The role of red blood cells and red blood cell-derived extracellular vesicles in anemia-associated endothelial dysfunction

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Isabella Maria Solga-Kamp
aus Mönchengladbach

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aus der Klinik für Kardiologie, Pneumologie und Angiologie
der Heinrich-Heine-Universität Düsseldorf
Klinikdirektor: Univ.-Prof. Dr. med. Malte Kelm

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Introduction

Endothelium

Blood vessels consist of three distinct layers: the innermost *tunica intima*, the middle *tunica media*, and the outer *tunica externa*. Based on their function, location, and size, blood vessels are classified into arteries, capillaries, and veins *(1)*. The endothelium is a single monolayer of cells that form the inner lumen of blood vessels as well as the lymphatic system *(2)*. The endothelium acts as a semi-permeable barrier that connects and regulates the exchange of various molecules, nutrients and proteins between the blood and surrounding tissues *(3)*. The main function of endothelium is the regulation and modulation of vascular tone, thereby influencing the overall function of the cardiovascular system *(4,5)*. In response to physical or chemical stimuli the endothelium synthesizes and releases various vasoactive compounds affecting vascular tone and growth *(6)*. Additionally, during vascular inflammation, the endothelium regulates the interactions with vessel walls and facilitates the adhesion of leukocytes, monocytes, and lymphocytes *(7)*.

Endothelium in vascular tone regulation

Vascular tone is defined as the degree of constriction of arteries relative to their maximal dilatory state. Vascular tone is maintained by vascular smooth muscle cells (VSMCs) under the influence of endothelium. Vascular tone is essential for regulation of blood pressure and organ perfusion *(8,9)*. In response to shear stress, endothelium produces both contractile and relaxing factors that regulates the vascular tone. The main contractile factors are endothelin (ET)-1 and thromboxane (TXA_2) *(10)*. The main endothelial relaxing factors are prostacyclin (PGI2), endothelium-derived hyperpolarization factor (EDHF) and nitric oxide (NO) *(10)*. The balance between both vasoconstrictors and vasodilators is crucial for maintaining vascular tone *(1)*.

The key signalling messenger/regulator of vasodilation produced by the endothelium is NO. It is synthetiszed by the 3 isoforms of the nitric oxide synthase (NOS): neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), endothelial NOS (eNOS, NOS3). The nNOS isoform is primarily expressed in neurons and synthesizes NO, which plays a crucial role in neurotransmission and neuromodulation *(11)*. In contrast, iNOS is induced by proinflammatory cytokines and immune modulators in different cell types such as endothelial cells, SMCs, cardiac myocytes, and macrophages and plays a crucial role in mediating inflammatory response *(11–13)*. The eNOS is primarily expressed in endothelial cells, and also in other cell types such as red blood cells (RBCs) and cardiomyocytes *(12,14)*. In the vascular system eNOS is the main isoform to regulate and maintain the vascular tone and thus blood pressure

(15). The deletion or pharmacological inhibition of eNOS leads to severe hypertension, indicating its crucial role in maintenance of blood pressure *(16)*.

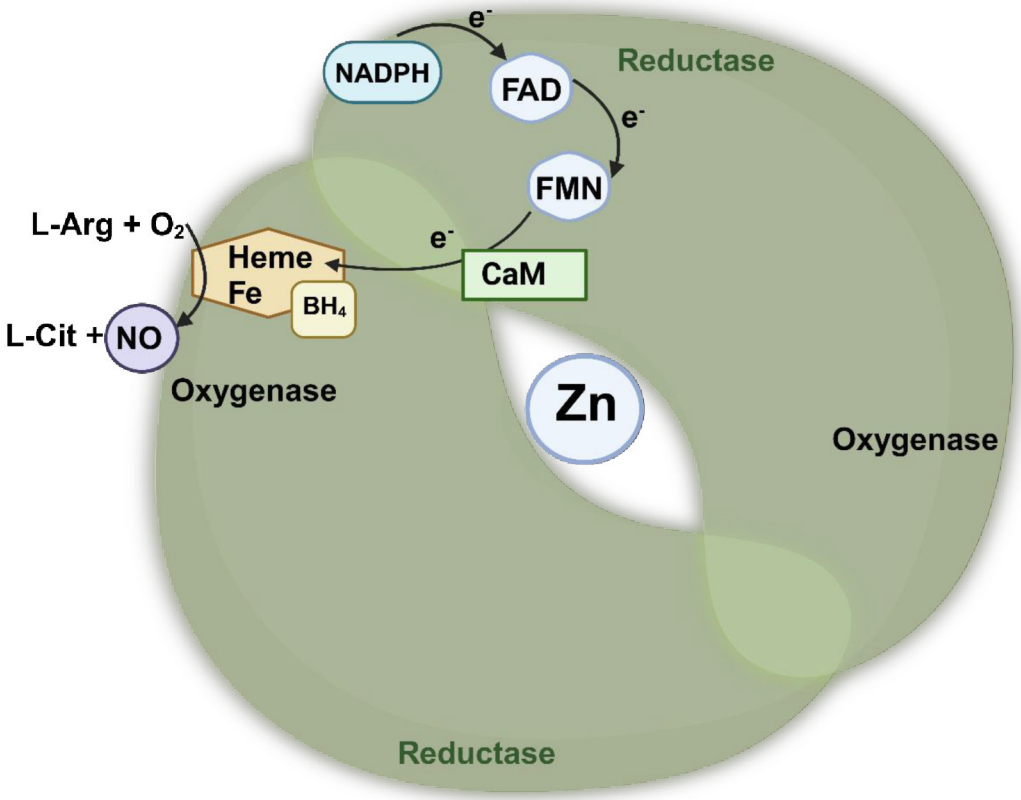

Figure 1: Schematic representation of endothelial nitric oxide synthase (eNOS) and synthesis of nitric oxide (NO). eNOS consists of two monomers stabilized by a zinc ion (Zn) and contains a central calmodulin (CaM)-binding region, a C-terminal reductase, and a N-terminal oxygenase domain. The dimeric structure enables the transfer of electrons from the reductase domain of one monomer through nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to the heme at the oxygenase domain of the other monomer.In presence of cofactor tetrahydrobiopterin (BH_4), eNOS uses substrate L-arginine (L-Arg) and O_2 and converts it into L-citrulline (L-Cit) and NO. The graphical illustration is based on Janaszak-Jasiecka et al. *(17),* modified and recreated using BioRender.

