mPCSK9 and then placed on HFD for 12 weeks. A secondary confirmatory model of atherosclerosis was done by retro-orbitally injecting mice with AAV8-D377Y-mPCSK9, subcutaneously implanting osmotic minipump (Azlet, CAT # 2004) delivering angiotensin II (Sigma-Aldrich, CAT #A9525) at a rate of 0.75 mg/kg per day, and feeding mice a HFD (Research Diets, CAT # D12336) for 4 weeks as previously described (Weiss *et al.*, 2001).

2.2.2.3 Endpoints

*2.2.2.3.1 Serum assays*

Blood was obtained via cardiac puncture following euthanasia for analysis of serum assays. Blood was then centrifuged at 6,000 RPM for 15 min to obtained serum. Free heme was obtaining by centrifuging fresh serum in <3kda Amicon Ultra-0.5 Centrifugal Filter columns (Sigma-Aldrich, CAT# UFC500396) for 1.5 hours at 10,000 RPM and measured using the QuantiChrom™ Heme Assay Kit (BioAssay Systems, CAT # DIHB-250).

*2.2.2.3.2 Atherosclerotic endpoints:*

*2.2.2.3.2.1 Cholesterol*

Total plasma cholesterol, triglycerides and high-density lipoprotein cholesterol (HDLc) were determined by enzymatic methods on the Beckman AU480 (Beckman Diagnostics, Brea, CA) automatic chemistry analyzer. Reagents, calibrators, and controls are obtained from Sekisui Diagnostics (Burlington, MA).

*2.2.2.3.2.2 Atherosclerotic lesion area:*

Mice were euthanized by $CO_2$ inhalation at prescribed time points (1 or 3 months). Heart and aorta were pressure-perfused with 0.9% sodium chloride solution, followed by pressure fixation at ≈100 mm Hg with a 4% formaldehyde solution. The hearts were embedded in paraffin and 5-µm-thick serial sections were prepared. Atherosclerosis was evaluated by measuring cross sectional atherosclerotic lesion area in the aortic root and total atherosclerotic lesion area (%) relative to luminal area *en face* (analyzed via Image J) in the descending thoracic and abdominal aorta (Weiss *et al.*, 2001). Histological analysis on the aortic root was used to evaluate lesion complexity through H&E and

Mason's trichrome staining. Analysis was carried out using American Heart Association guidelines: Data was randomized, predefined data analysis criteria, and 2 individuals to quantify lesions area independently (Daugherty *et al.*, 2017).

2.2.2.4 Statistical Analysis

Atherosclerotic lesion area in the aortic root and thoraco-abdominal aortic is expressed as mean ± SEM, tested for normality, and parametric test selected for analysis. All statistical analysis was carried out using Prism (Graphpad Software, La Jolla, CA) and significance was determined as indicated in the figure legends. Differences between two experimental groups were evaluated using unpaired Student t-test. Two-way ANOVA test was used for comparing more than two groups of data with tukey HSD post hoc test. Normality was tested using Kolmogorov-Smirnov test.

## 2.3. Results

### 2.3.1 Beta Thalassemia's Underlying Pathophysiology leads to Accelerated Atherosclerosis.

We report that the underlying pathophysiology in BT mice clearly leads to accelerated atherosclerosis. We saw an increase in total lesion area (%) relative to luminal area via aortic *en face* analysis in both BT mice sexes compared to their respective WT counterparts (WT female: 10.6 ± 2.9 % vs. BT Female: 23.3 ± 1.5 %, $p < 0.001$; and WT male: 28.1 ± 2.4 % vs. BT male: 45.5 ± 2.4 %, $p < 0.001$, Figure 2.2). Atherosclerosis was higher in both BT and WT male mice compared to their respective female counterparts. Atherosclerosis in BT male mice was found throughout the entirety of the aorta, while WT male aortic plaque accumulation was largely regionalized to the

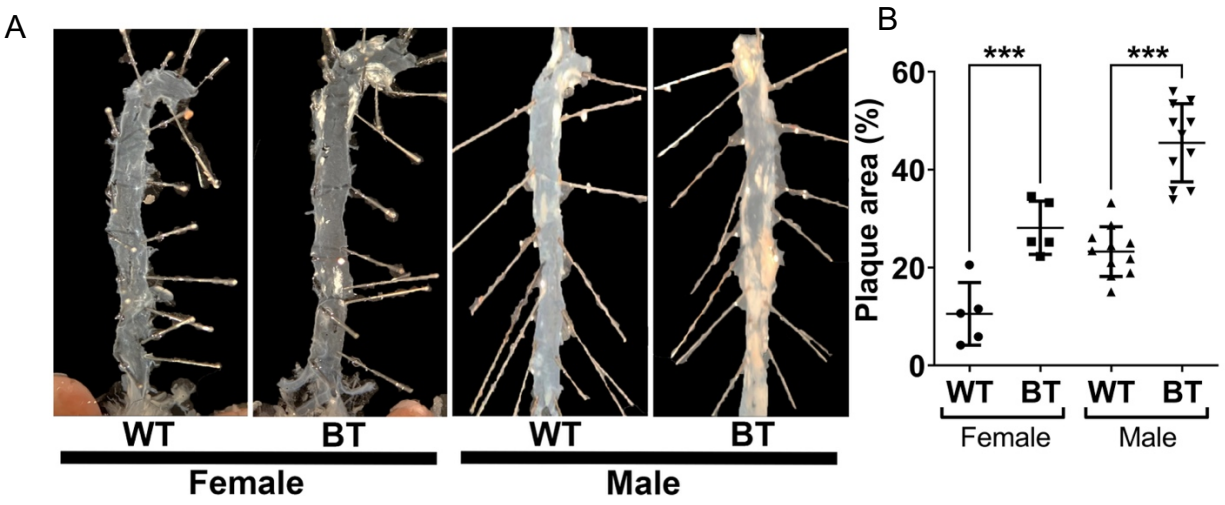


**Figure 2.2. Aortic enface Demonstrates Accelerated Atherosclerosis in BT Mice.** (A) Representative aortas of WT and BT female/male mice, (B) Plaque accumulation % analyzed relative to total luminal area. Values represent mean ± SEM. ***P<0.001, n=5-11.

aortic arch. There was no significant difference in the distribution (thoracic or abdominal) of atherosclerosis between WT and BT female mice. Similarly, we saw an increase in atherosclerotic lesion area via aortic root analysis in both BT sexes compared to their respective WT counterparts (WT female: 136.4 ± 13.6 $\mu m^2$ $x10^3$ vs. BT female: 241.7 ± 22.1 $\mu m^2$ $x10^3$, $p < 0.05$, and WT male: 304.6 ± 30.9 $\mu m^2$ $x10^3$ vs. BT male: 444.7 ± 38.1 $\mu m^2$ $x10^3$, $p < 0.01$, Figure 2.3). As shown in figure 2.4, both WT and BT developed less total cholesterol (total cholesterol WT: 690.5 ± 32.2 mg/dl vs. BT: 526.8 ± 51.8 mg/dl, $p < 0.01$). Low-density lipoprotein and high-density lipoprotein were increased and decreased respectively in BT mice compared to WT mice.

2.3.2 Secondary Model Confirms Accelerated Atherosclerosis in Beta Thalassemia

To confirm these findings, we also preformed studies in an aggressive short-term model of atherosclerosis (AAV-PCSK9 gain of function + angiotensin II infusion + HFD for 1 month). Similarly, to our main atherosclerotic model (AAV-PCSK9 gain of function + HFD for 3 months), both BT male and female mice developed more severe atherosclerosis as indicated by aortic *en face* analysis compared to their respective WT counterparts (Figure 2.5). In fact, atherosclerosis and subsequent aneurysmal formation was so severe (Figure 2.5) that it led to a 56.6% mortality rate in our BT mice compared to 12.5% in our WT mice (Figure 2.5). Due to high mortality and aneurysm formation not being a common phenotype in BT, we focused our studies only on our main model of atherosclerosis (AAV-PCSK9 gain of function + HFD for 3 months) (Farmakis *et al.*, 2004).

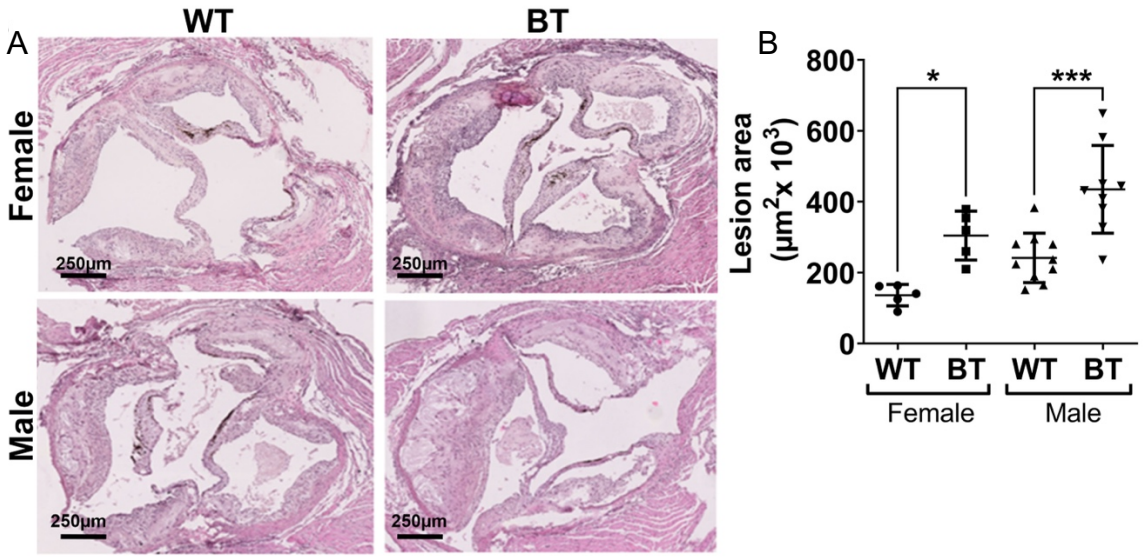


**Figure 2.3. Aortic Root Plaque Accumulation Demonstrates Accelerated Atherosclerosis in BT Mice.** (A) Representative H&E aortic root stains of WT and BT female/male mice, (B) Analysis of aortic lesion area $\mu m^2X10^3$.Values represent mean ± SEM. (n=5-11). *P<0.05; ***P<0.001, n= 5-11.

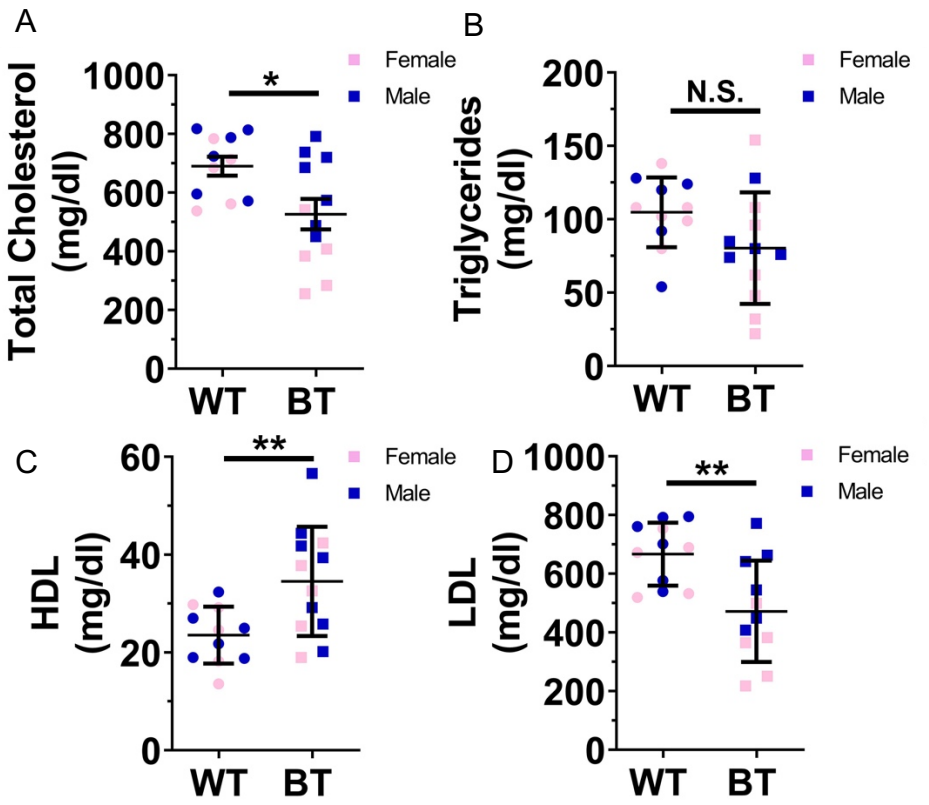


**Figure 2.4. Serum Lipid Levels are Unchanged.** (A) Serum total cholesterol, (B) Serum triglycerides, (C) Serum HDL, (D) Serum LDL. Male depicted in blue and female depicted in pink. Values represent mean ± SEM. *P<0.05; **P<0.01, n=5-11.

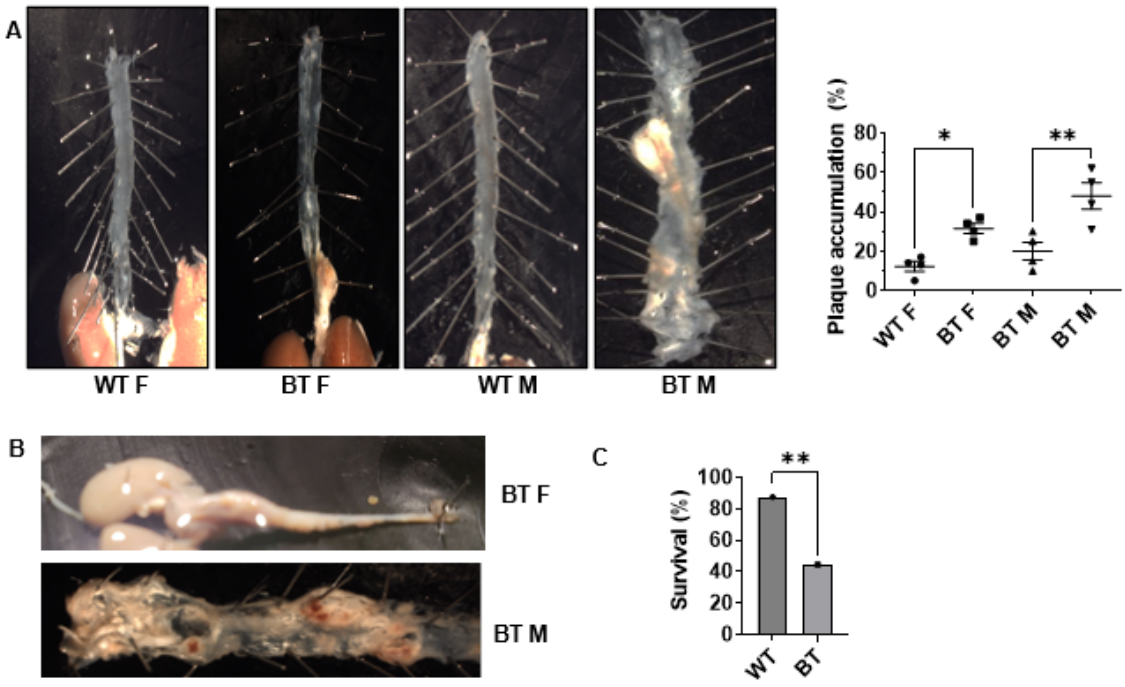


**Figure 2.5. Secondary Model of Atherosclerosis Confirms Accelerated Atherosclerosis in BT.** Short-term model of accelerated atherosclerosis with angiotensin II infusion confirms accelerated plaque accumulation in BT mice. (A) Representative aortic en face analysis (B) Representative severe aneurysms. (C) Percent survival in WT and BT mice. Values represent mean ± SEM. *P<0.05; **P<0.01. n=4.

2.3.3 Beta Thalassemia is a Hemolytic Anemia with Intravascular Hemolysis.

At baseline, serum hemopexin levels were severely depleted in BT mice (WT: 6.2 ± 0.2 mg/ml vs. BT: 0.5 ± 0.1 mg/ml, $p < 0.01$, Figure 2.6). In addition, BT mice had an increase in free heme compared to their wild type littermates (WT: 0.06 ± 0.02 mM vs. BT: 0.2 ± 0.04 mM, $p < 0.05$, Figure 2.7).

## 2.4 Discussion

BT patients have a variety of poorly understood and complex cardiovascular complications such as ineffective erythropoiesis, hypercoagulability, leg ulcers, altered lipid profiles, and impaired flow-mediated dilation of brachial arteries (Cao and Galanello, 2010; Needs *et al.*, 2022). To our knowledge, 12 clinical association studies from 2009-2022 have shown an increase in carotid-intimal media thickness in both BT major and BT intermedia with and without chelation history (Tantawy *et al.*, 2009; Hahalis *et al.*, 2011; Dogan and Citak, 2012; Gursel *et al.*, 2012a; Aa *et al.*, 2015; Hahalis *et al.*, 2016; Merchant *et al.*, 2016; Sherief *et al.*, 2017; Ahmad Ibrahim *et al.*, 2021a; Soltani *et al.*, 2021; Efat *et al.*, 2022; Kumaravel *et al.*, 2022). However, it is not known if there is a direct relationship between BT and atherosclerotic disease. From a cardiovascular risk perspective, BT patients traditionally have hypocholesteremia with low LDL level (Maioli *et al.*, 1989; Haghpanah *et al.*, 2010), lower blood viscosity due to decreased hematocrit (Crowley *et al.*, 1992; Nader *et al.*, 2019; Sloop *et al.*, 2002), and a lower incidence of hypertension (Hashemi *et al.*, 2007). On the other hand, BT patients have increased iron levels (from both transfusion and increased gastrointestinal iron absorption) (Taher and Saliba, 2017; Krittayaphong *et al.*, 2018), increased platelet factor 3 activity (Timan *et al.*, 1993), and increased triglycerides with low HDL

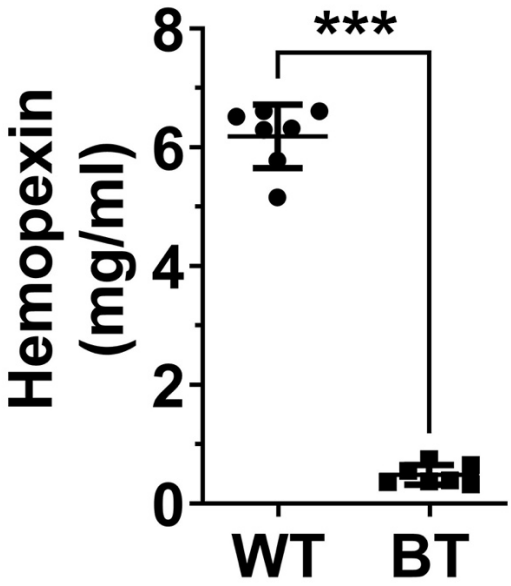


**Figure 2.6. Hemopexin Levels are Exhausted in BT Mice.** Baseline serum hemopexin (HPX) levels in WT and BT mice. Values represent mean ± SEM. *P<0.001; **P<0.01, n=7.