All three NOS isoforms are homodimers, consisting of two monomers with an oxygenase and reductase domain, stabilized by cofactor tetrahydrobiopterin (BH_4), heme and zinc-thiolate cluster at the dimer interface *(18,17,19)*. The activated NOS enzymes transfer electrons from nicotinamide-adenine-dinucleotide phosphate (NADPH), through the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in the reductase domain, to the heme group in the oxygenase domain. This process facilitates the reaction of oxygen with L-arginine, resulting in the conversion of L-arginine to L-citrulline and the production of NO *(19,20)*. This reaction is mediated in the presence of co-substrates molecular oxygen and NADPH, along with co-fac-

tors FAD, FMN and BH_4 and regulated by intracellular calcium (Ca^{2+}) concentration *(19)*. Increased intracellular Ca^{2+} concentration leads to the binding of calmodulin (CaM), which favors the electron transfer *(19,20)*. Both eNOS and nNOS are considered as Ca^{2+}-dependent since they require an increased concentration of Ca^{2+} for activation through calmodulin (CaM) binding. In contrast, iNOS is considered Ca^{2+}-independent because it binds to CaM even under resting conditions *(20)*. Synthesized NO diffuses into VSMCs and induces vasodilation. This reaction is triggered by binding to the soluble guanylate cyclase (sGC), which converts the guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Enhanced cGMP levels in VSMCs reduce intracellular Ca^{2+} concentration, resulting in vasodilation of VSMCs *(21,22)*.

The activation of eNOS and the release of NO is regulated by different mechanisms. The major stimuli of activation is the exposure to either mechanical shear stress or to chemical stimulants such as acetylcholine, serotonin, thrombin and bradykinin *(23)*. Shear stress is known to activate eNOS through phosphorylation of different phosphorylation sites (e.g. serine phosphorylation trough Ser1177, Ser633, Ser615, Ser114 and threonine phosporylation trough Thr495, and tyrosine phosporylation trough Tyr81, Tyr 657), which is essential to mediate flow-mediated dilation (FMD). In addition hormones like estrogen and insulin are also known to stimulate eNOS activity trough the activation of phosposites (e.g. Ser1177 and Tyr 657) *(24,25)*. The activity of eNOS is also regulated by factors that affect the availability of substrates and cofactors essential for its function, such as oxidative stress levels and associated BH_4 deficiency. *(26–28,17)*. The deficiency of substrates, co-factors, and increased oxidative stress leads to the inhibition of eNOS activity and NO production, resulting in ED *(17)*.

Endothelial dysfunction

ED is described as the decrease in eNOS activity and thus reduced NO bioavailability, which is often described as early hallmark of cardiovascular disease (CVD) *(17)*. Different diseases such as coronary artery disease (CAD), heart failure, diabetes and atherosclerosis are known to be associated with ED *(29)*. Beside a decrease or loss of vasodilation, inflammation, vascular remodelling, platelet aggregation and SMC proliferation are the pathological consequences of ED *(30)*. The main cause of ED is eNOS uncoupling resulting in the production of superoxide (O_2^-) instead of NO *(31)*. The eNOS uncoupling is mainly mediated by oxidative stress, tetrahydrobiopterin (BH_4) levels, L-arginine deficiency, accumulation of asymmetrical dimethylarginine (ADMA), and S-glutathionylation *(17)*.