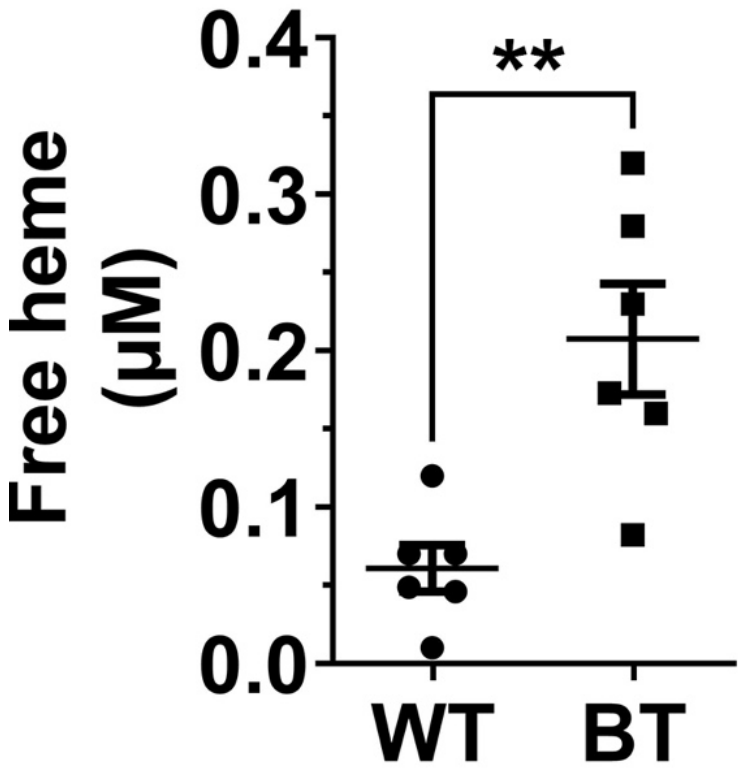


**Figure 2.7. BT has Elevated Free Heme Levels.** Baseline free heme in the serum (n=6). Values represent mean ± SEM. **P<0.01. n=6.

(Tantawy *et al.*, 2009). Traditionally, myocardial infarction is not common in BT, but In 2004, the first incidence of myocardial infarction was reported in BT with subsequent sporadic case reports in 2009 and 2023 (Fridlender and Rund, 2004b; El Rassi et al., 2009; Premawardhena et al., 2023). In 2016, Hahallis et Al. showed that coronary artery calcium score was not significantly changed, but carotid intimal thickness was elevated suggest a disparate rate of atherosclerosis progression between carotid and coronary arteries among thalassemic patients (Hahalis et al., 2016). The average life expectancy has increased from 16 years in 1964 to 40-49 years in 2011 with 37%, and 89% respective survival at average life expectancy (Dhanya et al., 2020). As a result, BT disease presentation is a changing landscape that has more comorbidities due to an ageing population such as supraventricular arrhythmias, left ventricular dysfunction, and pulmonary hypertension (Farmakis et al., 2020) It is clear that BT has potentially very complex cardiovascular effects on the vasculature, but it is unclear if BT has a direct, causal role in the pathogenesis of atherosclerosis.

Our results are striking that in a mouse model of BT intermedia, we saw markedly accelerated atherosclerosis. We found increased atherosclerosis in both male and female BT mice as measured by both enface and aortic root analysis in 2 models of atherosclerosis. BT mice had more than a 1.5-fold increase in plaque accumulation in aortic *enface* and the plaque accumulation was robust and present throughout the entire aorta. Unsurprisingly, atherosclerosis was more prevalent in male mice, a common finding others have reported which may be due to innate differences between males and females or due to decreased PCSK9 expression in female mice (Vozenilek *et al.*, 2018). Both WT and BT mice developed similar degrees of hypercholesteremia in our studies.

Although the main hallmark of BT is ineffective erythropoiesis, our results and clinical evidence indicates that BT underlying pathophysiology leads to increased intravascular hemolysis. We found that at baseline, the BT mice exhibited an increase in free heme and a decrease in HPX. This finding is similar to observations in humans with BT (Vinchi, Vercellotti, *et al.*, 2016; Kiger *et al.*, 2019b).

A few limitations to our study are that we had to stagger in our mice to each experimental group over the course of several years due to mice breeding issues. Our mice had the same 3-month endpoints, but the ideal experimental setup would have been to have all mice on the atherosclerotic experiments concurrently to limit environmental biases. In addition, our BT mouse model is a heterozygous model that only reflects BT intermedia; we cannot study BT major because the mice are neonatal fatal (Kumfu *et al.*, 2017). Despite $Hbb^{th3/+}$ being the most widely accepted mouse model of BT, it is only a model of BT intermedia, and we will not be able to understand the complete impact of no beta globin gene production such as found in BT major (Kumfu *et al.*, 2017). Realistically, BT patient have regular blood transfusion, splenectomy, and iron chelation (explored in chapter 4) that could also impact atherosclerosis in BT but were not investigated. Lastly, the progression of atherosclerosis in mouse models may not be the same as in humans (Fan *et al.*, 2015).

In these experiments, we have established an animal model that provides a viable system to investigate the link between BT and atherosclerosis and convincingly demonstrates that BT causes increased atherosclerosis. We performed long- and short-term model of atherosclerosis in both male and female BT mice compared to their respective WT counterparts. Our main model of atherosclerosis (AAV-PCSK9 gain of

function + HFD for 3 months), gave a good background to signal ratio while avoiding confounding factors such as survival bias seen in our short-term model. Using these models, we have gathered conclusive evidence that beta-thalassemia accelerates the advancement of atherosclerosis. Moreover, we have discovered that although females have a lower atherosclerotic disease burden, BT's impact on atherosclerosis remains similar. Furthermore, we also recapitulated human findings that BT is clearly a hemolytic anemia that leads to increase free heme and decreased hemopexin.

# Chapter 3: Overexpressing Hemopexin in Hemolytic Mice on a Long-Term Model of Atherosclerosis

## 3.1 Introduction to HPX experiments

### 3.1.1 Introduction

The heme molecule is composed of a porphyrin ring and an iron ion that is coordinated to the ring (Guo *et al.*, 2022). The porphyrin ring is a flat, planar structure composed of four pyrrole subunits that are linked by methine bridges as shown in figure 3.1 (Nagy *et al.*, 2010). The four nitrogen atoms in the pyrrole subunits coordinate to the iron ion in the center of the ring. The iron ion in heme can exist in two oxidation states: Fe(II) / ferric and Fe(III) / ferrous. Heme is found in many different proteins, including hemoglobin, myoglobin, and cytochromes. In hemoglobin and myoglobin, heme is responsible for binding and transporting oxygen, while in cytochromes, it participates in electron transport reactions. Heme is an important molecule that is a porphyrin ring with an iron ion, participates in oxygen transport, and found inside red blood cells.

The underlying pathophysiology of BT leads to excess levels of free heme in the vasculature and subsequent oxidative stress/inflammation. In BT, the reduced production of beta globin gene leads to excess pairs of alpha-hemichromes that leads to erythrocyte instability and increased hemolysis as depicted in figure 3.2 (Cao and Galanello, 2010; Needs *et al.*, 2022). Typically, heme levels are well regulated; however, in condition such as in hemolytic anemia, the body can be exposed to excess levels of free heme (Morris, 2008a). Excess free heme can generate ROS directly through Fenton chemistry (Voskou *et al.*, 2015) or downstream TLR4 signaling (Figueiredo *et al.*, 2007; Lin *et al.*, 2012) further described in chapter 1.4.2.2.

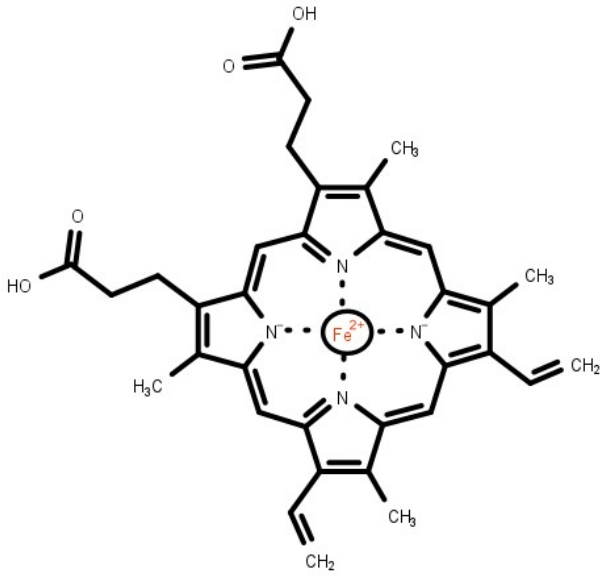


**Figure 3.1. Heme Molecule.** A heme molecule with a ferric iron coordinated to a porphyrin ring.

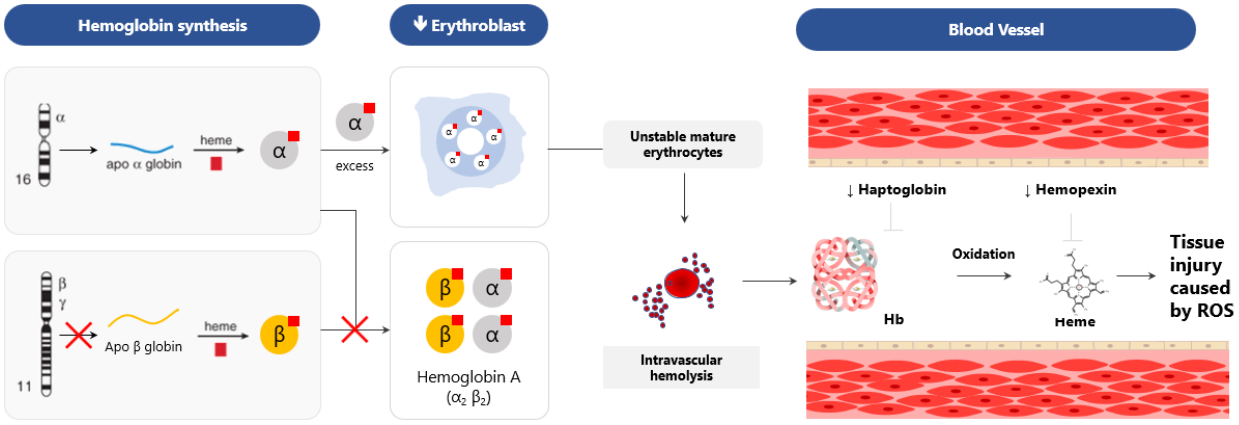


**Figure 3.2. BT's Underlying Pathophysiology Leads to Increased Hemolysis.** Reduced production in the beta globin gene leads to increased unpaired alpha- hemichromes that cause erythrocyte instability and subsequent hemolysis. Consequently, the vasculature is exposed to high levels of free heme and depleted levels of hemopexin.

Hemopexin (HPX), heme's endogenous scavenger, prevents heme toxicity, but in hemolytic anemias such as BT, hemopexin is exhausted which results in more than a ten-fold decrease compared to controls (Vinchi, Vercellotti, *et al.*, 2016). Excess free heme leads to increased ROS formation and hemopexin depletion.

The role of heme is well documented in cardiovascular diseases such as atherosclerosis, heart failure, myocardial ischemia-reperfusion injury, degenerative aortic valve stenosis, and cardiac iron overload (Guo *et al.*, 2022). Heme can mediate injury by various methods such as intercalating into lipid membranes due to its hydrophobic nature, generating ROS that leads to DNA damage, and promoting lipid peroxidation (Schmitt *et al.*, 1993; Higdon *et al.*, 2012; Guo *et al.*, 2022). Here, we investigate the contribution of heme to the progression of atherosclerosis in BT mice and a drug induced model of hemolysis. Furthermore, we used an AAV-HPX therapy strategy to determine the relative importance of heme in BT-mediated accelerated atherosclerosis.

The effect of heme, heme-related proteins, and heme decomposition metabolites is complex. Summarized in figure 3.2 and discussed in Chapter 1.4.2, free heme, free hemoglobin, and iron are pro-inflammatory, while heme oxygenase metabolites, such as biliverdin/CO, and scavenging proteins such as hemopexin decrease heme-mediated effects and are anti-inflammatory (Morris, 2008a). Heme can lead to LDL oxidation, TLR4 actitation, and downstream activation of the Fenton reaction via iron oxidation (Lin *et al.*, 2012).

3.1.2 Heme and Atherosclerosis

Heme contributes to atherosclerosis by acting as pro-oxidant in many different cell types. Globally, heme promotes the generation of active alkoxyl (RO•)/peroxyl (ROO•) radicals via Fenton chemistry, stimulates lipid peroxidation, and activates TLR4 signaling.

Erythrocyte metabolites such as heme and oxidized LDL molecules accumulate in atherosclerotic plaques. *Nagy et al.* showed in ex-vivo human aortic tissue samples the accumulation of red blood cell, ferri-hb and free heme, all of which can promote complicated atherosclerotic lesion formation (Nagy *et al.*, 2010). Free heme can also promote atherogenesis by increasing reactive lipid metabolites such as oxidized LDL. Free heme spontaneously inserts into LDL particles and promotes oxidation (Jeney *et al.*, 2002). During hemolysis, hemoglobin is released and eventually oxidized in order from Ferro-Hb then Ferryl- Hb to Ferri Hb (also known as methemoglobin) described in more detail in chapter 1.4.2.1 (Bunn and Jandl, 1966). Ferri-Hb and free heme both lead to LDL oxidation while the other Hb moieties do not alter LDL oxidation (Jeney *et al.*, 2002). Furthermore, oxidized-LDL leads to increases oxidation of Ferro-Hb to Ferri-Hb (Nagy *et al.*, 2010).

In endothelial cells (ECs), Balla et al. showed that heme does not directly cause cytotoxicity, but ECs loaded with heme are exceedingly susceptible to oxidative stress induced by either hydrogen peroxide, oxidants produced enzymatically (xanthine/xanthine oxidase), or activated leukocytes (Balla *et al.*, 1991). Ultimately, heme leads to ECs activation by increasing adhesion molecules and EC permeability (Silva *et al.*, 2009; Potor *et al.*, 2013). In human umbilical vein endothelial cells, heme in a dose-dependent manner was able to increase HO-1, ferroportin, ICAM, E-selectin, and iNOS

that could be quenched in a 1:1 ratio by hemopexin (Vinchi *et al.*, 2013). Others have shown in HUVECs that heme leads to increase P-selectin, von Willebrand factor, soluble VCAM, and interleukin 8 expression which can also be reversed by hemopexin (Gentinetta *et al.*, 2022b). Heme has been shown to increase NLRP3 inflammasome and subsequent production of Interleukin-1 beta in ECs (Erdei *et al.*, 2018). In a model of SCD mice, heme triggers TLR4 signaling, endothelial cell activation, and vaso-occlusion (Belcher *et al.*, 2014).

In vascular smooth muscle cells (VSMC), heme activates NADPH oxidase-mediated ROS formation and downstream redox-sensitive mitogen-activated protein kinase that leads to concentration-dependent migration and proliferation of VSMCs (Moraes *et al.*, 2012). More recently, heme has also been shown to induce endoplasmic reticulum stress in human aortic smooth muscle cells through all classical endoplasmic reticulum-related pathways (ATF-4 expression, X-Box Binding Protein 1, and ATF6 cleavage) (Gáll *et al.*, 2018). However, downstream heme decomposition products including carbon monoxide and biliverdin can inhibit the proliferation of VSMCs during chronic low dose hemin administration (Chang et al., 2008). In the presence of heme, HPX administration is able to decrease VSMCs proliferation, while HO-1 inhibition leads to increased VSMC proliferation (Moraes *et al.*, 2012). The levels of heme, heme-decomposition products, and heme scavenger affect VSMCs proliferation and ROS formation.

Heme affects macrophages by promoting phenotype switching, HO-1 expression, and activation of TLR4 signaling. Macrophages are important to the development of atherosclerosis because they deposit in the sub endothelium and convert to “foam cells”

(Libby *et al.*, 2019). Classically, macrophages are divided into class 1 and class 2 that have pro-inflammatory and anti-inflammatory effects, respectively (Guo *et al.*, 2022).

In vitro, peritoneal macrophages from HO-1 knockout mice compared to controls had more ROS generation, MCP-1/IL-6 expression, and increased foam cell formation when treated with oxLDL (Orozco *et al.*, 2007). Heme directly activates TLR4 signaling in macrophages that leads to increased TNF$\alpha$ expression, oxidative burst, and neutrophil recruitment (Figueiredo *et al.*, 2007). New classes of macrophages such as Mhem (M hemoglobin) have been described in the atherosclerosis literature (Boyle *et al.*, 2009). Mhem is associated with intraplaque hemorrhage and phenotypically categorized by CD163 expression (the hemglobin:haptoglobin receptor as described in 1.4.2), HO-1, IL-10 secretion, and low levels of human leukocyte antigen-DR (Boyle, 2012). The role Mhem plays in atherosclerosis is controversial (Guo *et al.*, 2022). Researchers found that Mhem have no lipid retention which is contrary to what is typically found in macrophage differentiated foam cells, and they promote cholesterol efflux by upregulating activating transcription factor 1 (Boyle, 2012; Finn *et al.*, 2012). On the other hand, Guo et al. found that Mhem induces plaque accumulation by increasing ferritin, endothelial permeability, and VCAM expression (Guo *et al.*, 2018). These studies show that heme actives multiple pro-inflammatory pathways in macrophages to promote a proatherogenic environment.

### 3.1.3 Model of hemolysis

There are several genetic, drug, and infection-related models to induce hemolysis. A few of the most popular genetic models classes are hemolytic anemias (e.g. sickle cell-Townes, BT- $HBB^{th3/+}$), autoimmune hemolytic anemias (e.g. New Zealand black, Playfair and Marshall–Clarke, HOD transgenic mice, HL transgenic mice), and inherited blood

disorders (e.g.spherocytosis) (Vinchi *et al.*, 2013; Belcher *et al.*, 2014; Kumfu *et al.*, 2017; Howie and Hudson, 2018). Administration of hemin, ferric iron heme usually coordinated to a chloride ion, and Phenylhydrazine (PHZ) are two of the most popular pharmacological ways to induce elevated levels of free heme in the vasculature (Paul *et al.*, 1999; Kazunobu Ishikawa *et al.*, 2001). Both hemin and PHZ are traditionally used for short models of hemolysis, but they have also been used in long-term models of subclinical hemolysis (Paul *et al.*, 1999; Fernandez *et al.*, 2001a; Graw *et al.*, 2022). In addition, there are several factors to consider when selecting an appropriate model: such as anemia severity, animal safety, technical feasibility, and the ability to recapitulate the human disease. For this proposal, we have used BT intermedia mice (HBBth3/+) to recapitulate BT human disease as it the most widely accepted mouse model of BT. We have selected a PHZ model as a complimentary drug-induced model of hemolysis to evaluate the contribution of heme and phenotype reversal with HPX therapy due to its technical simplicity, modularity, and previous use in long-term models of atherosclerosis. Furthermore, PHZ has been accepted as a “partial model for BT” because it recapitulates the RBC blood cell hemodynamic properties (Ramot *et al.*, 2008).

**3.2 Methods**

Male Mice (n=6-12) were treated with PCSK9 overexpressing AAV and 3-months of the Paigen high fat diet as described in 2.2. Mouse colonies were established, maintained, and bred in-house at the Emory University Department of Animal Resources or acquired from Jackson laboratory. Serum assays and atherosclerotic endpoints were described in chapter 2.2.

### 3.2.1 Phenylhydrazine

C57BL/6 mice from Jackson laboratory (CAT# 000664) were used in these experiments. Three groups of mice were utilized: controls (no additional treatment), PHZ (treated with Phenylhydrazine), and PHZ+HPX (treated with Phenylhydrazine and HPX therapy). For our drug-induced model of intravascular hemolysis, mice received a weekly intraperitoneal injection (25 mg/ kg in 0.1 ml of phosphate buffered saline) of fresh Phenylhydrazine (Paul *et al.*, 1999; Fernandez *et al.*, 2001a).

### 3.2.2 Beta Thalassemia

BT ($Hbb^{th3/+}$) mice (Jax # 000996) on a C57BL/6 background were used as a model of BT-intermedia (Kumfu *et al.*, 2017). Aged match C57BL/6 wild type (WT) littermates were used as normal controls. Three groups of mice, all treated with the PCSK9 AAV, were utilized: WT, BT, and BT + HPX (BT mice treated with HPX therapy).

### 3.2.3 Hemopexin therapy

Increased expression of hemopexin was achieved using an AAV encoding the human hemopexin gene. AAV8-mouse hemopexin expression tagged to GFP and carrying ApoE/hATT1e was generated by Vector Biosystems. Inc (Malvern, PA). This AAV preferentially targets the mouse liver. In the HPX-treated mice, 4E11 GC AAV-Hemopexin was co-injected retro-orbitally with PCSK9.

### 3.2.4 Assays

#### *3.2.4.1 Iron assays*

Both total Iron (directly from serum) and non-transferrin bound iron (after centrifuging serum in <3kda Amicon Ultra-0.5 Centrifugal Filter columns) was measured

using an Iron Assay Kit (Sigma-Aldrich, CAT# MAK025). Hematocrit was measured via the microhematocrit method as previously described (Mondal and Lotfollahzadeh, 2022).

*3.2.4.2 Hydrogen peroxide detection*

Extracellular $H_2O_2$ from aortas was measured using the Amplex Red assay kit (Invitrogen, CAT # A22188) as previously described (Lewis *et al.*, 2022). Fluorescence was detected on a fluorescence plate reader (Ex/Em= 530/580 nm) with background fluorescence subtracted. Fluorescence was normalized to dry tissue weight.

*3.2.4.3 Polymerase Chain Reaction*

Total RNA was extracted from homogenized aortas using the RNeasy Mini Kit (Qiagen, CAT # 74004) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA and purified with QiaQuick (Qiagen, CAT# 28104). Gene expression was quantified on an Applied Biosystems StepOnePlus Real-Time PCR System. IL-6, TNFα and 18S Quantitect Primer Assays were purchased from Qiagen. Gene expression was normalized to the housekeeping gene 18S, and expressed relative to the average untreated wildtype (AA) value using the comparative cycle threshold (Ct) method with the formula: Fold change= $2^{-\Delta\Delta Ct}$.

3.2.5 Statistical Analysis

Atherosclerotic lesion area in the aortic root and thoraco-abdominal aortic is expressed as mean ± SEM, tested for normality, and parametric test selected for analysis. All statistical analysis was carried out using Prism (Graphpad Software, La Jolla, CA) and significance was determined as indicated in the figure legends. Differences between two experimental groups were evaluated using unpaired Student t-test. Two-way ANOVA test

was used for comparing more than two groups of data with tukey HSD post hoc test. Normality was tested using Kolmogorov-Smirnov test.

**3.3 Results**

3.3.1 Phenylhydrazine-mediated Hemolysis leads to Accelerated Atherosclerosis that can be Reduced with Hemopexin Therapy

Initially, we wanted to determine a dose of PHZ that led to similar hematocrit levels found in our BT mice. Our BT mice have a hematocrit value of 32.3 ± 0.5 % compared to 44.2 ± 0.6 % found in their respective WT counterparts as shown in figure 3.3. We conducted a pilot experiment by injecting 25 mg/kg of PHZ twice a week for a month that led to similar hematocrit levels as seen in our BT (figure 3.4). However, an initial pilot experiments on our model of atherosclerosis (PCSK9 overexpression and Paigen high fat diet for 3-months) saw a >70% mortality rate by 2 months in mice treated with PHZ twice a week. As a result, we adjusted our dose to one injection of PHZ per week. Hematocrit was significantly reduced in our PHZ groups compared to our littermate controls (control: 44.8 ± 0.6 % vs. PHZ: 31.2 ± 1.8 % or PHZ + HPX: 33.2 ± 0.6 %, $p < 0.01$). This was similar to what was observed in the BT mice (BT: 32.7 ± 0.4 %, Figure 3.5). In addition, as shown in figure 3.6, PHZ treatment also resulted in significant splenomegaly similar to what is typically observed in BT mice due to extramedullary hematopoiesis (Kumfu *et al.*, 2017).

Aortic *en face* analysis showed increased plaque accumulation in the PHZ group compared to controls (control: 20.3 ± 2.2 % vs. PHZ: 41.9 ± 4.9 %, $p < 0.05$, Figure 3.7). Aortic root total lesion area was increased in the PHZ group compared to controls (controls: 274.6 ± 20.9 $\mu m^2$ $x10^3$ vs. PHZ: 470.0 ± 58.7 $\mu m^2$ $x10^3$, $p < 0.05$, Figure 3.7).

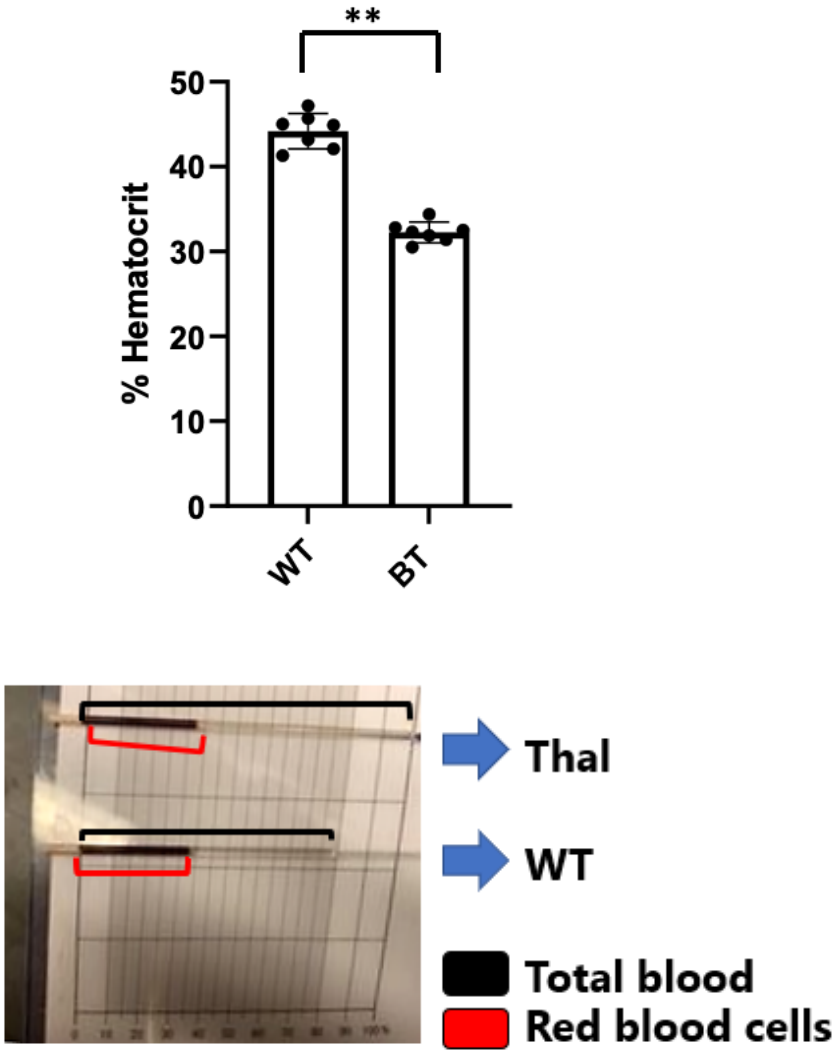


**Figure 3.3. Determining Hematocrit Levels in BT.** Blood was collected via cardiac puncture with a 21-gauge needle. Hematocrit was determined via microhematocrit method by centrifuging whole blood in a capillary tube at 13000 RPM for 12 minutes. **P < 0.01, n=7.

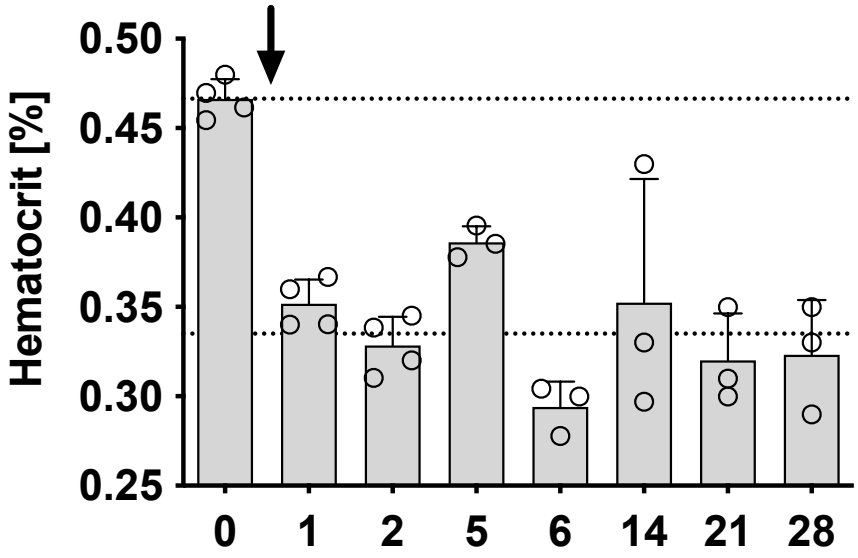


**Figure 3.4. PHZ Administration in Mice Lead to a Sustained Decrease in Hematocrit.** Bi-weekly injections of 25mg/kg of PHZ lead to decreased hematocrit levels. Hematocrit was determined via microhematocrit method by centrifuging whole blood in a capillary tube at 13000 RPM for 12 minutes. n=4.

Hemopexin therapy in PHZ-treated mice led to a decrease in plaque accumulation via aortic enface (PHZ: 41.9 ± 4.9 % vs. PHZ + HPX: 23.8 ± 3.1 %, $p < 0.05$, Figure 3.7). Hemopexin therapy in PHZ-treated mice led to a decrease in plaque accumulation via total lesion area of the aortic root (PHZ: 470.0 ± 58.7 $\mu m^2$ $x10^3$ vs. PHZ+ HPX: 292.5 ± 28.0 $\mu m^2$ $x10^3$, $p < 0.05$, Figure 3.7). Free heme levels were increased in PHZ-treated mice compared to controls (controls:0.11 ± 0.01 μM vs. WT ± PHZ: 0.34 ± 0.04 μM, $p < 0.01$, Figure 3.8), while PHZ mice treated with HPX showed decreased free heme levels when compared to PHZ mice (control + PHZ +HPX: 0.22 ± 0.03 μM, $p < 0.05$, Figure 3.8). Similar to BT findings, HPX levels were decreased in PHZ mice compared to controls (controls: 6.3 ± 0.6 mg/dl vs. PHZ: 0.3 ± 0.1 mg/dl, $p < 0.01$, Figure 3.9), HPX therapy was exhausted due to elevated levels of free heme, but functionally, HPX was still successful therapeutically due to decreased in-vivo levels of free heme and subsequent decreases in plaque accumulation. In figure 3.10, we see total cholesterol levels are unchanged in PHZ mice treated with HPX therapy.

### 3.3.2 Beta Thalassemia leads to Accelerated Atherosclerosis that can be Reduced with Hemopexin Therapy.

To determine the contribution of free heme to increased atherosclerosis in BT, we studied the effect of enhanced expression of hemopexin (HPX), free heme's endogenous scavenger, on the development of atherosclerosis. AAV-mediated HPX expression led to a significant decrease in plaque accumulation in BT male mice by aortic *enface* analysis (BT: 45.5 ± 2.4 % vs BT + HPX: 31.7 ± 2.5 %, $p < 0.01$, Figure 3.11). In addition, we saw decreased plaque lesion area via aortic root analysis in the BT mice overexpressing HPX

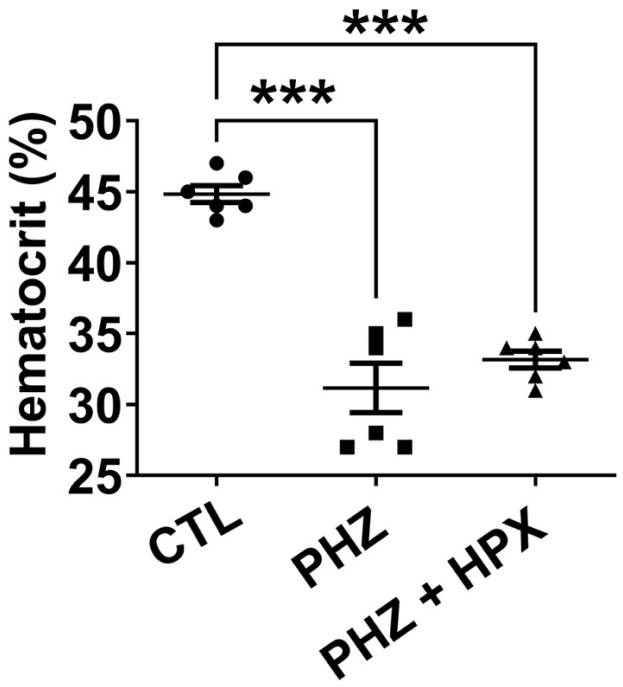


**Figure 3.5 Hematocrit Levels Were Decreased at 3-Months After Treatment With PHZ.** Blood was collected via cardiac puncture with a 21-gauge needle. Hematocrit was determined via microhematocrit method by centrifuging whole blood in capillary tube at 13000 RPM for 12 min. ***P < 0.001, n=6.

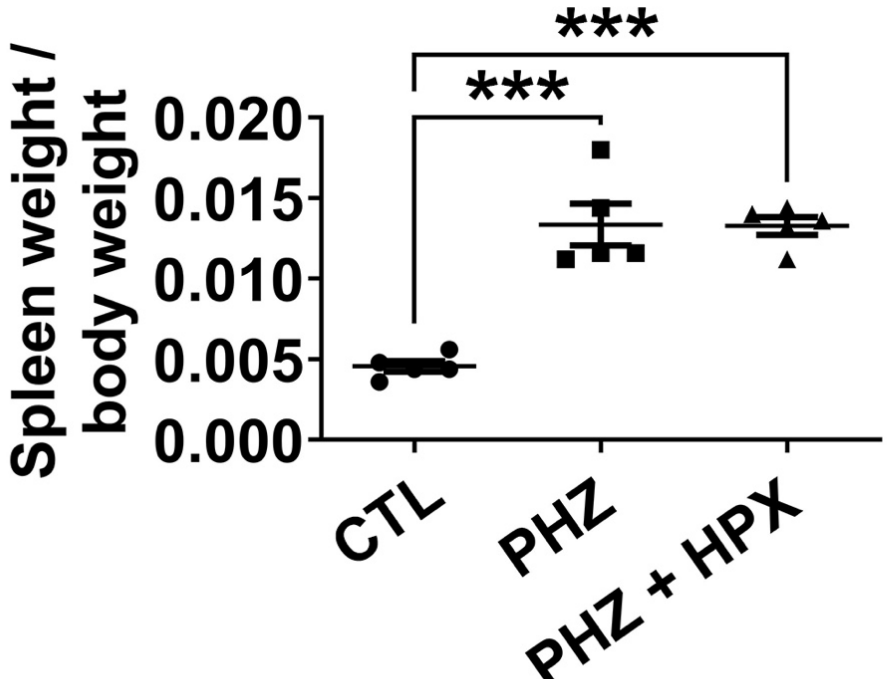


**Figure 3.6. Splenomegaly Indicative of Extramedullary Hematopoiesis Due to Intravascular Hemolysis.** Spleens were perfused at 3 months and normalize to body weight. **P < 0.001, n=5.