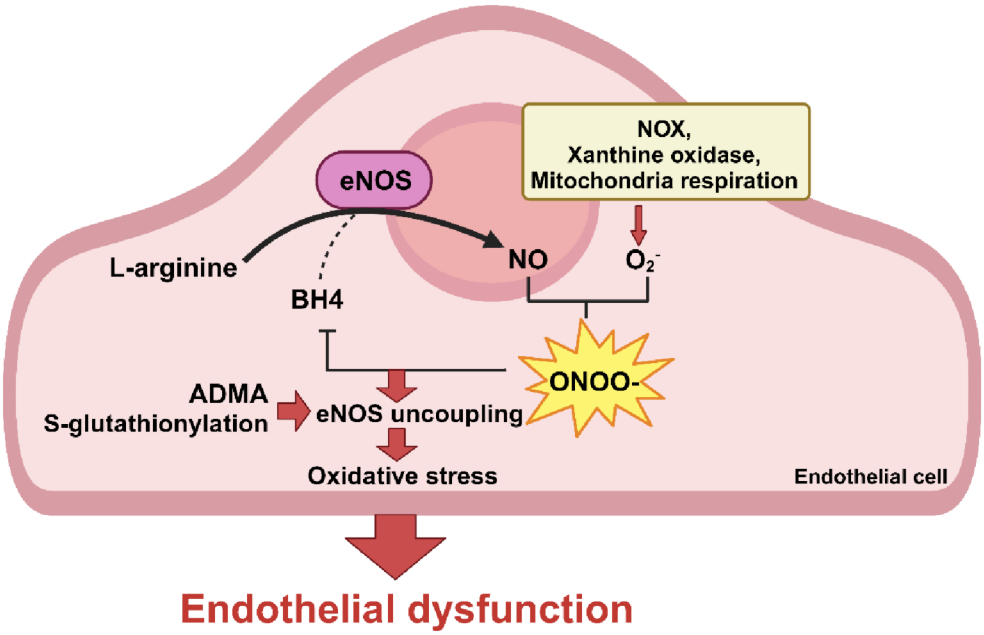

Figure 2: Schematic representation of eNOS uncoupling leading to oxidative stress and endothelial dysfunction. In the presence of cofactor tetrahydrobiopterin (BH_4), eNOS synthetizes nitric oxide (NO) from L-Arginine. The eNOS uncopling is mainly mediated by superoxide anion (O_2^-) generated from various sources such as NO metabolites (NOx), xanthine oxidase and mitochondria respiratory chain in the endothelium. The O_2^- anions interact with NO, resulting in the formation of peroxynitrite $ONOO^-$, which further leads oxidation of BH_4 to BH_2. The limitation of BH_4, and other factors such as asymmetrical dimethylarginine (ADMA) and eNOS S-glutathionylation contribute to eNOS uncoupling. This results in reduced NO production and endothelial dysfunction (ED). Illustration based on Huynh et al. *(32)* and recreated with BioRender.

Cardiovascular risk factors like diabetes, hypertension, hypercholesterolemia, obesity and smoking are known to cause enhanced ROS production, leading to oxidative stress *(33,17)*. This further results in oxidation of BH_4 to BH_2 leading to a reduction in the BH_4 levels in the endothelium, which promotes eNOS uncoupling *(34)*. Furthermore, the interaction between O_2^- and NO results in peroxynitrite ($ONOO^-$) formation, enhancing the oxidation of BH_4, further promoting eNOS uncoupling *(35)*. Additionally, ROS generated by enzymes such as NADPH oxidase, xanthine oxidase, and mitochondrial respiratory chain complexes is known to induce ED by uncoupling the eNOS enzyme in pathological conditions *(36)*.

Since ED plays a key role in the development of CVD, it is crucial to have a reliable tool to assess ED in patients within a clinical setting *(37)*. The FMD refers to shears stress-mediated endothelial-dependent vasodilation response due to blood flow which is mainly mediated by nitric oxide released from the endothelium *(38)*. In humans, FMD is assessed by inducing transient ischemia using a cuff and performing ultrasound-guided imaging of the brachial artery *(39)*. FMD is a non-invasive method for the assessment of endothelial function, which is a predictor for the grade and development of CVD *(40–42)*. Different pathologies and diseases, including MI, atherosclerosis, diabetes, hypertension and coronary artery disease have been shown to be associated with impaired FMD responses in patients *(43)*.

Reactive oxygen species and inflammation in ED

Increased ROS have been identified as the primary contributor to ED. ROS are small chemically reactive ions and molecules, derived from oxygen metabolism, generated within the biological system *(44,45)*. ROS molecules consist of free radicals such as superoxide anion ($O_2^{-}\cdot$), hydroxyl racial (HO·), nitric oxide (NO·), and lipid radicals, as well as other molecules like hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl), also having oxidizing effects *(30)*. The potential enzymatic sources for ROS formation in the vascular system are mitochondrial respiration, arachidonic acid pathway enzymes lipoxygenase and cyclooxygenase, cytochrome p450s, xanthine oxidase, NADH/NADPH oxidases, NO synthase, peroxidases, and other hemoproteins *(30)*.

The physiological role of ROS includes regulation of aging, immunity, function as redox messenger, and is essential in maintaining endothelial homeostasis and SMC contraction *(46,47,44)*. Under pathological conditions enhanced ROS production leads to cardiovascular damage by causing defects in cell regeneration, lipid peroxidation, protein degradation, DNA damage, mitochondrial injury, and disruptions in energy metabolism *(48)*.

Inflammation and oxidative stress concur with each other and plays curial role in immune modulation. The disruption of normal physiological functions activates immune cells, leading to systemic inflammation characterized by elevated ROS levels *(49)*. Excessive ROS generation further amplifies inflammation, contributing to the development of various inflammatory disease *(50)*. In endothelium, apart from eNOS uncoupling, ROS contributes to endothelial activation, which is known to be evidenced by the upregulation of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), E-Selectin, and P-Selectin *(51)*. These molecules are often described as markers of endothelial inflammation, and are known to promote the adhesion of leucocytes *(52)*. Endothelial cells are also activated by various stimuli, such as cytokines interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-a), in response to infections or tissue injury *(53)*. Dysregulated inflammation and increased ROS formation are the key contributors of endothelial dysfunction leading to the development of CVD. Both are associated with pathological conditions such as atherosclerosis, diabetes mellitus and endothelial dysfunction, which are known risk factors for acute myocardial infarction (AMI) *(54)*.

Acute myocardial infarction

AMI is one of the leading causes of sudden death globally. A blockage in the coronary arteries deprives the heart muscle of blood and oxygen supply leading to the death or injury of cardiomyocytes, resulting in necrosis *(55)*. Approximately 70% of fatal AMI cases are caused by occlusions due to atherosclerotic plaques, making atherosclerosis the leading cause of AMI

(56). Atherosclerosis is a progressive disease associated with ED *(57)*. Dysfunctional endothelium leads to increased vasoconstriction, platelet aggregation, and enhanced endothelial permeability, allowing low-density lipoproteins (LDL) to accumulate and damage the coronary arterial wall *(57,58)*. This triggers endothelial inflammation and promotes further plaque formation *(58)*. The rupture of the atherosclerotic plaque leads to thrombus formation and platelet aggregation, creating an occlusive thrombus, which clogs the artery and reduces oxygen delivery to the myocardium. This reduction in oxygen supply causes myocyte death and impairs heart function *(56,59)*. In the clinical setting laboratory screening for troponins and electrocardiogram (ECG) is used to detect the AMI based on changes in the ST- T- waves and Q- waves *(60,61)*.

The death of cardiac cells in the infarcted region triggers the activation of inflammatory and healing processes, which are crucial for the subsequent decline in heart function and the prognosis of heart failure *(62,63)*. The healing and repair process after AMI is generally divided into three phases: the inflammatory phase, the proliferative phase, and the maturation phase, including different stage of immune cell infiltration and scar formation *(59)*.

Coronary artery disease and different cardiovascular risk factors like smoking, hypertension, diabetes mellitus, and obesity are the main cause for AMI *(56)*. These risk factors are associated with ED, which further promotes the development of atherosclerosis, influencing the overall prognosis after AMI *(64)*. Furthermore, anemia has been recognized as an independent predictor of adverse outcomes and is another risk factor for AMI *(65)*.

Anemia

Anemia is described as a pathological condition associated with reduced hemoglobin (Hb) or hematocrit (HCT) concentration and a decreased number of circulating RBCs, resulting in a reduced oxygen carrying capacity in the blood *(66)*. This leads to a decreased ability of erythrocytes to transport oxygen (O_2) to tissues and/or changes in the morphology of RBCs, which impairs their normal oxygen-carrying function leading to tissue hypoxia *(67,68)*. According to World Health Organization (WHO) guidelines male patients are considered anemic if their Hb levels fall below 13.0 g/dL, while female patients are considered anemic if their Hb levels fall below 12.0 g/dL. The primary mechanisms of anemia are categorized into two groups: 1. Increased RBC consumption due to intravascular hemolysis or extravascular blood loss, and 2. Deficient, or ineffective erythropoiesis, resulting mainly from nutritional deficiencies, inflammation, or genetic disorders *(69,70)*.

Hospital-acquired anemia (HAA) plays a significant role in the clinical setting, as it is associated with increased hospital stay, morbidity and mortality in these patients, especially patients in

the intensive care unit (ICU) *(71,72)*. HAA is defined as anemia developed during hospitalization with normal Hb values upon admission, reducing during hospitalization *(73)*. HAA is caused due to blood sampling for laboratory tests or clinical interventions such as invasive procedures (e.g. catheters), inflammation, or bleeding from injury sites. *(72,71)*.
Anemia is diagnosed in 20–30% of patients with CVD and is considered to be an independent risk factor due to its association with increased morbidity and mortality in these patients *(74,75,67)*. Serious cardiovascular complications such as bleeding, thromboembolic events, stroke, hypertension, arrhythmias, and inflammation are associated with anemia *(67)*. In addition, clinical studies demonstrated anemia as co-morbidity playing an important role in the outcome of AMI patients. The presence of anemia, in combination with AMI, often results in a poor prognosis due to an increased risk of cardiac events *(76,77).* Anemia in AMI may impair the oxygen supply through the blood *(76,78)*. Further studies have demonstrated that anemic patients are at a higher risk of major bleeding, arrhythmias, and heart failure following an AMI. As a result, these patients experience increased morbidity and mortality rates after AMI *(78,79)*. It was also previously demonstrated that acute blood-loss anemia induces RBC dysfunction by decreasing nitric oxide NO bioavailability, increasing ROS formation, compromising membrane integrity, and enhancing NO scavenging by free plasma hemoglobin *(80)*.

Red blood cells and their role in the cardiovascular system

The primary function of blood is the transport of gases, nutrients, hormones, and the removal of waste materials from the body. RBCs are particularly essential for transporting O_2 and carbon dioxide (CO_2) between the lungs and the rest of the body *(81)*. In addition to their role in gas exchange, RBCs play a role in viscosity and blood flow, the maintenance of vascular homeostasis through the regulation of the NO pool, and redox regulation *(82,67)*. The ability of deformation is an important property of RBCs for the adaption to mechanical stress and flow *(83)*. The cellular composition, membrane properties, and molecular construction of RBCs enable them to deform, allowing them to pass through all types of vessels, even small capillaries *(84)*. The concentration and deformability of RBCs and composition of plasma proteins play an important role in the blood viscosity and flow *(85)*.

RBCs modulate vascular tone through their NO scavenging properties, regulating NO bioavailability (*14*). Further, they release other vasoactive factors such as adenosine triphosphate (ATP), sphingosine-1-phosphate (S1P), and cyclic guanosine monophosphate (cGMP) making them a crucial player in maintaining blood flow and vascular homeostasis *(67,86–88)*. Additionally, RBCs are known to express endothelial nitric oxide synthase (eNOS) contributing to the production of NO *(14)*. RBCs function as reservoirs of oxidative NO products, such as nitrite and nitrate, facilitating the controlled release and bioactivation of NO under varying physiological conditions *(67,89).* In addition, RBCs are known to store NO in the form of S-nitrosothiols

(RSNOs), which are formed when NO reacts with thiol groups in proteins, creating a stable form of NO storage *(90,91)*. Furthermore, hemoglobin in RBCs is known to bind NO directly, resulting in nitrosylhemoglobin, which is another storage form of NO *(67).* These NO metabolites are known to influence the circulating NO pool, blood pressure regulation, and cardioprotection *(14).*

RBCs possess a redox regulation system that includes sources of ROS production and both non-enzymatic and enzymatic antioxidant defense systems *(82)*. Key enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, along with antioxidants like glutathione and ascorbic acid, play a crucial role in protecting RBCs from oxidative damage *(92,93)*. Methemoglobin, the oxidized form of Hb, serves as primary source of ROS formation in RBCs *(94)*.