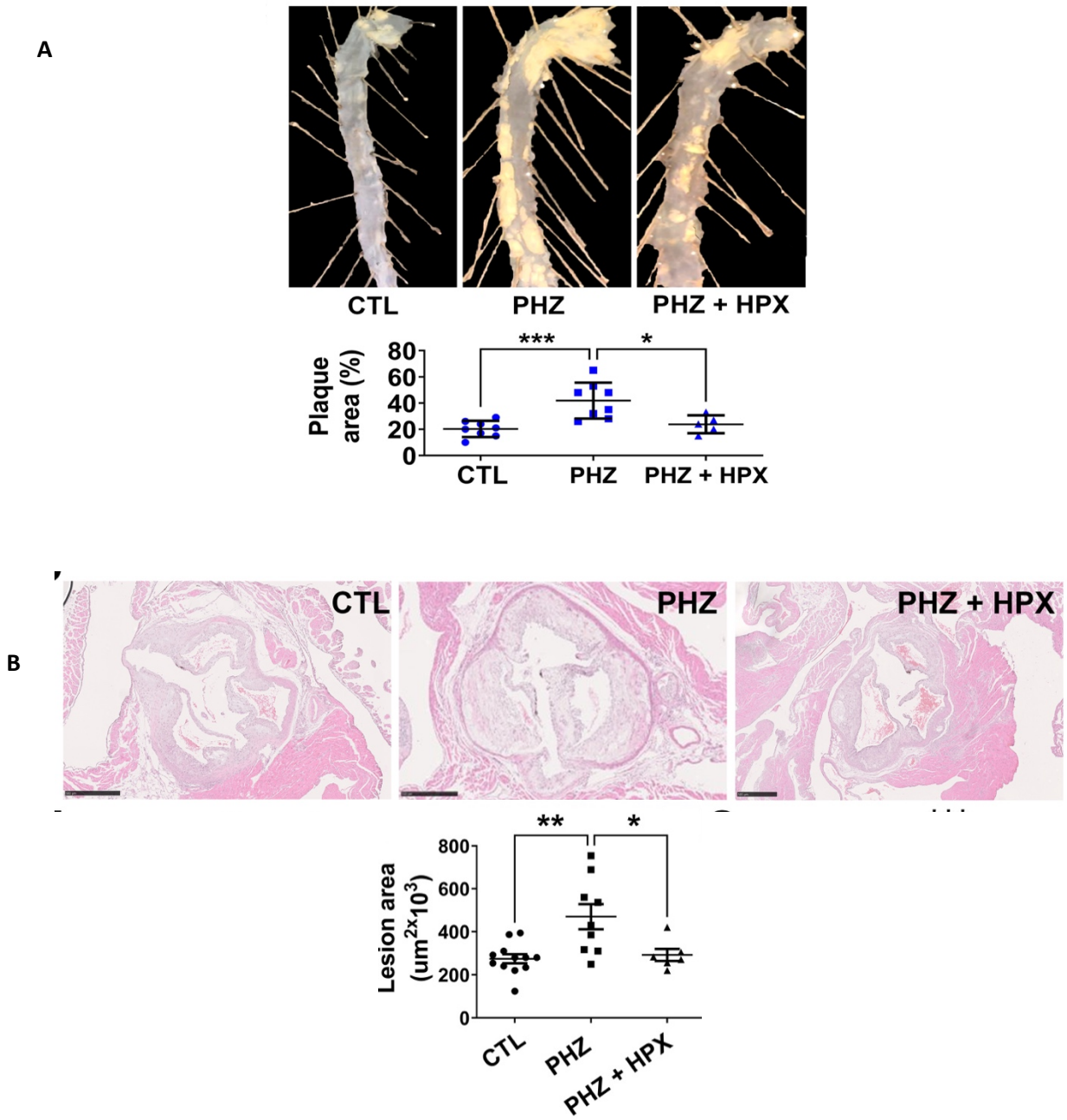


**Figure 3.7. Phenylhydrazine Leads to Accelerated Atherosclerosis**. (A) Representative aortic *en face* for treated mice and plaque accumulation in mice (n=6-11) and % plaque accumulation relative to total luminal area. (B) Representative hematoxylin and eosin (H&E) of the aortic root and total lesion area $\mu m^2 x 10^3$ at the aortic root. Values represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.01 n=6-11.

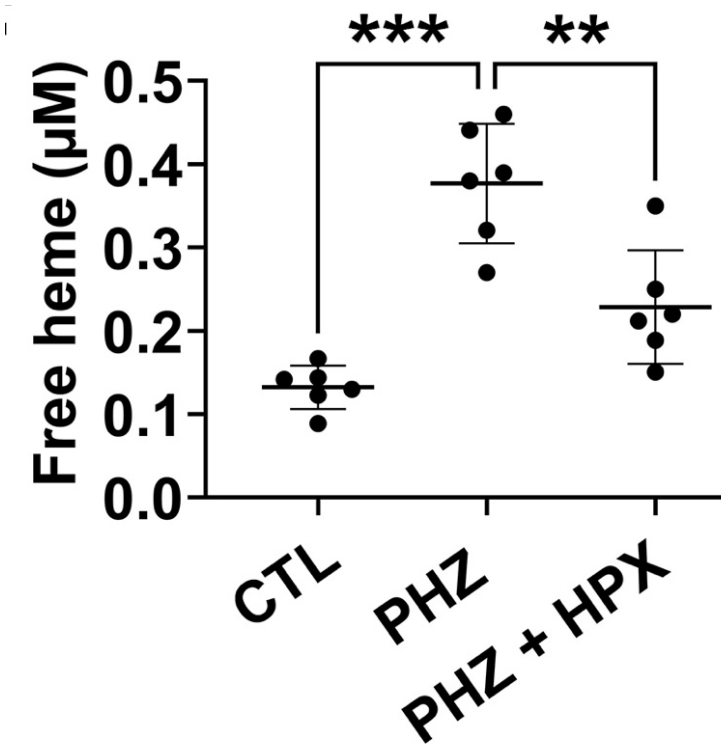


**Figure 3.8. Phenylhydrazine Leads to Increased Free Heme that was Decreased by HPX.** Serum free heme levels at 3-months. Free heme was measured using QuantiChrom™ Heme Assay Kit. **P < 0.01; ***P < 0.001, n= 5-7.

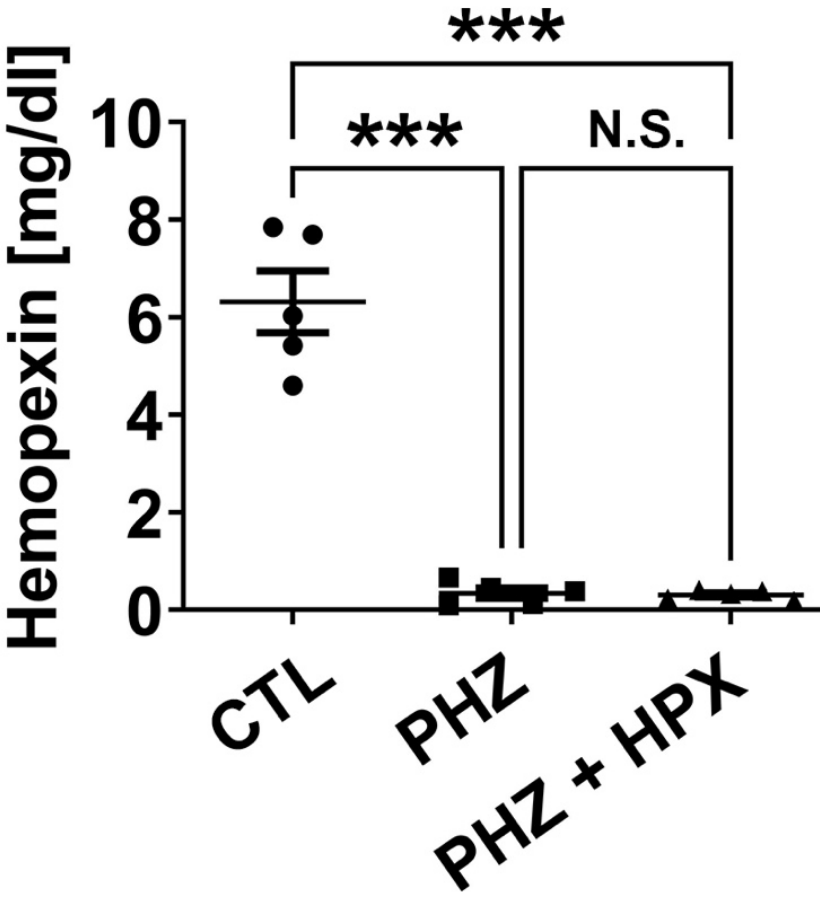


**Figure 3.9. HPX Levels are Depleted in PHZ mice.** Serum HPX was measured by ELISA. ***P < 0.001, n=5-7.

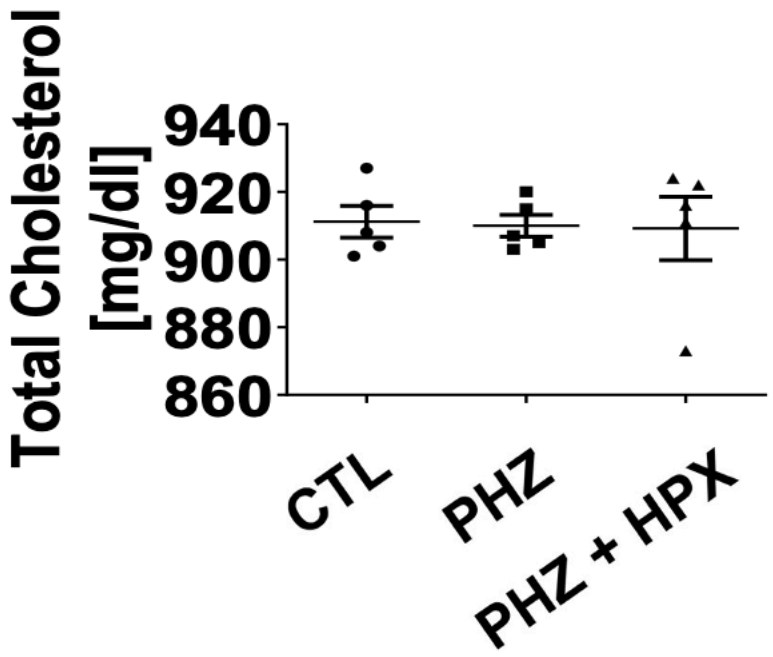


**Figure 3.10. Total Cholesterol Levels are Unchanged in PHZ Mice with HPX Therapy**. Blood was collected via cardiac puncture with a 21-gauge needle. Serum total cholesterol, triglycerides, HDL, and LDL were measured by enzymatic methods on a Beckman AU480 (Beckman Diagnostics, Brea, CA) automatic chemistry analyzer. n=4-5.

mice compare to BT mice (BT: 444.7 ± 38.1 $\mu m^2$ $x10^3$ vs BT + HPX: 318.8 ± 28.6 $\mu m^2$ $2x10^3$, $p < 0.05$, Figure 3.12). Furthermore, BT aortic root histology (Mason's trichrome) indicated hemopexin therapy decreased necrotic area (BT: 193.4 ± 8.5 $\mu m^2 x10^3$ vs. BT + HPX: 107.3 ± 5.9 $\mu m^2 x10$, $p < 0.05$, Figure 3.13). Furthermore, BT aortic root histology indicated hemopexin therapy decreased collagen staining in BT mice (BT: 186.6 ± 11.1 $\mu m^2 x10^3$ vs. BT + HPX: 85.4 ± 6.4 $\mu m^2 x10^3$, $p < 0.01$, Figure 3.13). HPX therapy resulted in a two-fold increase in HPX protein expression (BT: 0.8 ± 0.1 mg/ml vs. BT HPX 1.9 ± 0.3 mg/ml, Figure 3.14). HPX therapy resulted in a decrease in serum free heme levels in the BT mice (BT: 0.2 ± 0.02 μM vs. 0.09 ± 0.02 μM, $p < 0.05$, Figure 3.15) Furthermore, HPX therapy decreased aortic hydrogen peroxide levels (Figure 3.16) and aortic IL-6, TNFα, and HO-1 via RT-qPCR (Figure 3.18). HPX therapy did not change aortic antioxidant level (SOD-1, Catalase, GPX-1) as seen in Figure 3.16. These data demonstrate that hemopexin therapy decreases atherosclerotic plaque accumulation, ROS levels, and inflammatory gene expression in BT supporting our hypothesis that free heme is a major mediator of BT-mediated accelerated atherosclerosis.

3.3.3 Hemopexin Therapy paradoxically Increases Atherosclerosis in WT mice.

At 3 months, WT mice had increased plaque in both aortic enface (WT: 22.27 ± 1.5 % vs WT + HPX: 32.2 ± 2.0 %, $p < 0.01$, Figure 3.19) and aortic root area (WT: 241.7 ± 22.1 $\mu m^2$ $x10^3$ vs WT + HPX: 463.0 ± 63.6 $\mu m^2$ $2x10^3$, $p < 0.05$, Figure 3.19). WT mice administered AAV-Hemopexin control vector had no significant change in aortic *en face* plaque area (Figure 3.20).

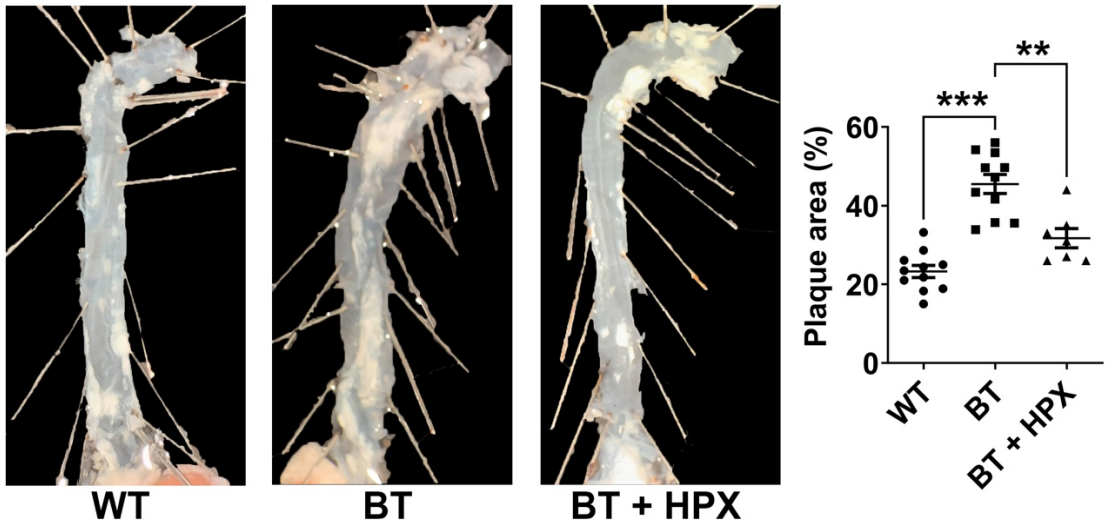


**Figure 3.11. HPX Therapy Reduced Advanced Atherosclerosis in BT Mice via Aortic en face Analysis.** Representative aortic *en face* plaque area in WT and BT mice and % plaque area relative to total luminal area. Values represent mean ± SEM. **P<0.01,***P<0.o01, n=6-10.

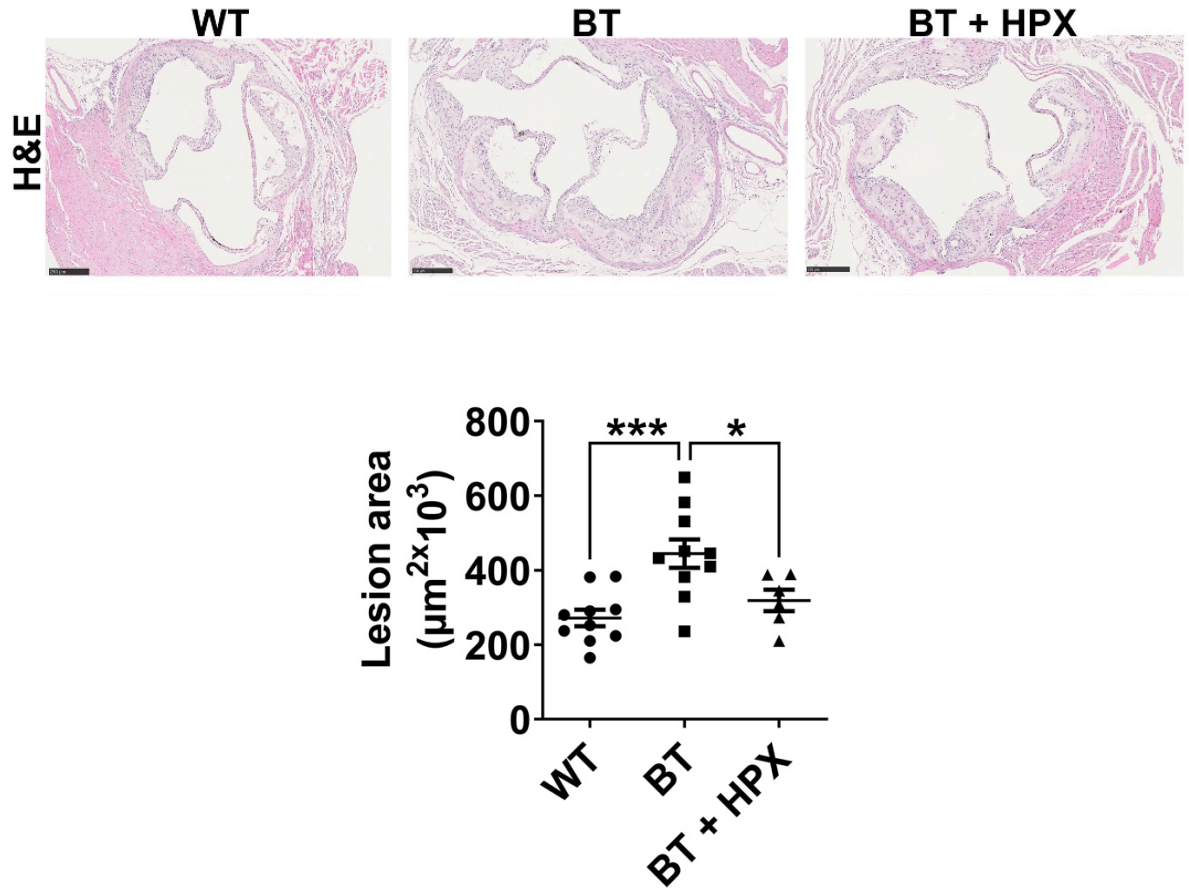


**Figure 3.12. HPX Therapy Reduced Aortic Root Atherosclerosis in BT Mice.** Representative hematoxylin and eosin (H&E) staining of the aortic root and total lesion area $\mu m^2 x10^3$ at the aortic root. Values represent mean ± SEM. *P<0.05, ***P<0.001, n=6-10.