RBC dysfunction

Under various pathological conditions, RBCs exhibit altered functions, including enhanced adhesion capabilities, increased production of ROS, elevated arginase activity, and changes in protein content and enzymatic activities *(82)*. In addition, dysfunctional RBCs undergo hemolysis, releasing cell-free Hb, which act as potent NO scavenger altering the NO bioavailability *(95)*. Changes in RBC morphology with increased stiffness and changes in membrane viscosity lead to altered RBC deformability *(82)*. Impaired deformability often is associated with diabetes mellitus, hypertension and hypercholesterolemia *(96)*. Further studies have shown that pathological conditions like diabetes, hypercholesterolemia and dyslipidaemia are associated with RBC dysfunction affecting endothelial function. Two proposed mechanisms are the upregulation of arginase 1 and increased ROS formation in RBCs influencing endothelial function *(97,98)*. Furthermore, previous studies have demonstrated that the cardioprotective properties of RBCs are diminished due to RBC dysfunction in anemia *(99)*. However, the exact mechanism underlying RBC dysfunction and its contribution to poor outcomes following CVD and AMI remains unclear.

Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are defined as heterogeneous membranous nanoparticles with a lipid bilayer *(100)*. Recent studies demonstrated that EVs play a crucial role in intercellular communication *(84)*. Based on their origin and size EVs are classified into three different major subtypes. Exosomes are the smallest particles (30-100 nm), and are formed by inward budding of multivesicular bodies *(101)*. By the fusion of the multivesicular bodies with the plasma membrane, exosomes are released into the extracellular space *(102,103)*. The second group of

EVs are microvesicles (100-1000 nm), which are secreted by direct outward budding or shedding from the plasma membrane *(102,103)*. This process is often stimulated by increased intracellular calcium levels or other cellular signals, which promote cytoskeletal rearrangements and membrane budding *(104)*. The formation of microvesicles will be discussed in more detail. The third subtype are the apoptotic bodies (1-5 µm), which are released during apoptosis from apoptotic cells *(103,105)*.

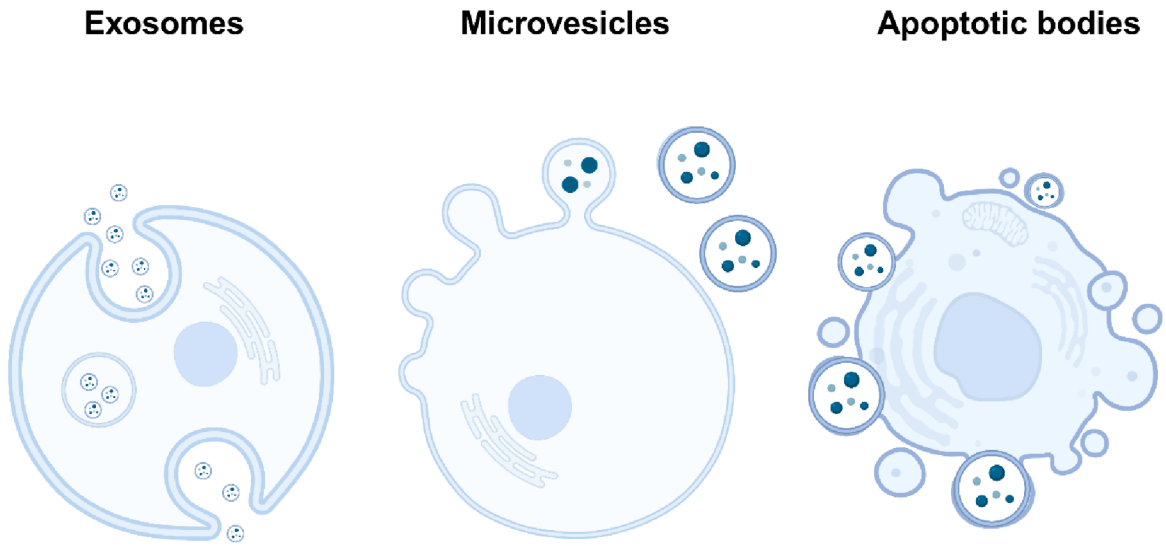

Figure 3: Schematic presentation of the different subytypes of extracellular vesicles and their release. Exosomes are released into the extracellular space by the fusion of multivesicular bodies (MVB) with the plasma membrane. Microvesicles are released from direct budding of the plasma membrane. Apoptotic bodies are released during apoptosis from apoptotic bodies. Illustration based on Villysson et al. *(103)* modified and recreated with BioRender.

The content and, consequently, the function of EVs vary depending on the parental cell and the stimulus that triggers their formation *(106,107)*. EVs are rich in proteins, including surface receptors, membrane and soluble proteins but also different lipids *(100)*. They also contain nucleic acids and different types of ribonucleic acid (RNA) including mRNA, microRNA and long noncoding RNA *(108)*. Various stimuli such as shear stress, cell injury, cytokines, ATP (Adenosintriphosphate) depletion and calcium influx, trigger EV formation *(109)*. EV synthesis is regulated by activation and inactivation of different enzymes, which are essential for membrane stability. The three major enzymes influencing the organisation of the phospholipid membrane are flippase, floppase and scramblase *(110)*. Under resting conditions, the membrane protein flippase translocates phospholipids from the exoplasmic to the cytoplasmic leaflet of membrane *(111)*. An increase in cytosolic calcium concentration inhibits flippase activity, while floppases and scramblases facilitate the transfer of phosphatidylserine from the inner leaflet to the outer leaflet of the lipid bilayer. This process results in the exposure of negatively

charged phospholipids on the cell surface, leading to the loss of membrane phospholipid asymmetry *(112,113)*. Proteases are another group of enzymes that play a crucial role in maintaining membrane stability. Among them, calpains are particularly notable; they are activated by calcium influx and facilitate the breakdown of the connections between the cytoskeleton and membrane proteins. These processes disturb the membrane stability, thus favouring EV formation *(110)*. Another proposed mechanism for EV formation involves the interactions between actin and myosin, which lead to the contraction of cytoskeletal structures *(114)*.

After EVs are secreted by the donor cell, their uptake by recipient cells depends on the presence of specific receptors or proteins on their surface and how these interact with corresponding molecules on the recipient cell *(103)*. Various proteins and protein-protein interactions are known to contribute to this complex process. Tetraspanins, lectins, proteoglycans, and integrins have been identified to play a role in the binding and internalization of EVs into recipient cells *(115)*. EV uptake can occur through several mechanisms including endocytosis, phagocytosis, membrane fusion and micropinocytosis *(116,117)*. One proposed endocytic uptake mechanism is clathrin-mediated endocytosis (CME), where clathrin-coated vesicles form and facilitate the uptake of EVs by disrupting the cell membrane *(116)*. Another mechanism is macropinocytosis, which involves actin-mediated membrane invaginations that envelop and internalize EVs *(118,116)*. Additionally, EVs are taken up by phagocytosis or through direct fusion with the recipient cell membrane, creating a pore for EV entry. This membrane fusion process involves proteins such as soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) and Sec1/Munc-18 *(118)*.

In recent years, EVs have become an important focus in the CVD research field. Various studies have shown that cardiovascular risk factors such as diabetes, hypertension, and hypercholesterolemia are associated with elevated levels of circulating endothelial and platelet-derived EVs *(119)*. Increased levels of EVs have also been observed in conditions like coronary artery disease and heart failure. *(119–121)*.

Red blood cell-derived extracellular vesicles

Studies have shown that red blood cell-derived extracellular vesicles (REVs) play a crucial role in various pathological conditions, including sickle cell anemia, thrombosis, CVD, diabetes, cancer, and inflammation *(109,122,123)*. RBCs are known to lose about 20% of their volume, including Hb and membrane components, through vesiculation over their 120-day lifespan *(84)*. This process is proposed as a RBC aging mechanism that removes harmful components, such as denatured Hb, the C5b-9 complement attack complex, band 3 neoantigens, and immunoglobulin G. This removal helps maintain RBC viability during their maturation *(124,125)*.

REVs contain various enzymes involved in redox homeostasis including glutathione S ransferase, thioredoxin, peroxiredoxin-1 and -2 *(123)*. REVs are further implicated in processes such as oxidative stress, inflammation, thrombosis, and foam cell formation. These processes can lead to vascular inflammation, increased endothelial damage and dysfunction, and the development of coronary heart disease *(109)*. In addition, REVs are also proposed to impact NO bioavailability and potentially play a role in vasoregulation due to their NO scavenging properties. It is suggested that REVs scavenge NO slower than extracellular Hb, but faster than Hb encapsulated within RBCs *(123)* thus modulating NO bioavailability. The role of REVs in anemia and anemia-associated pathologies remains unknown.

Overall aim and hypothesis

Clinical studies have shown that anemia is not only a co-morbidity or “bystander” but also an independent risk factor for AMI. The anemic patients with AMI show higher rates of morbidity and mortality. However, the exact mechanisms by which anemia influences complications and worsens outcomes in these patients remain largely unknown. Given that ED plays a central role in the development of various CVD and is associated with multiple cardiovascular risk factors, it also contributes to the progression of coronary artery disease (CAD).

In this thesis, we aim to investigate the effects of anemia on ED, with a particular emphasis on the role of RBCs. We hypothesize that:

1. Chronic anemia is associated with endothelial dysfunction due to increased oxidative stress in the endothelium **(Chapter 2).**
2. Anemia aggravates systemic endothelial dysfunction after acute myocardial infarction **(Chapter 3).**
3. Red blood cell-derived extracellular vesicles promote endothelial dysfunction in anemia **(Chapter 4)**.

In our recent study, we demonstrated that acute blood-loss anemia induces RBC dysfunction by reducing NO bioavailability, increasing ROS formation, and reducing membrane integrity. In addition, we showed a compensatory but transient increase in cardiac and vascular adaptations during acute anemia. However, in chronic anemia, it remains unclear whether the vascular complications are related to RBC dysfunction or also involve systemic alterations in endothelial function, leading to ED. The aim of our investigations in **Chapter 2** will primarily focus on the effects of chronic blood-loss anemia over 6 weeks on endothelial function, with particular emphasis on a newly established chronic blood-loss anemia mouse model. This chapter will specifically explore the potential role of RBCs in mediating ED and the underlying mechanisms.

Clinical studies have shown that anemia worsens the prognosis of AMI, but the underlying mechanisms remain unclear. ED is a common feature in nearly all CVDs and significantly affects patient outcomes. However, the impact of anemia on endothelial function after AMI has not yet been investigated. Therefore, in **Chapter 3** we aim to investigate the effect of both acute and chronic anemia in mediating ED after AMI, as well as the underlying signaling mechanisms involved. In this chapter, considering potential vascular heterogeneity, we will assess vascular function in the aorta, femoral artery and in saphenous arteries, which represents large, medium and small sized arteries. In accordance with the findings in **Chapter 2,** we will investigate the possible role of dysfunctional RBCs in mediating ED, as well as the contributions of ROS and inflammation. Additionally, we aim to investigate whether ROS could be a potential therapeutic target by using N-acetylcysteine (NAC), a ROS scavenger, to target the

ED in anemia post AMI. To achieve this, both acute and chronic blood-loss anemia mouse models will be studied, with and without induction of ischemia/reperfusion.

Based on our findings from **Chapter 2** and **3**, we aim to further investigate the potential mechanism by which dysfunctional RBCs effect the endothelial cells. One potential mechanism we hypothesise to play a significant role in ED is mediated through EVs released from RBCs. EVs have gained more attention in recent years and have already been shown to play a crucial role in different diseases including CVDs. RBC-derived EVs (REVs) have also been proposed to play a role in pathological conditions such as sickle cell anemia, inflammation and diabetes. Based on these studies and our findings, in **Chapter 4**, we aim to investigate the potential role of anemic REVs in mediating ED. We will characterize REVs in anemia using various methods and further explore their content by performing proteomics analysis of REVs and plasma-derived EVs (PLEVs) from both anemic and non-anemic stable coronary artery disease patients. Additionally, we plan to advance our investigations by conducting co-incubation studies of human EVs from anemic and non-anemic patients with HUVECs and murine aortas to examine potential EV-mediated changes in endothelial cell pathophysiology.