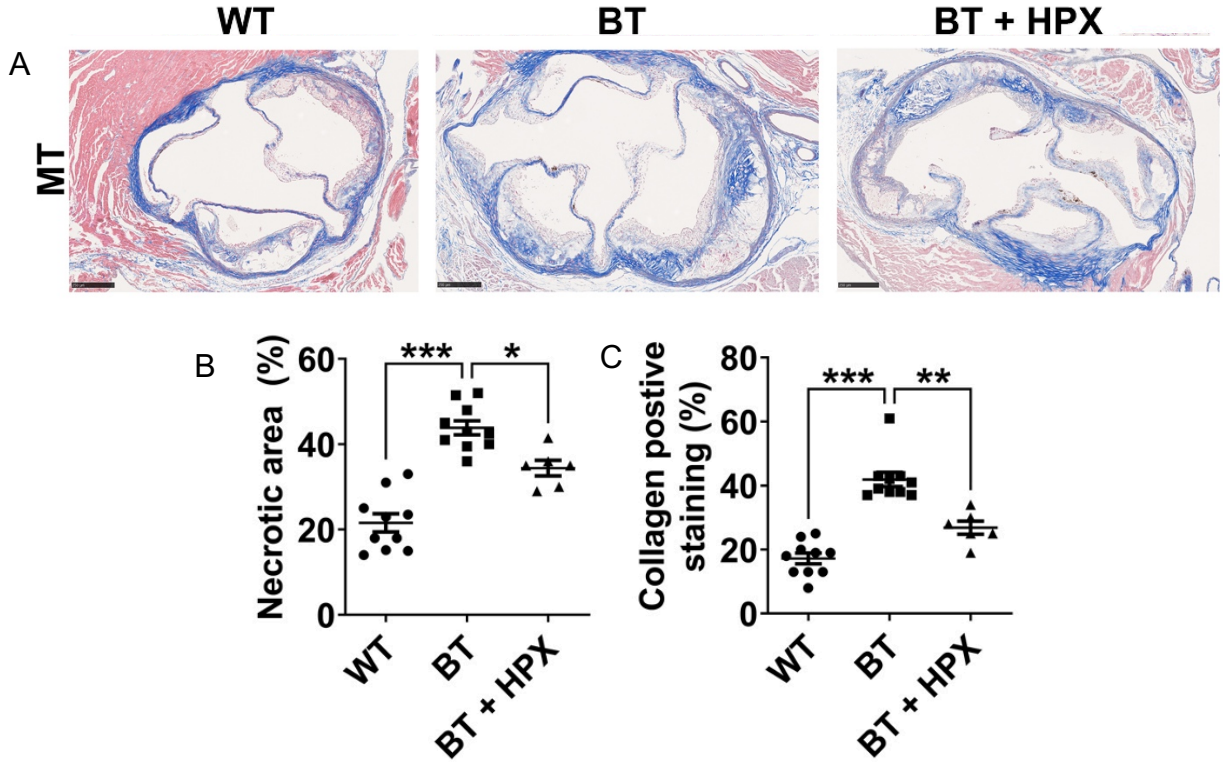


**Figure 3.13. HPX Therapy Reduced Plaque Complexity in BT mice.** (A) Representative trichrome staining of the aortic root, (B) Necrotic area $\mu m^2 x 10^3$, (C) Collagen positive staining $\mu m^2 x 10^3$. Values represent mean ± SEM. *P<0.05; **P<0.01, n=6-10.

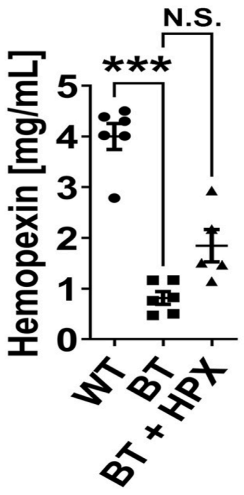


**Figure 3.14. HPX therapy Increases HPX Levels in BT Mice.** Serum HPX was measured by ELISA. Values represent mean ± SEM. ***P < 0.001, n=5-7.

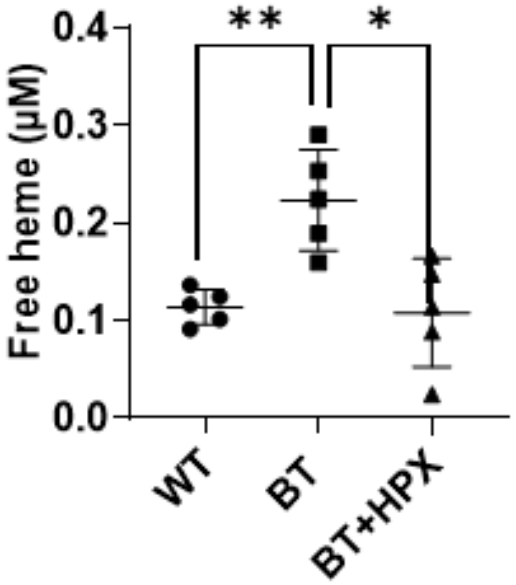


**Figure 3.15. HPX Therapy Decreases Free Heme in BT.** Serum free heme levels at 3-months. Free heme was measured using QuantiChrom™ Heme Assay Kit. Values represent mean ± SEM. *P < 0.05;**P < 0.01, n=5-7.

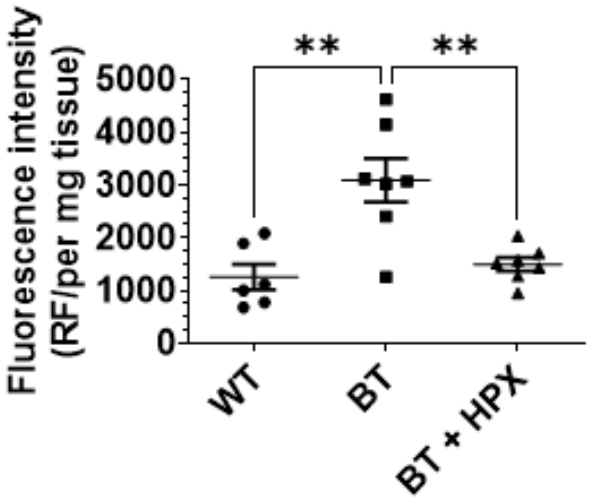


**Figure 3.16. HPX therapy decreases hydrogen peroxide level in BT mice.** Aortic hydrogen peroxide levels measured via fluorescence intensity using the Amplex Red assay. Values represent mean ± SEM. **P < 0.01, n=6-7.

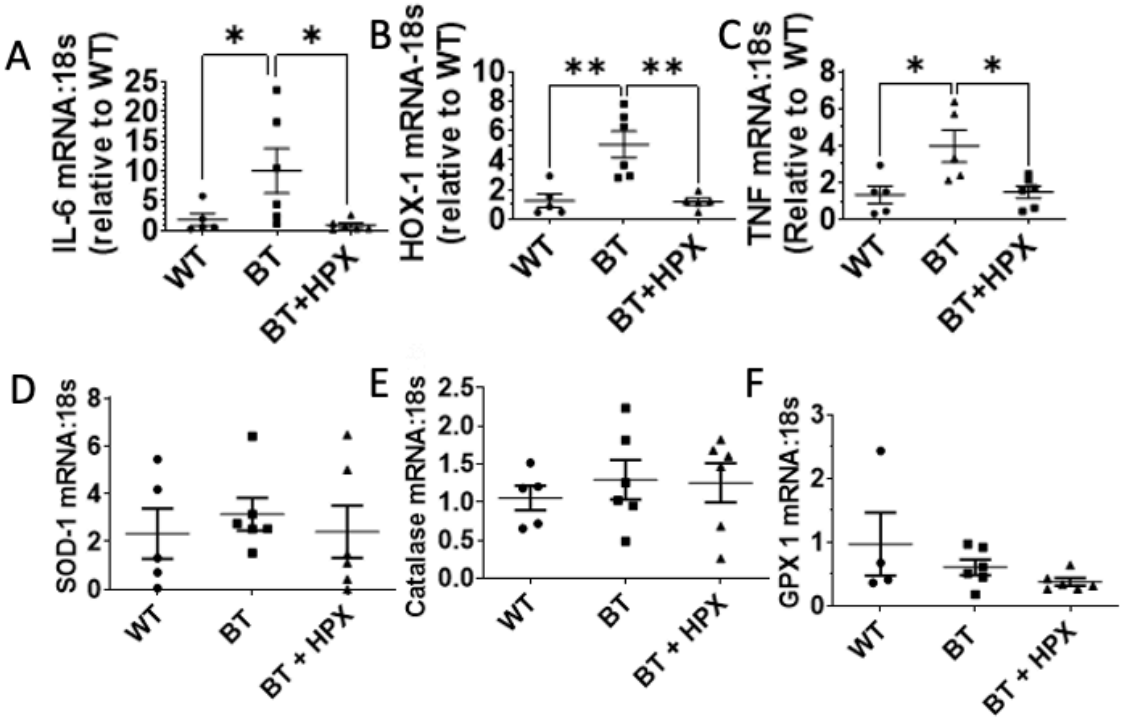


**Figure 3.17. Aortic Gene Expression.** Aortic mRNA expression of proinflammatory cytokines and antioxidants at 1 month. (A) Interleukin-6 (IL-6), (B) Heme oxygenease-1 (HOX-1), (C) Tumor Necrosis Factor-$\alpha$ (TNF$\alpha$), (D) Superoxide dismutase type-1 (SOD-1), (E) Catalase, (F) GPX-1. Values represent mean ± SEM. $P < 0.05$; $^{**}P < 0.01$, n=5-7.

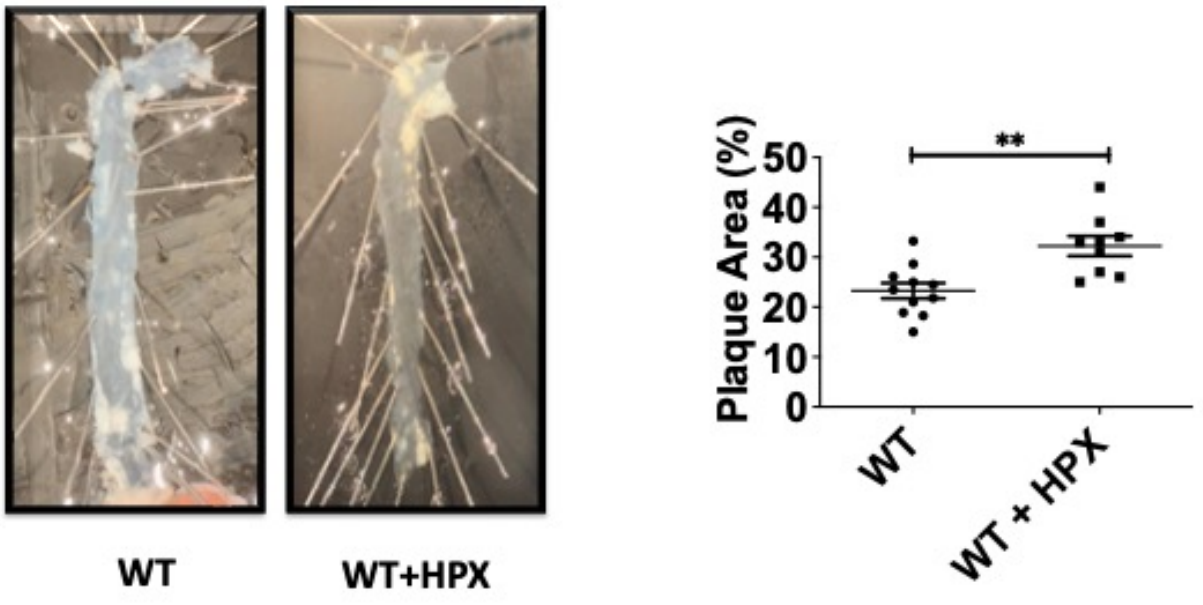


**Figure 3.18. HPX Therapy Increased Atherosclerosis in WT Mice via Aortic en face Analysis.** Representative aortic *en face* plaque area in WT and WT + HPX mice and % plaque area relative to total luminal area. Values represent mean ± SEM. P<0.01, n=10.

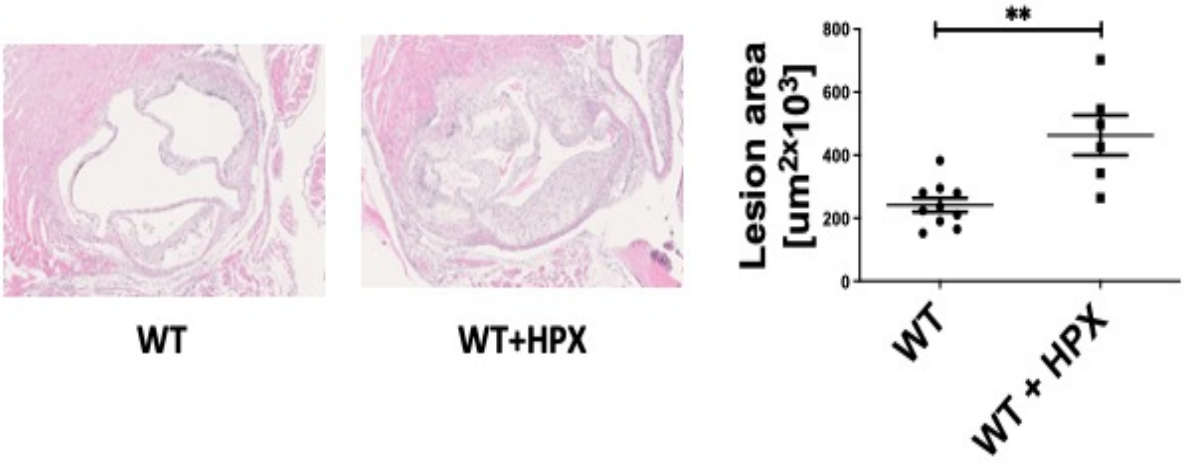


**Figure 3.18. HPX Therapy Increased Atherosclerosis in WT Mice via Aortic en face Analysis.** Representative aortic *en face* plaque area in WT and WT + HPX mice and % plaque area relative to total luminal area. Values represent mean ± SEM. P<0.01, n=10.

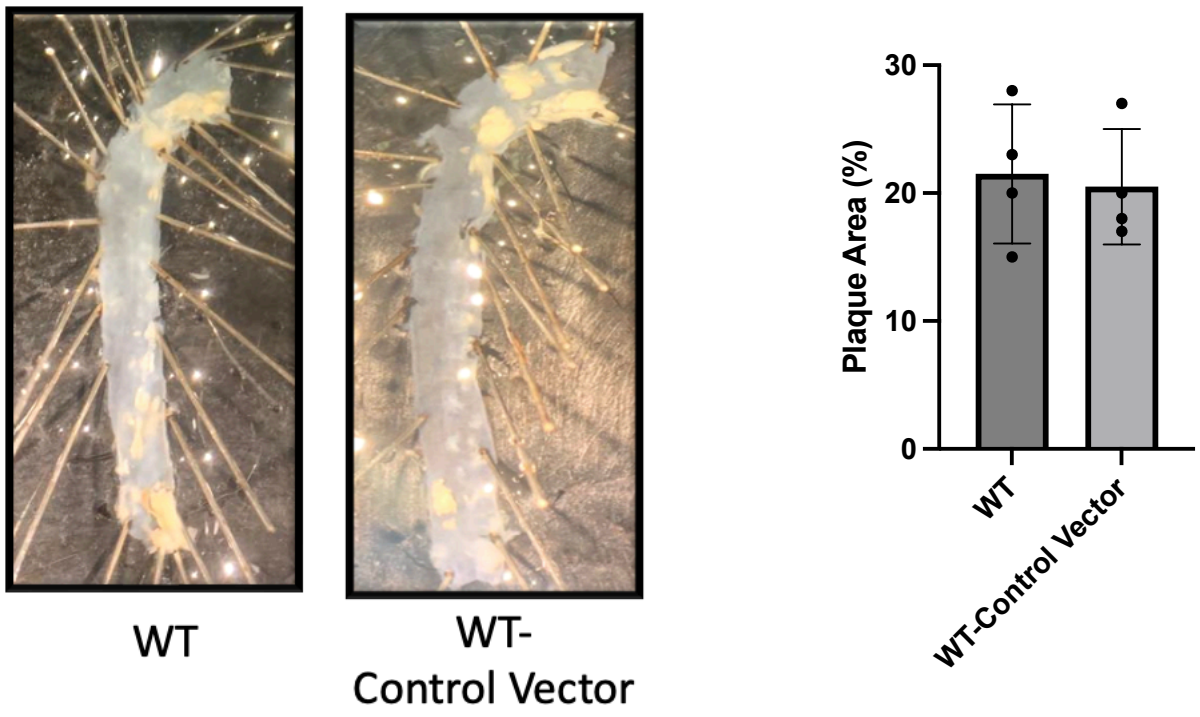


**Figure 3.19. AAV- HPX Control Vector Did Not Affect Atherosclerosis.** Representative hematoxylin and eosin (H&E) staining of the aortic root and total lesion area $\mu m^2 x 10^3$ at the aortic root. Values represent mean ± SEM. *P<0.05, **P<0.01, n=4.

### 3.4 Discussion

The potential pathological cause of accelerated atherosclerosis in BT is multifactorial and could be due to anemia, compensatory responses to the anemia, dyslipidemia, or as a result of hemolysis of red blood cells. Here, we are showing that endogenous intravascular hemolysis in BT contributes to accelerated atherosclerosis. We corroborated these findings by using a model of Phenylhydrazine-induced hemolysis which also led to accelerated atherosclerosis.

HPX therapy led to decreased plaque area in both BT and PHZ induced model of intravascular hemolysis. Typically, hemopexin is administered with intraperitoneal injections, but this is a costly and time-consuming process (Vinchi *et al.*, 2013; Vinchi, Costa da Silva, *et al.*, 2016; Gentinetta *et al.*, 2022b). We are the first, to our knowledge, to investigate an AAV mediated approach to overexpress HPX and to show the effect of hemopexin therapy in a mouse model of atherosclerosis. BT is a multifaceted disease that could have several causes for atherosclerosis such as anemia or hypercoagulablity (Needs *et al.*, 2022). We used a PHZ drug induced model of hemolysis to mechanistically compare the results of HPX therapy in BT, a hemoglobinopathy with known intravascular hemolysis. Both PHZ and BT accelerated atherosclerosis that could be reversed by HPX therapy. AAV-mediated HPX expression successfully decreased free heme levels in both BT and PHZ-treated mice. At three months, HPX levels in BT mice and PHZ treated mice with AAV-HPX had no relative change compared to their respective controls. BT mice and PHZ mice had increased intravascular hemolysis noted by a 122% and 170% mean free heme change from baseline compared to WT. Both BT and PHZ models are an extensive model of hemolysis that leads to high levels of heme. The excessive levels of

heme can exhaust hemopexin level, but HPX therapy still led to decreased plaque area and free heme levels in treated mice. HPX therapy decreased plaque area and reduced free heme levels in both PHZ and BT mice.

Elevated levels of heme have been shown to have a clear proatherogenic affect, but the in-vivo compensatory mechanisms are nuanced and incompletely understood. Heme has been shown to increase permeability and vascular adhesion in endothelial cells, SMC proliferation, and LDL oxidization. Furthermore, heme and other erythrocyte products are found in severe human atherosclerotic plaques (Nagy *et al.*, 2010). Free heme is catabolized by heme oxygenase in the liver to iron, a polyporphin ring, carbon monoxide, and bilirubin. The progression of atherosclerosis caused by heme and heme degradation/scavenging pathway is complex. Previously, hemopexin (Mehta *et al.*, 2016) knockout led to increased atherosclerosis in mice presumably by decreasing the detoxifying of free heme in the vasculature. Heme-oxygenase has been clearly shown to provide an atheroprotective effect by detoxifying heme and increasing anti-atherogenic carbon monoxide, and bilirubin (Yet *et al.*, 2003a; Morita, 2005). There is no data on haptoglobin knockout mice on atherosclerosis, but mice and human with variants of haptoglobin (HP-2-2), that express less haptoglobin, have more atherosclerosis (Levy *et al.*, 2007). In 2001, Ishikawa et al., placed 4-test groups of 10-week LDL-receptor knockout mice on two-different high fat diets (western/high cholesterol with cholate) for 6-week. The 4-test groups were: “mice given intraperitoneal injections of hemin (25 mg/kg body weight) to induce HO-1, hemin and desferrioxamine (10 mg/kg body weight) to induce HO-1, Sn-protoporphyrin IX (7.5 mg/kg body weight) to inhibit HO-1, or saline as a control” (Kazunobu Ishikawa *et al.*, 2001). Paradoxically, both mice treated with hemin

and hemin desferrioxamine on both high fat diets had less atherosclerosis than the control group, and the desferrioxamine provided no additional benefit. In earlier studies with PHZ-mediated intravascular mediated hemolysis in mice, decreased atherosclerosis was observed, while other studies using PHZ, administration in rabbits showed increased atherosclerosis. Our PHZ-induced model of hemolysis used a lower total drug exposure of only 1 weekly 25mg/kg injection in PCSK9 overexpressing mice with HFD for 12 weeks as compared to 1 injection every 48 hrs in $ApoE^{-/-}$ mice with a HFD for 10 weeks. We originally tried a higher dosage of two weekly injections, but we had an unacceptably high mortality rate. Based on our experience, PHZ mediated hemolysis levels were cleared fully by 3 days, and as a result, our model was more reflective of transient hemolysis than a chronic hemolysis which provides an additional perspective to the effects of hemolysis. Taken together, the results are perplexing and suggest the in-vivo compensatory response of heme-oxygenase and hemopexin are playing a crucial role which are not fully understood. In addition, anemia can potentially trigger other compensatory mechanisms that may impact atherosclerosis. The influence of erythropoietin on atherosclerosis is uncertain and technically difficult due to variability in responses to erythropoietin (Paul *et al.*, 1999). Although some evidence suggests that erythropoietin may protect against atherosclerosis by stabilizing plaque (Wu *et al.*, 2020), a typical genetic variation in the promoter region that increases expression has been linked to accelerated disease progression in peripheral artery disease (Renner *et al.*, 2020). In 2013, studies with bone marrow transplantation of SCD, a different hemoglobinopathy, into ApoE knockout mice showed decreased atherosclerosis. SCD mice had a paradoxical decrease in plaque area (Wang *et al.*, 2013). On the other hand,

autoimmune hemolytic models of anemias have been shown to decrease plaque area, but increase plaque vulnerability (Santiago-Raber *et al.*, 2020b). Excess levels of heme have led to clear tissue damage such as acute kidney injuries (Gentinetta *et al.*, 2022b). Further investigation is needed to understand the in-vivo compensatory mechanisms. Globally, free heme leads to a proatherogenic environment with complex compensatory mechanisms.

In our study, we observed a perplexing outcome in which WT mice treated with HPX exhibited a significant increase in the accumulation of atherosclerotic plaque. We conducted experimental tests on our empty vector used in conjunction with AAV-HPX and found no significant alteration in atherosclerotic plaque accumulation. We observed that HPX therapy reduced the mRNA levels of heme oxygenase-1 (HOX-1) in the aorta, corroborating findings reported by other research groups, while it is increased in the liver (Vinchi, Vercellotti, et al., 2016). HOX-1 has been demonstrated to impact atherosclerosis, as evidenced by studies utilizing heme-oxygenase knockout mice, which exhibited increased atherosclerosis (Yet et al., 2003b; Gough et al., 2006). These collective findings emphasize the need for a comprehensive understanding of the compensatory mechanisms within the hemolysis pathway and the broader role of hemopexin in the context of iron regulation.

These results open the possibility for several future routes of investigation. We could compare the benefits of intraperitoneal HPX administration vs. AAV-HPX therapy in our model or other disease models such as acute kidney injury. In addition, we could characterize the understanding of compensatory mechanism more deeply in our PHZ model and hemin dose response curve by varying time, erythropoietin levels, and a

careful analysis of proteins in the heme degradation pathway. A few limitations to our study are that we analyzed the effects of HPX on atherogenesis, but not established lesions. A future direction could study HPX therapy after initial lesion formation. Here, we only studied the direct effect heme mediated ROS generation. In addition, we have not evaluated other potential mechanisms of heme including heme oxidation and TLR4-mediated ROS formation (Belcher *et al.*, 2014).

In summary, this work demonstrates that free heme is playing a causal role in the development of atherosclerosis and reveals hemopexin as a potential therapeutic approach.

# Chapter 4: Deferiprone Decreases Atherosclerosis in Beta Thalassemic Mice,but Does Not Provide an Additive Benefit with Hemopexin Therapy.

## 4.1 Introduction

Iron is an essential trace element that plays numerous crucial roles in human physiology such as oxygen transport and energy metabolism (Cornelissen *et al.*, 2019). Iron is a d-block transition metal that has extensive redox potential to both give and acquire electrons with ease. Iron can exist in ferric ($Fe^{2+}$) and ferrous ($Fe^{3+}$) form. Iron is a critical component of hemoglobin, myoglobin, and iron sulfur-proteins (Naito *et al.*, 2021). Typically, iron is well regulated in the body, but iron overload and iron deficiency can lead to worsened health outcomes (Kempf and Wollert, 2020). In BT, there is iron overload due to increased iron absorption and blood transfusion (Needs *et al.*, 2022). As a result, iron chelation is standard therapy in BT to avoid negative outcomes such as myocardial siderosis (Cao and Galanello, 2010). Here, we will study the impact of using iron chelation with a pharmacological agent to “scavenge” free iron, on the progression of atherosclerosis in BT.

### 4.1.1 Key Proteins and Pathways

A summary of key iron proteins and pathways are summarized in figure 4.1. A few of the key protein in the iron pathway are:

1. Transferrin: Transferrin (Tf) is a glycoprotein that binds to iron and transports it through the bloodstream to cells throughout the body (Li *et al.*, 2010a). It is synthesized in the liver and circulates in the bloodstream, where it binds tightly and selectively to iron. Upon binding to iron, the protein undergoes a conformational alteration that boosts its binding affinity to transferrin receptors located on cell surfaces (Li *et al.*, 2010a). Once transferrin docks with transferrin receptors on the cell surface, the complex is taken up by receptor-mediated endocytosis, a process

in which the transferrin-receptor complex is internalized into the cell (Hvidberg *et al.*, 2005). The iron is then released from transferrin. Transferrin plays a pivotal role in maintaining iron homeostasis by delivering iron to cells requiring iron while preventing iron overload in cells that do not.

2. Ferritin: Ferritin is a 24-compartment spherical container in which ferric iron is stored as a ferrihydrite mineral (Krittayaphong *et al.*, 2018). This protein is responsible for storing iron in cells. It is found in all cells, but particularly in liver cells, and plays a critical role in maintaining iron homeostasis (Kempf and Wollert, 2020). Serum ferritin is a diagnostic tool used to monitor iron levels. Ferritin is upregulated in several disease states such as infection or end-stage kidney disease (Krittayaphong *et al.*, 2018).
3. Ferroportin: Ferroportin (FPN) is a protein that is responsible for exporting iron from cells into the bloodstream (Suh and Jeon, 2018). Hepcidin binds to ferroportin and causes it to be internalized, reducing the amount of iron that is released from cells (Nagy *et al.*, 2010).
4. Heme: Heme is a molecule that contains iron and is a critical component of hemoglobin, the protein in red blood cells that carries oxygen throughout the body. Heme is released during hemolysis and absorbed from the small intestine (Suh and Jeon, 2018, p. 2).
5. Heme oxygenase: Heme oxygenase is a family of enzymes responsible for breaking down heme into its constituent parts, including iron, biliverdin, and carbon monoxide (Araujo *et al.*, 2012).

6. Hepcidin: Hepcidin is the “master regulator” of iron metabolism (Nagy *et al.*, 2010). Hepcidin is a hormone produced in the liver that negatively regulates FPN. Hepcidin binds to FPN and induces its internalization and degradation, thereby reducing the release of iron from cells and lowering the amount of iron in the bloodstream (Suh and Jeon, 2018). The precise mechanism by which hepcidin induces the degradation of ferroportin is not fully understood, but it is thought to involve the recruitment of other proteins to the site of ferroportin, such as human homeostatic iron regulator protein (HFE) and transferrin receptor 2 (TFR2) (Vyoral and Jiri Petrak, 2017). In addition to its role in regulating ferroportin, hepcidin has also been shown to have other effects on iron metabolism. For example, hepcidin can inhibit the activity of the iron-exporting protein, ferrochelatase, leading to decreased heme synthesis and lower levels of iron in the bloodstream (Vyoral and Jiri Petrak, 2017). Hepcidin can also directly inhibit the uptake of iron by enterocytes in the gut, further reducing the absorption of iron from the diet.

   Divalent metal transporter 1 (DMT1) Divalent metal transporter 1 is a transmembrane protein that is found in the apical membrane of duodenal enterocytes, and in other tissues, including the liver, spleen, and bone marrow (Suh and Jeon, 2018). In the duodenum, DMT1 is responsible for the uptake of dietary iron by enterocytes. DMT1 can transport both ferrous (Fe2+) and ferric (Fe3+) iron, but ferrous iron is the preferred substrate. DMT1 has been found to be dysregulated in several diseases, including iron deficiency anemia, hemochromatosis, and neurodegenerative disorders such as Alzheimer's disease (Suh and Jeon, 2018).

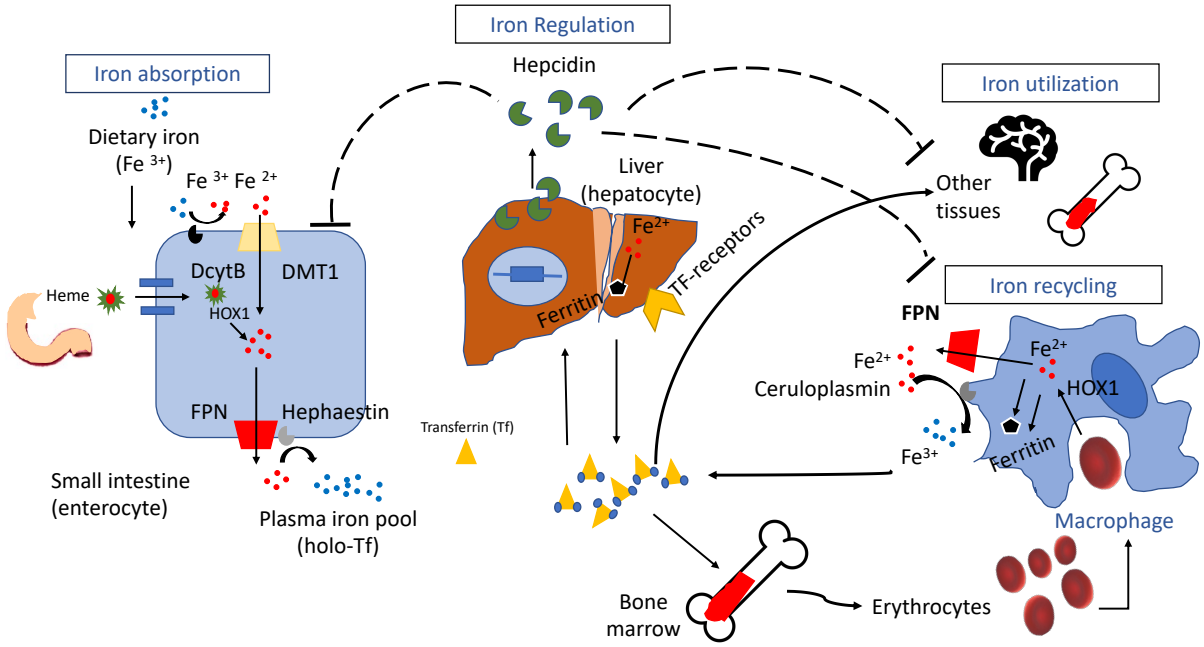


**Figure 4.1. Iron Regulation Pathway.** Iron is absorbed in the small intestine through Divalent metal transporter 1 (DMT1) receptors and exported in circulation through Ferroportin. Transferrin and ferritin are the main depots for storage of iron in the circulation and tissue receptively. Hepcidin negatively regulates DMT1. Adapted from (Girelli *et al.*, 2016).

7. Ceruloplasmin/Hephaestin: Both are proteins, that catalyze the conversion from Ferrous to Ferric iron (Suh and Jeon, 2018).

### 4.1.2 Iron and Atherosclerosis

Iron is an important micronutrient for hemoglobin development, but in excess, iron can lead to free radical generation (Nagy *et al.*, 2010). Ferrous iron can promote the Fenton reaction which converts hydrogen peroxide to hydroxyl radical (Voskou *et al.*, 2015). Atherosclerosis lesions accumulate iron and locally generated ROS can oxidize LDL cholesterol (Sharkey-Toppen *et al.*, 2014). Iron is primarily stored as transferrin in circulation and ferritin in tissue to prevent free iron toxicity. Transferrin bound iron is the main binding protein that allows for delivery into other cells via the transferrin receptor. Transferrin saturation (TS) is used to analyze iron burden (e.g. high TS is iron overload and low TS is Iron deficiency) (Kempf and Wollert, 2020). A list of iron parameters and their associated risk are described in table 4.1.

The role of iron in atherosclerosis is incompletely understood (Cornelissen *et al.*, 2019; Kempf and Wollert, 2020). In 1981, Sullivan et al. proposed the hypothesis that men and postmenopausal woman are a greater risk of atherosclerosis due to higher levels of iron (Sullivan, 1981). The past 40 years of assessing the effect of iron on atherosclerosis has returned inconsistent results (Sullivan, 2007; Kautz *et al.*, 2013). Iron has been studied in animal models (genetic iron overload, exogenous iron overload, iron restriction) and epidemiological human studies (Vinchi, 2021). Iron concentration is higher in human atherosclerotic plaque than in healthy arterial tissue (Stadler *et al.*, 2004). In his refined “Iron hypothesis”, *Sullivan et al.* suggest the presence of macrophages containing high levels of iron within the plaque play a significant role in promoting atherosclerosis

(Sullivan, 2009). Clinical studies have consistently confirmed that iron deficiency leads to a higher risk of coronary artery disease, myocardial infarction, and other cardiovascular diseases, but there is no clear causal mechanism (Kempf and Wollert, 2020). On the other hand, iron overload has had more conflicting results (Suh and Jeon, 2018; Cornelissen *et al.*, 2019; Vinchi, 2021). When analyzing humans with genetic modulation that leads to increased iron levels, such as hereditary haemochromatosis (HH), there have been mixed clinical results: no change (von Haehling *et al.*, 2015), decreased risk (Gill *et al.*, 2017), and increased risk of cardiovascular event (Grammer *et al.*, 2019) have all been reported. Non-transferrin bound iron and vascular iron deposition are the more consistent metric of increased risk of cardiovascular diseases, while other metrics such as ferritin have led to discrepant results (Vinchi, 2021). Recently, the risk of cardiovascular disease due to iron levels has been described as a “J”/“U” shaped curve in which the lowest and highest iron levels are associated with increased cardiovascular risk (Kempf and Wollert, 2020).

*Vinchi et al*. found that in a model of HH mouse, carrying a heterozygous loss-of-function mutation of Fpn (FpnC326S), crossed on an apolipoprotein E ($ApoE^{-/-}$) background leads to an increase in non-transferrin bound iron (NTBI) and enhanced atherosclerotic plaque area (Vinchi, 2021). Iron infusion has led to increase in plaque area in $ApoE^{-/-}$ mice (Marques *et al.*, 2019) and rabbits (Araujo *et al.*, 1995). In contrast, other animal studies show a decrease in plaque area in the flowing experimental conditions:

1. Dietary iron overload in $ApoE^{-/-}$ mice (Kirk *et al.*, 2001)

2.“Flat iron” mice, a classical model of FPN disease/HH, crossed onto an ApoE$^{-/-}$ background (Kautz *et al.*, 2013)

3. Hepcidin-null (Hamp-null) mouse onto an LDLR$^{-/-}$ background (Malhotra *et al.*, 2019).

These differences have been hard to reconcile, but several factors such as: different backgrounds (ApoE$^{-/-}$/LDLR$^{-/-}$), different LDL levels in models, macrophage iron load vs tissue iron load, differing levels of NTBI, a potentially independent role of hepcidin to FPN axis, and age (Vinchi, 2021) could have contributed to these disparate results. Globally, iron does affect the cardiovascular system in both high and low levels of iron, but the mechanisms by which it does so are complex and poorly understood.