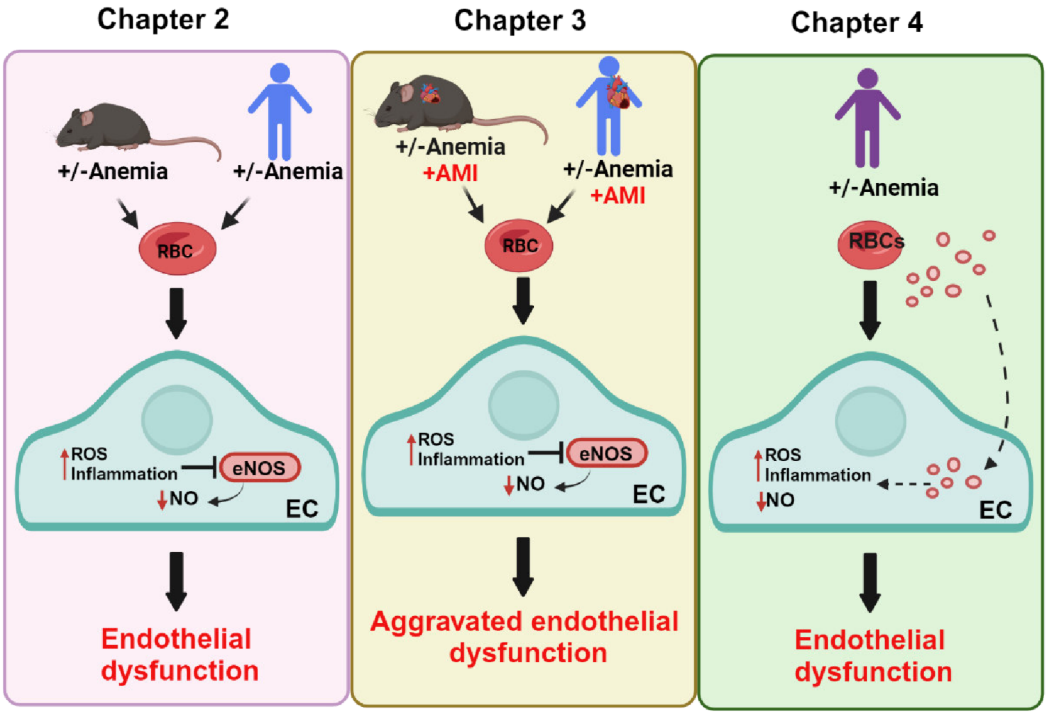

Figure 6: Graphical abstract of the three main scientific chapters of this thesis. In **Chapter 2** we hypothesize, that chronic anemia is associated with endothelial dysfunction (ED) due to increased oxidative stress in the endothelium. In **Chapter 3** we hypothesize that both acute and chronic anemia aggravate systemic ED after acute myocardial infarction (AMI). Finally, in **Chapter 4**, we investigate the potential role of red blood cell-derived extracellular vesicles (REVs), considering the hypothesis that REVs promote ED in anemia. Illustration created with BioRender.

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Chapter 2

Chronic anemia is associated with systemic endothelial dysfunction

Ramesh Chennupati1 *†, **Isabella Solga1†** , Patricia Wischmann1† , Paul Dahlmann , Feyza Gül Celik , Daniela Pacht , Aslıhan Şahin , Vithya Yogathasan , Mohammad Rabiul Hosen , Norbert Gerdes, Malte Kelm and Christian Jung*

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† Contributed equally
(Adapted version)

Abstract

Background

In acute myocardial infarction and heart failure, anemia is associated with adverse clinical outcomes. Endothelial dysfunction (ED) is characterized by attenuated nitric oxide (NO)-mediated relaxation responses which is poorly studied in chronic anemia (CA). We hypothesized that CA is associated with ED due to increased oxidative stress in the endothelium.

Methods

CA was induced by repeated blood withdrawal in male C57BL/6J mice. Flow-Mediated Dilation (FMD) responses were assessed in CA mice using ultrasound-guided femoral transient ischemia model. Tissue organ bath was used to assess vascular responsiveness of aortic rings from CA mice, and in aortic rings incubated with red blood cells (RBCs) from anemic patients. In the aortic rings from anemic mice, the role of arginases was assessed using either an arginase inhibitor (Nor-NOHA) or genetic ablation of arginase 1 in the endothelium. Inflammatory changes in plasma of CA mice were examined by ELISA. Expression of endothelial NO synthase (eNOS), inducible NO synthase (iNOS), myeloperoxidase (MPO), 3-Nitrotyrosine levels, and 4-Hydroxynonenal (4-HNE) were assessed either by Western blotting or immunohistochemistry. The role of reactive oxygen species (ROS) in ED was assessed in the anemic mice either supplemented with N-Acetyl cysteine (NAC) or by in vitro pharmacological inhibition of MPO.

Results

The FMD responses were diminished with a correlation to the duration of anemia. Aortic rings from CA mice showed reduced NO-dependent relaxation compared to non-anemic mice. RBCs from anemic patients attenuated NO-dependent relaxation responses in murine aortic rings compared to non-anemic controls. CA results in increased plasma VCAM-1, ICAM-1 levels, and an increased iNOS expression in aortic vascular smooth muscle cells. Arginases inhibition or arginase1 deletion did not improve ED in anemic mice. Increased expression of MPO and 4-HNE observed in endothelial cells of aortic sections from CA mice. NAC supplementation or inhibition of MPO improved relaxation responses in CA mice.

Conclusion

Chronic anemia is associated with progressive endothelial dysfunction evidenced by activation of the endothelium mediated by systemic inflammation, increased iNOS activity, and ROS production in the arterial wall. ROS scavenger (NAC) supplementation or MPO inhibition are potential therapeutic options to reverse the devastating endothelial dysfunction in chronic anemia.