### 4.1.3 Iron Chelators

Chelation therapy is a fundamental treatment for individuals with BT who frequently receive blood transfusions (Casu *et al.*, 2016; Ali *et al.*, 2021). Iron chelators have exceptionally high affinities for iron, allowing them to extract iron from intracellular storage locations such as ferritin and hemosiderin (Hoffbrand *et al.*, 2012; Needs *et al.*, 2022). Ferritin and hemosiderin are responsible for storing scavenged "free" iron in the body (Grammer *et al.*, 2019). Currently, there is “insufficient evidence to determine the effectiveness or ineffectiveness of chelation therapy in improving clinical outcomes of people with atherosclerotic cardiovascular disease” (Villarruz-Sulit *et al.*, 2020). A systematic review of the use of the iron chelator EDTA, a synthetic amino acid and polydentate heavy metal chelator, found that 17 trials showed improved clinical outcomes while 7 had no statistical benefit (Ravalli *et al.*, 2022).

| Iron Status | | | | Risk of CVD mortality/disease severity | | |
|---|---|---|---|---|---|---|
| **Iron Parameter** | **Normal Range** | **Iron Deficiency** | **Iron Overload** | **No risk** | **Increased** | **References** |
| **Serum Iron [µg/dL]** | 65–170 | <50 | >170 | 90–130 | <90; >130 | (Grammer *et al.*, 2019) |
| **Tf Saturation [%]** | 20–55 | <20 | >55 | 25–40 | <25; >40 | (Grammer *et al.*, 2019) |
| **Serum ferritin [ng/mL]** | 20–300 | <20 | >1000 | 150–350 | <150; >350 | (Grammer *et al.*, 2019) |
| **NTBI [µM]** | <1 | Undetectable | >0.5 ; BT 0.5-10; HH 0.1–5 | <1 | Undetectable; >1 | (Vinchi *et al.*, 2020) |
| **LPI [µM]** | <0.2 | Undetectable | >0.2; BT 0.5–10; HH 0.2–2 | <0.1 | Undetectable; >0.1 | (Vinchi *et al.*, 2020) |

**Table 4.1. Range of Iron Parameters and its Associated Risk for Cardiovascular Disease.** *Grammer et al.* reported a range of iron parameters, such as serum iron, transferrin saturation, serum ferritin, NTBI, and LPI, for individuals with normal, iron deficiency, and iron overload. Non-transferrin bound iron (NTBI) and liable plasma iron (LPI) values were extrapolated from the preclinical study conducted by *Vinchi et al*. A J/U shape risk association was found in which the iron overload and iron deficiency lead to increased cardiovascular risk. The abbreviations used in the text include CVD for cardiovascular disease, HH for hereditary hemochromatosis, and BT for Beta Thalassemia. Adapted from (Vinchi, 2021)

Other iron chelators have been used clinically and in experimental settings. Deferoxamine is an iron chelator therapy that was used since the 1970s, but due to its short half-life, it is administered via subcutaneous infusion overnight several times a week (Marques *et al.*, 2019). In addition, Deferoxamine is charged and cannot readily enter cells (Neufeld, 2006). Deferiprone (DFP) is a hydroxypyridineone that was first used in humans in 1987 and is a bidentate chelator which scavenges 1 iron molecule with 3 DFP molecules (Neufeld, 2006). An advantage of DFP is that that it is not charged and can penetrate membranes to help remove toxic iron from tissues (Sripetchwandee *et al.*, 2019). Deferasirox is also another uncharged oral iron chelator, N-substituted bis-hydroxyphenyltriazoles with a longer half-life, and a prominent gastrointestinal side effect. DFP has been shown to have superior ability to reduce myocardial iron load and is more commonly used in hemolytic anemias such as BT (Wahidiyat *et al.*, 2018).

**4.2. Methods**

Iron chelation therapy is a mainstay in BT. Both transfusion-dependent and non-transfusion dependent BT lead to an increase in iron levels due to constant blood transfusion and an increase in iron absorption leading to iron overload (Hoffbrand *et al.*, 2012; Krittayaphong *et al.*, 2018; Needs *et al.*, 2022). Deferiprone (DFP) is an iron chelator that removes iron from ferritin and hemosiderin in intracellular stores, the primary stores of scavenged “free” iron in the body (Sripetchwandee *et al.*, 2019). To gain a better understanding of the consequences of excessive iron in BT, we used deferiprone (DFP), an iron chelator, in the presence and absence of hemopexin therapy. We aim to understand the functional impact of iron on atherosclerosis in BT distinct from that of heme and reveal potential implications for iron chelation in BT. WT, BT, BT mice

administered with DFP, and BT mice co-administered with DFP + HPX therapy (as described in chapter 3.2) were placed on the atherosclerotic model as described in section 2.2. Deferiprone (DFP, Sigma-Aldrich, CAT # Y0001976) was administered at 1.25 mg/ml in the drinking water as previously described (Casu *et al.*, 2016). Atherosclerotic endpoints and serum assays were described in section 2.2.

In addition, non-transferrin bound iron (after centrifuging serum in <3kda Amicon Ultra-0.5 Centrifugal Filter columns) was measured using an Iron Assay Kit (Sigma-Aldrich, CAT# MAK025).

**4.3 Results**

**4.3.1 Deferiprone reduces atherosclerosis in Beta Thalassemia.**

To determine if traditional iron chelation therapy is effective in ameliorating the proatherogenic effects of BT, we employed 4 treatment groups (WT, BT, BT + DFP, BT + DFP + HPX) that we used in our model of atherosclerosis as described in chapter 2. BT + DFP and BT + DFP + HPX mice had decreased plaque area via aortic *en face* analysis compared to BT (BT 50.12 ± 1.78 %| vs. BT + DFP: 35.2 ± 2.0 % vs. BT + DFP + HPX: 34.4 ± 1.9 %, $p < 0.01$, Figure 4.2). BT+ DFP and BT + DFP + HPX mice had decreased plaque area via aortic root lesion area analysis compared to BT (BT 349.8 ± 33.5 μm x $10^3$ vs. BT + DFP: 216.5 ± 8.9 μmx$10^3$ vs. BT + DFP + HPX: 224.1 ± 14.52 μmx$10^3$, $p < 0.01$, Figure 4.3). BT + DFP + HPX did not provide an additive benefit over BT + DFP when compared to BT. Serum Iron levels were decreased in BT + DFP compared to BT but were not significantly decreased in BT + DFP + HPX (Figure 4.4). Non-transferrin and non-hemopexin bound iron levels indicated decreased chelation of iron in both

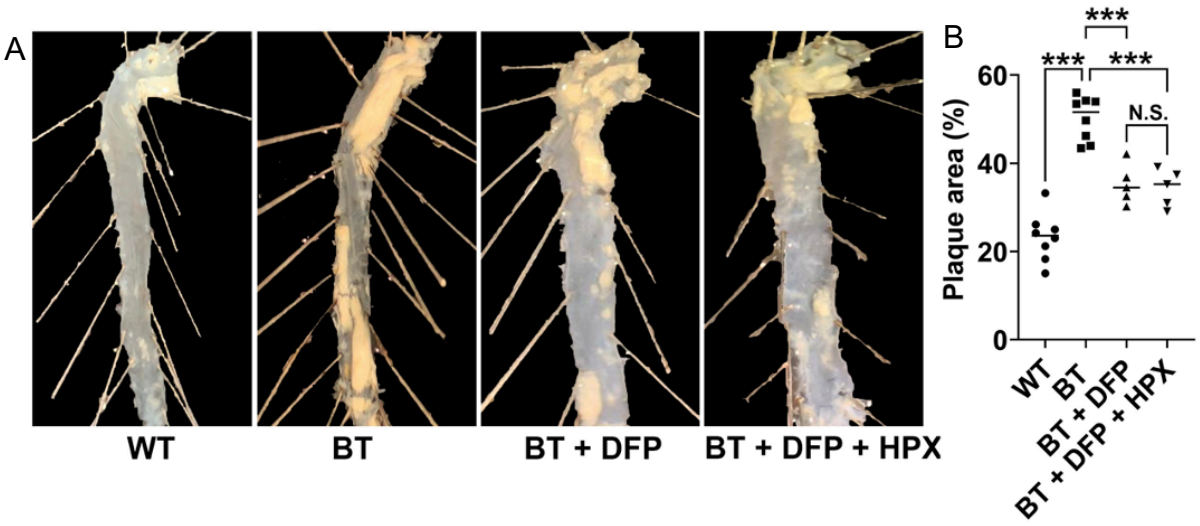


**Figure 4.2. Aortic en face Analysis Demonstrates Decreased Plaque Area with DFP Treatment, but no Additive Benefit with HPX Therapy.** (A) Representative aortic enface for WT, BT, BT with Deferiprone, and BT with Deferiprone and HPX therapy. (B) Analysis of percentage plaque area relative to total luminal area. Values represent mean ± SEM. ***P <0.001.

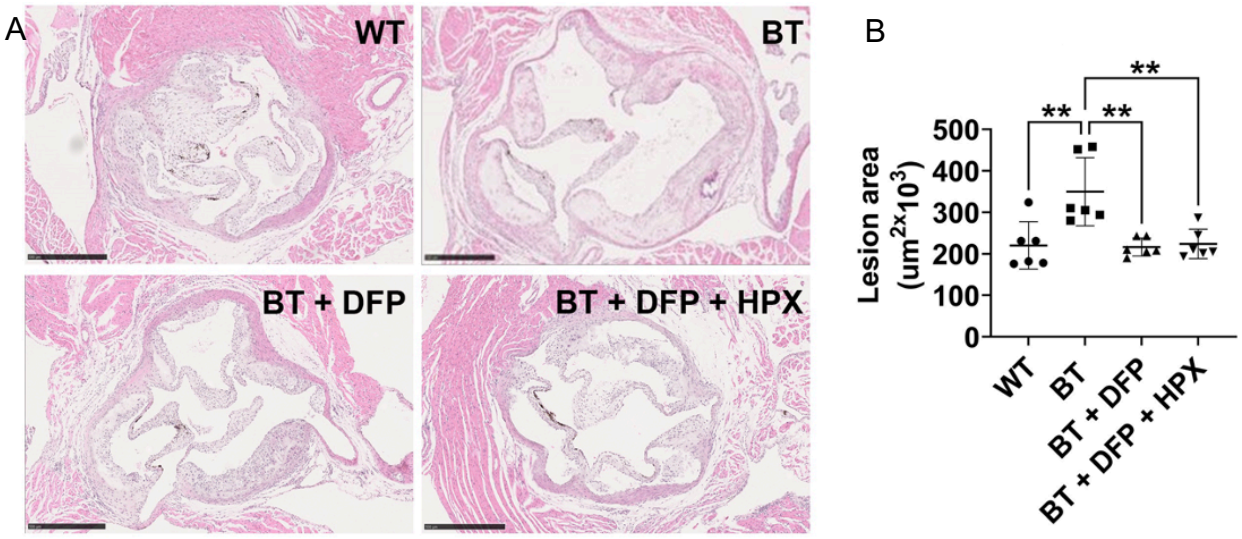


**Figure 4.3 Aortic Root Histology Analysis Demonstrates Decreased Plaque Area with DFP treatment, but no Additive Benefit with HPX Therapy.** (a) Representative aortic root H&E histology of WT, BT, BT with Deferiprone, and BT with Deferiprone and HPX therapy. (b) Analysis of total lesion area μm x $10^3$. Values represent mean ± SEM. **P <0.01.

BT + DFP and BT + DFP + HPX compared to BT (BT: 1.97 ± 0.2 µg/dl vs. BT+DFP: 1.32 ± 0.2 µg/dl,, and BT+DFP+HPX: 1.06 ± 0.3 µg/dl, $p < 0.01$, Figure 4.4)

**4.4 Discussion**

Iron chelation therapy is a mainstay of therapy in BT due to high iron levels caused by regular blood transfusions and increased iron absorption (Taher and Saliba, 2017). The role that iron plays in atherosclerosis is controversial; both iron deficiency and overload have been associated with enhanced atherosclerosis (Nagy *et al.*, 2010; Sharkey-Toppen *et al.*, 2014; Cornelissen *et al.*, 2019). Recently, non-transferrin bound iron (NTBI) and iron tissue accumulation has been highlighted as the most important contributors to a pro-inflammatory phenotype that generates ROS via Fenton chemistry (Cornelissen *et al.*, 2019). Here, we have shown that DFP is able to successfully decrease plaque levels in BT mice. HPX therapy did not provide an additive benefit suggesting that HPX therapy does not lead to a toxic increase in free labile iron.

Iron chelation therapy successfully decreased free iron and NTBI. Iron chelation with HPX therapy showed a significant decreased in NTBI, but not in total iron. One possible explanation is that HPX therapy leads to “artificially” increased hemopexin bound iron in the circulation which blocks heme mediated ROS formation. Iron chelation therapy decreased serum iron levels and plaque accumulation but did not provide an additive benefit to hemopexin therapy. These data, in light of published literature, suggest that free iron is mechanistically downstream of heme. This works lays a foundation to explore the interplay between iron and heme/hemopexin in BT. Future work could be aimed at understanding the global iron axis in BT. For instance, to determine if HPX therapy affects

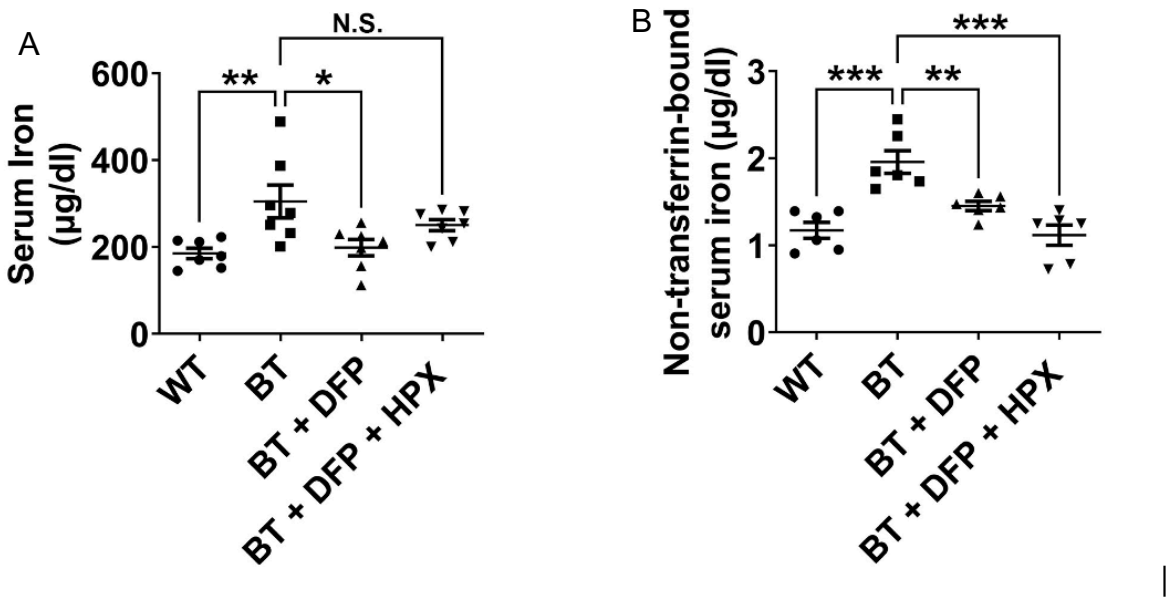


**Figure 4.4 Iron Chelation Decreases Serum Iron Levels.** (A) Serum Iron levels. (B) Non-transferrin-bound iron levels**.** Values represent mean ± SEM. *P <0.05, **P <0.01, ***P <0.001. n=6.

transferrin saturation and changes in iron proteins such as hepcidin, ferroportin, and DMT1.Ultimately, our data corroborate recent findings that in mice models of genetic overload that increases in free iron lead to increased atherosclerosis and suggest that iron chelation can be used to decrease atherosclerosis progression in BT (Vinchi *et al.*, 2020).

Iron chelation is a mainstay therapy in BT. Here, we highlight the importance of iron chelation to improve atherosclerotic plaque accumulation in a mouse model of BT intermedia. This work also further highlights the need for large scale randomized clinical trials of iron chelators testing the effect of cardiovascular outcomes and the potential anti-atherogenic benefit for iron chelation in BT.

# Chapter 5: Discussion/Future Directions

## 5.1 Overview

The data presented in Chapter 2 of this dissertation clearly demonstrate that BT's underlying pathophysiology leads to accelerated atherosclerosis. We presented aortic root and aortic *enface* atherosclerotic plaque analysis that proved that accelerated atherosclerosis was present in both BT male and female mice compared to their WT counterparts. In chapter 3, we showed that free heme plays an important mechanistically relevant role in the progression of atherosclerosis in BT. HPX, heme's endogenous scavenger, therapy reduced free heme and plaque accumulation in both BT mice and in a PHZ-induced model of intravascular hemolysis. In chapter 4, we showed that deferiprone mediated-iron chelation decreased plaque accumulation in our BT mice. In summary, we show that the underlying pathophysiology of BT leads to accelerated atherosclerosis that is mediated by free heme in the circulation that can be decreased by HPX therapy and iron chelation.

## 5.2 Does Beta Thalassemia underlying pathophysiology lead to accelerated atherosclerosis?

Between 2009 and 2022, 12 clinical studies have shown that carotid-intimal media thickness is elevated in individuals with both BT major and BT intermedia, regardless of whether or not they have a history of chelation (Tantawy *et al.*, 2009, 2015, 2017; Hahalis *et al.*, 2011, 2016; Dogan and Citak, 2012; Gursel *et al.*, 2012b; Merchant *et al.*, 2016; Sherief *et al.*, 2017; Ahmad Ibrahim *et al.*, 2021b; Soltani *et al.*, 2021; Kumaravel *et al.*, 2022). Carotid artery intimal medial thickness (CAIMT) is widely used a surrogate marker for atherosclerosis (ONUT *et al.*, 2012). CAIMT is measured through the B-mode of ultrasound which provides a simple, non-invasive, and reliable approach to monitor

atherosclerosis (Sherief *et al.*, 2017). CAIMT in the <25$^{th}$, 25$^{th}$-75$^{th}$ , and >75$^{th}$ percentile predict decreased, average, and increased risk respectively for cardiovascular disease (ONUT *et al.*, 2012).

Traditionally, the vasculopathies of BT were underappreciated due to the premature death of the BT population, but as the BT population ages, BT patients present with more cardiovascular comorbidities (Farmakis *et al.*, 2020). Age, chelation history, transfusion history, and splenectomy are confounding factors that could affect the interpretation of human studies (Farmakis *et al.*, 2020). The literature on the effect of BT on cardiovascular disease is perplexing. CAIMT levels in both BT major and intermedia are consistently increased (Sherief *et al.*, 2017; Ahmad Ibrahim *et al.*, 2021b). Human studies show that the rate of vascular events is approximately 4 times higher in BT intermedia compared to BT major (Hahalis et al., 2016). This could be due to survivorship bias, differing lipid profiles, anemia levels, transfusion history, chelation history, or an entirely different disease paradigm. As the BT major population ages, we will gain greater understanding in the survivorship bias that might exist in BT major that had an average life expectancy of 16 years in the 1970's and increased to 40-49 by 2011 (Farmakis *et al.*, 2020).

There is one report of decreased peripheral artery disease, tested using Ankle Brachial Index, (a less precise method compared to ultrasound) in BT intermedia patients in their mid 60's (Vanini *et al.*, 1993). A more recent study done with ultrasound of CAIMT and Ankle Brachial Index in BT intermedia patients found that patients had increased CAIMT but no differences by Ankle Brachial Index which potentially suggests disparate locations of atherosclerotic plaque susceptibility (Nassef *et al.*, 2020). Myocardial

infarction is not common in BT, but in 2004, the first incidence of myocardial infarction was reported in BT with subsequent sporadic case reports in 2009 and 2023 (Fridlender and Rund, 2004a; El Rassi *et al.*, 2009; Premawardhena *et al.*, 2023). MI in transfusion dependent thalassemia (TDT) is higher than that in non-transfusion dependent thalassemia (NTDT) with a prevalence of 2.11% TDT compared to 0.71% in NTDT (Helmi *et al.*, 2018). Untreated BT major is not commonly seen due to disease severity and treatment necessity, but TDT, most commonly in BT major, consistently shows worse cardiovascular outcomes. Thus, it is hard to delineate the true effect of BT major or transfusion dependent thalassemia in CVD without considering the side effects of extensive transfusion such as iron accumulation.

BT patients have decreased total lipid levels, but have a dyslipidemia which results in an increased atherogenic index (log[Triglycerides/HDL] (Cao and Galanello, 2010; Sherief *et al.*, 2017). BT intermedia patients also do not display advanced coronary artery calcium scores, a sign of mature atherosclerosis that occurs in older adults, compared to healthy controls, but in that study, the BT patients were not appropriately age-matched and were on average 7 years younger than the controls (Hahalis *et al.*, 2016). A larger clinical study of over 1500 BT patients showed a clear 1.5 fold increase in CAD risk compared to healthy controls, and BT intermedia subjects were interestingly at the highest risk (Chen *et al.*, 2015). CAIMT is uniformly increased in BT major/intermedia with increased risk in transfusion dependent thalassemia.

In chapter 2, our work saw a robust increase in plaque accumulation in a mouse model of BT intermedia which corroborates the majority of the clinical evidence. These results were so striking with the aortic enface analysis highly significant differences (P<

0.001) in both BT males and females compared to their WT counterparts. In addition, aortic root histology was also significant (P< 0.01), but there was greater variability. Traditionally, aortic root and aortic enface have similar plaque deposition in mice models, but differences have been previously reported potentially due to different embryological origins (Surman *et al.*, 2021). Our atherosclerotic experiments were appropriately powered for statistical analysis and included both female and male animals, showed no significant difference in serum lipid profiles, were confirmed in a secondary model of atherosclerosis, and overall showed consistent results over the course of 4 years of study. Taken together, this work definitively shows that the underlying pathophysiology in BT leads to accelerated atherosclerosis.

An excellent future direction would be aimed at investigating the different confounding factors that might act synergistically to increase the risk of atherosclerosis in BT. Unfortunately we cannot study the effects of BT major because these mice are neonatally lethal (Kumfu *et al.*, 2017). Thus, we cannot address the difference that might arise between BT major and BT intermedia. In chapter 4, we explored the effects of iron chelation but there are many factors such as age, splenectomy, and blood transfusion that could affect atherosclerotic plaque accumulation. On example is the finding that $ApoE^{-/-}$ mice with splenectomies have more plaque accumulation compared to controls (Rezende *et al.*, 2011). Splenectomies are extremely common in BT, but the effect on atherosclerotic plaque development in BT is unknown. This work further highlights the need to screen the at-risk population in BT for atherosclerosis, and that even in BT intermedia, patients could be at increased risk for atherosclerosis.

### 5.3 Is intravascular hemolysis a prominent feature of Beta Thalassemia?

The impact of intravascular hemolysis has been well-document in hemoglobinopathies (Morris, 2008a; Belcher *et al.*, 2014). In 2009, *Kato et al.* and *Gladwin et al.* proposed intravascular hemolysis plays a critical pathological role in sickle cell disease (SCD), and several investigators have explored related effects of intravascular hemolysis such as TLR4 activation (Belcher *et al.*, 2014), xanthine oxidase expression (Schmidt, 2022), pulmonary hypertension (Hsu *et al.*, 2007), and the role of hemopexin therapy (Vinchi *et al.*, 2013). Likewise, appreciation for the pathological effects of intravascular hemolysis is increasing in other hemoglobinopathies such as BT (Cao and Galanello, 2010; Voskou *et al.*, 2015; Needs *et al.*, 2022).

Human data have shown that BT patients have a 2-fold increase in free heme and a 10-fold decrease in hemopexin (Vinchi, Vercellotti, *et al.*, 2016; Kiger *et al.*, 2019a). In fact, free heme levels are higher in BT patients than those reported in SCD patients (Kiger *et al.*, 2019a). Our data clearly show that increased free heme was present in BT in each of our heme experiments both at baseline and in our atherosclerotic model. As a result, hemopexin levels were also significantly decreased at baseline and in our atherosclerotic model.

### 5.4 Is hemopexin therapy a promising therapeutic in Beta Thalassemia for atherosclerosis?

Over the past decade, hemopexin (HPX) therapy has been investigated in hemoglobinopathies, sepsis, and acute kidney injury models (Vinchi, 2021). A full list is available in table 1.4. Currently, CSL889, a plasma-derived hemopexin, is in a phase-1 clinical trials in SCD (www.clinicaltrials.gov identifier NCT04285827) based on pre-clinical

evidence showing improved endothelial activation and decreased heme-mediated vaso-occlusion (Gentinetta *et al.*, 2022a). Traditionally, hemopexin has been administered via intraperitoneal injections of purified proteins (Vinchi, Vercellotti, *et al.*, 2016). However, this is costly and impractical for long-term mice experiments. One mg of purified HPX costs $560 in Sigma-Aldrich (accessed on 05-12-23).

In this thesis, we showed that HPX therapy is efficacious in the context of atherosclerosis and AAV-HPX is a potential long-term alternative. In chapter 3, we saw that HPX therapy was able to successfully decrease atherosclerotic plaque levels in both BT and phenylhydrazine (PHZ) mediated hemolysis. After 3 months, our HPX was able to maintain decreased free heme levels. In our HPX experiments, HPX levels were similar in BT and PHZ mice treated with HPX therapy to their respective controls. This difference could potentially be explained by HPX consumption because our free heme levels were significantly decreased. We reported no significant mortality or side-effects in our mice treated with HPX. HPX therapy was able to decrease plaque accumulation in both a genetic (BT) and a pharmacological (PHZ) model of intravascular hemolysis.

Paradoxically, in our WT mice treated with HPX, we saw a significant increase in atherosclerotic plaque accumulation. Despite this still being perplexing, we have investigated the literature, performed control experiments, and propose future experiments to gain insights into this unexpected experimental finding. Initially reported by *Bakker et al.* in 2005, HPX has serine protease activity, but in 2106 *Lin et al,* found that HPX protease activity is negligible compared to serine protease enzymes and did not affect chemotaxis of neutrophils (Bakker *et al.*, 2005; Lin *et al.*, 2016). In addition, we experimentally tested our empty vector that was used by our AAV-HPX, but we saw

no significant change in atherosclerotic plaque accumulation.

We also showed that HPX therapy decreased serum non-transferrin bound iron levels. Iron has been shown to have a “U”/”J” curve for cardiovascular risk in which low and high iron levels are at greater risk. We used 4E11 GC of our AAV-HPX that lead to a 4-fold increase in HPX levels. In a future direction, we could perform a dose-response curve on HPX levels on our atherosclerotic model to see the effects on atherosclerosis. In addition, we showed that HPX therapy decreased heme oxygenase-1 (HOX-1) mRNA levels in the aorta; a finding that was also reported by other groups (Vinchi, Vercellotti, *et al.*, 2016). HOX-1 have been shown to affect atherosclerosis. Heme-oxygenase knockout mice have been shown to increased atherosclerosis (Yet *et al.*, 2003b; Gough *et al.*, 2006). Globally, this calls out the need to understand the compensatory mechanisms in the hemolysis pathway and understand the broader role hemopexin plays in the context of iron.

## 5.5 Implications for Accelerated Atherosclerosis in Beta Thalassemia and Future directions

### 5.5.1 Changing Landscape in Beta Thalassemia

Globally, this work illustrates how advancements in disease management and the changing landscape in a disease can highlight other important aspects of the disease. In 1970’s, the average life expectancy of BT was 16 years old, but the advancements in iron chelation and regular blood transfusion have increased average lifespans into the mid 40s (Farmakis *et al.*, 2020) .

After establishment of blood transfusion and iron chelation as the mainstay therapy in BT, BT patients present with less severe, and thus, the next generation of BT

literature highlights understanding the associated vasculopathy (Morris, 2008a). Bone marrow transplants are potential curative solutions, but they have limitations due to limited availability of major histocompatibility complex (MHC)-matched donors, the need for long-term immunosuppression, narrow application to the youngest patients and increased risk of immunological complications, as well as non-rejection mortality in older subjects with organ damage (John *et al.*, 2018; Needs *et al.*, 2022). As an illustrative comparison, the field of atherosclerosis initially highlighted the importance of lipids, but through the widespread success of statins, it is clear that there is a substantial, residual risk for cardiovascular disease that is independent of hypercholesterolemia. (Libby, 2021c; Björkegren and Lusis, 2022). Now, the field of atherosclerosis is growing to understand new perspectives on the disease such as various cell-specific contributions via techniques such as single-cell RNA sequencing and the effect of other comorbidities such as obesity and insulin resistance (Libby, 2021c). Furthermore, the field of atherosclerosis is teasing out the controversial role of key related concepts such as iron or oxidative stress that have limited clinical effectiveness despite preclinical evidence (Cornelissen *et al.*, 2019).

### 5.5.2 Are other hemolytic anemias associated with an increased risk for accelerated atherosclerosis?

The risk for atherosclerosis in other hemolytic anemia is incompletely understood and could be disease specific. In sickle cell disease, the risk for atherosclerosis is considered to be low, but poorly understood. Atherosclerosis is not a common cause of morbidity and mortality in SCD, but autopsy evidence does show that cerebral arteries, pulmonary arteries, and the splenic artery have plaque accumulation (Elsharawy *et al.*,

2009). The first report to our knowledge to show increased CAIMT in SCD was reported in 2017 (Kaddah *et al.*, 2017) with case reports in 2019 (Ayoola *et al.*, 2020), 2020 (Kadam *et al.*, 2019), and 2021 (Hanna *et al.*, 2021). In BT and SCD patients CAIMT levels were reported to be similar, but in SCD, CAIMT did not correlate with ferritin as has been showed in BT (Tantawy *et al.*, 2009; Hanna *et al.*, 2021). Paradoxically, in 2013, bone marrow transplant studies of SCD marrow into ApoE$^{-/-}$ mice showed decreased plaque formation (Wang *et al.*, 2013). However, these mice received bone marrow transplants (BMT) which has been shown to confound atherosclerotic endpoints. In BMTs, mice have to undergo radiation that has been shown to activate p53 (shown to decrease atherosclerosis), have difficulties in weight gain, and demonstrate intestinal barrier dysfunction that could affect atherogenesis (Aparicio-Vergara *et al.*, 2010). In addition, the authors did not show the aortic *enface* results*,* and the sickle cell mice transplanted onto the ApoE$^{-/-}$ background had significantly lower LDL levels ( WT:1.6 mmol/l vs SCD: 0.6 mmol/l). In addition SCD mice are an extremely fragile mouse model on a mixed genetic background that could confound results. Furthermore, a potential difference between BT and SCD is the significantly higher levels of iron accumulation found in BT patients (Coates and Wood, 2017).

In a future direction, analyzing the progression of atherosclerosis in other hemoglobinopathies or genetic hemolytic anemias such as auto-immune hemolytic anemia would be of great interest. For instance, *Chappell et al.* recently developed a more representative mouse model of alpha-thalassemia that could be used to see the progression of atherosclerosis (Chappell *et al.*, 2021). Hereditary spherocytosis and mechanical devices like heart valves and ventricular assist devices also lead to increased

hemolysis which would be interesting to test in our model of atherosclerosis (Agnieszka *et al.*, 2022; Zamora and Schaefer, 2023). Running experiments in our model of atherosclerosis in SCD mice will avoid previous results that used bone marrow transplants. Taken together, difference or similarities between these models could highlight important phenotypes in this disease paradigm.

5.5.3. What are the contributions of the other adaptive and pathological responses to hemolysis?

In this dissertation, we have explored the effects of HPX therapy, but a future direction could contribute to the understanding of other players in the intravascular pathway such as haptoglobin therapy, TLR4 activation, and the NO pathway. Haptoglobin binds to free hemoglobin and binds to CD163 receptors on macrophages where they are cleared (Nagy *et al.*, 2010). Compared to HPX, haptoglobin is a protein with a shorter half-life and it has been mostly explored for a role in septic shock (Graw *et al.*, 2016; Remy *et al.*, 2019). Haptoglobin therapy and heme therapy have been shown to operate in a similar pathway and are somewhat redundant (Belcher *et al.*, 2018). Haptoglobin is costly to purify, and recently, *Munoz et al.* showed in BT mice, that apohemoglobin-haptoglobin complex, a more cost-effective option, can improve liver function by decreasing ALT, AST, and ALP markers and reducing circulating serum iron concentration and transferrin saturation concentration. A future direction could explore the role of haptoglobin therapy or the combination of haptoglobin therapy with HPX in the setting atherosclerosis in BT.

Heme can also act as a ligand to activate TLR4 signaling. TAK-242 and bone marrow transplants of TLR4 knockout mice have been used to explore the role of TLR4

in hemglobinpathies (Belcher *et al.*, 2014). We performed inconclusive preliminary experiments with BT mice bone marrow transplants onto TLR4 knockout mice (n=2) on our model of atherosclerosis, but the BT mice bone marrow transplanted onto TLR4 knockout mice had no difference when compared to WT mice bone marrow transplanted onto TLR4 knockout mice and <5% plaque accumulation via aortic enface analysis. This is a known limitation of bone marrow transplantation studies in mouse models of atherosclerosis. A recommended future approach is to cross the mice onto the TLR4 knockout background, employ a more aggressive model of atherosclerosis, or to use pharmacological inhibition of TLR4 using theTLR4 inhibitor TAK-242.

Upon excess hemolysis, nitric oxide is limited because RBCs release arginase which can metabolize arginine, a necessary precursor of NO. In addition free hemoglobin can react with nitric oxide, and tetrahydrobiopterin ($BH_4$) leading to degradation of nitric oxide synthase-cofactors (Voskou *et al.*, 2015). We could explore the role of nitric oxide in the progression of atherosclerosis in BT by: 1. investigating the arginase levels in BT mice, arginine metabolites, and nitric oxide levels at baseline 2. Arginine supplementation (Lewis *et al.*, 2022) 3. Genetic knockout of the NOS family of enzymes (Casarotto *et al.*, 2018).

Heme oxygenase is critical to the progression of atherosclerosis, but the interpretation is complex. Heme oxygenase-1 (HO-1) is the inducible isoform of heme oxygenase. Pharmacological inhibition of HO-1 and genetic knockout of HO-1 on an $ApoE^{-/-}$ background led to increased atherosclerosis (Yet *et al.*, 2003b). Presumably, the reason is because proinflammatory heme in vasculature is detoxified by HO-1. Paradoxically, administering interparental injections of a low-dose of heme in $LDLR^{-/-}$ mice

has been shown to significantly decrease plaque accumulation (K. Ishikawa *et al.*, 2001). PHZ has been shown to increase HO-1, but has led to both increased and decreased plaque in different animal models (Paul *et al.*, 1999; Fernandez *et al.*, 2001b). Heme is pro-inflammatory, but heme is catabolized by heme oxygenase in the liver to both pro-(iron) and anti-inflammatory (CO, bilirubin) byproducts.

There are several unknowns left to be understood in this compensatory paradigm of heme oxygenase. A couple of unknowns are the following: Is there a threshold at which the detoxifying effects of HO-1 outweigh the proinflammatory effects of heme, is there a difference in the compensatory response to chronic hemolysis vs. transient hemolysis and the subsequent effect on atherosclerosis, are there differences in tissue HO-1 compartmentalization of heme in atherosclerotic plaque, and what is the impact of iron overload with increased HO-1 activity? We showed that mice on our PHZ-induced model of intravascular hemolysis (once weekly 25 mg/kg) had increased atherosclerosis in our PCSK9 overexpression with high fat diet atherosclerotic model atherosclerotic model, while *Paul et al.* showed decreased plaque accumulation in a mouse model of ApoE$^{-/-}$ (1 25mg/kg injection ever 48 hours). Other PHZ models have shown increased atherosclerotic plaque levels, but these experiments were done in rabbits (Fernandez *et al.*, 2001b). Our model was a more transient model of hemolysis which could affect the compensatory response of HO-1. A future experiment could evaluate different timing and doses of heme on the progression of atherosclerosis. Due to the number of permutations, a shorter atherosclerosis model such as the partial carotid ligation model might be more useful (Nam *et al.*, 2009). Since iron is produced downstream of heme-oxygenase, a future direction would explore the roles of transferrin, ferritin, divalent metal transporter 1,

and hepcidin in the presence of excess free heme, HPX therapy, and iron overload in BT mice.

### 5.6. Summary

In summary, this work definitively shows for the first time that BT leads to accelerated atherosclerosis. Intravascular hemolysis is a prominent feature in BT and resulting increases in free heme are mechanistically relevant to the acceleration of atherosclerosis in BT. Excess free heme generated by a PHZ- induced model of hemolysis also led to increased atherosclerosis supporting this role for free heme. We showed that AAV-HPX therapy leads to decreased free heme and atherosclerotic plaque levels in BT mice and the PHZ-induced model of hemolysis. In addition, deferiprone-mediated iron chelation therapy led to deceased plaque accumulation in BT mice but provided no additive benefit to HPX therapy and vice versa suggesting that the ultimate effect of heme on atherosclerosis progression is mediated by iron. Future directions require increasing our understanding of the compensatory effects of hemolysis, understanding if there is a U/J shape benefit to hemopexin in which too little or too much hemopexin is detrimental, gaining a broader understanding of the relationship between iron and hemopexin, and the impact of other factors such as splenectomy that could contribute to atherosclerosis in BT.

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