Keywords: anemia, endothelial dysfunction, nitric oxide, reactive oxygen species, n-acetyl cysteine, myeloperoxidase

Introduction

Endothelial dysfunction (ED) is central to a plethora of cardiovascular diseases (CVD). ED is defined by the inability to maintain vascular homeostasis due to decreased nitric oxide (NO) production (1, 2). NO is a major vasodilator and anti-inflammatory signalling molecule, which plays an important role in vascular tone and blood pressure regulation. Decreased NO bioavailability disrupts the non-thrombogenic intimal surface, which promotes adhesion and aggregation of platelets and monocytes to the activated endothelial surface (3). The bioavailability of NO mainly depends on the availability of eNOS' substrate, arginine, and coupling state of eNOS. Arginases are catabolizing enzymes of arginine, which share the common substrate with eNOS, and are known to limit NO bioavailability (4, 5). Both isoforms of arginase (1 and 2) are expressed in endothelial cells of mice and humans (6). Endothelial arginase 1 is known to contribute to ED in coronary arteries of diabetic patients (7), and animal models of hypertension, ischemia/reperfusion, and aging (8, 9). In addition, endothelial arginase 2 upregulation and associated NO dysregulation have been reported in diabetes, aging (10), and hyperlipidemia (11). Inhibition of arginases led to increased NO-bioavailability and consequently improved the endothelium-dependent relaxation responses in different disease states (8).

The bioavailability of NO depends on the coupling state of eNOS, which relies on several factors such as oxidative depletion of tetrahydrobiopterin (BH4), S-glutathionylation, reactive oxygen, and nitrogen species (12). Concomitantly, uncoupled eNOS in the NO generation system leads to the production of superoxide anions (O2 −) rather than NO (13). Thus, superoxide anions are not only associated with reduced NO production but also enhance pre-existing oxidative stress. Additionally, peroxynitrite is known to cause depletion of cofactor BH4, which leads to uncoupling of eNOS and thus, reduced NO-bioavailability (14). Recent studies also showed that myeloperoxidase (MPO), which is upregulated in endothelial cells during inflammation, limits NO-bioavailability by producing reactive oxidants such as hypochlorous acid (15).

Anemia is frequently observed in patients with CVD such as acute and chronic coronary syndromes as well as heart failure. Approximately 30 to 40% of patients with acute myocardial infarction (AMI) have evident anemia upon admission (16), or during hospitalization (17). It is well-studied that anemia alone or in combination with other morbid conditions leads to poor prognosis in AMI (18). These studies have concluded that dysfunctional RBCs in anemia influ-

ence the severity of CVD. We recently showed that acute blood-loss anemia transiently increases FMD due to an acute increase in shear stress and cardiac output. Simultaneously acute anemia induces RBC dysfunction by reducing NO bioavailability, increasing ROS formation, reducing membrane integrity, and increasing NO scavenging by free plasma hemoglobin (19). However, it is unclear whether the complications related to chronic anemia are restricted to RBC dysfunction or also associated with systemic alteration in endothelial cells that may result in ED.

In this study, we evaluated the systemic ED in a newly established murine blood-loss chronic anemia model. Additionally, we also investigated whether the RBCs from anemic patients affect the endothelial function using co-incubation with aortic rings followed by endothelial function analysis. To our knowledge, this is the first study to evaluate systemic ED in a blood-loss anemia murine model.

Materials and methods

Animals

All procedures were approved and performed in accordance with the guidelines of LANUV (Landesamt für Natur, Umwelt- und Verbraucherschutz Nordrhein-Westfalen, Germany). Mice were treated by following the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123). The approved permits for the animal experiments are 84-02.04. 2020.A073 and 84-02.04.2018.A234. The C57Bl/6J (wild type, WT) mice were obtained from Janvier Labs (Saint-Berthevin Cedex, France). Mice with endothelial ablation of arginase 1 (ARG1) were generated by crossing $Arg1^{fl/fl}$ (C57BL/6-Arg1tm1Pmu/J, Jackson laboratory) mice with tamoxifen-inducible Cdh5-creERT2 [Tg(Cdh5-cre/ERT2)1Rha, MGI:3848982] mice which were kindly provided by Prof Dr E. Lammert (Heinrich-Heine-University of Düsseldorf). To induce the recombination, the mice were injected with 50 mg/Kg body weight tamoxifen (Sigma) for 5 consecutive days. The resulting mice that lack ARG1 in their endothelium $Arg1^{fl/fl}$/Cdh5-cre/ERT2-pos are named as EC-Arg1-KO in the manuscript. The respective Cre-negative littermates were used as controls. All mice were housed in standard cages (constant room temperature and humidity, 12hr light/dark cycles) and had free access to standard pelleted chow and tap water.

Anemia induction

To induce anemia, WT, EC-Arg1-KO and respective control mice were divided into two groups, a sham group without anemia and a group with induced chronic anemia. To induce chronic anemia, a repetitive mild blood withdrawal (by <20 g/L changes in Hb) from the facial vein was performed every third day for a time period of six weeks. At the end of the 6th week mice which showed hemoglobin levels < 10 g/L, are considered for experiments. The hematological parameters after anemia induction are represented in Supplementary Figure S1.

Supplementation of NAC: Male (WT) mice, which were 10–11 week-old, were induced with chronic anemia. After two weeks of anemia induction, anemic and respective sham mice were given 1% (w/v) NAC (Sigma) through drinking water for 4 weeks. After 6 weeks of anemia, mice were sacrificed and tissues were collected.

Flow-mediated dilatation assessment

The 10–11 week-old C57BL/6J male mice were used for the assessment of flow-mediated dilation (FMD) responses. The measurements were performed before, and after 2-, 4-, 6, -weeks of anemia induction. FMD responses were assessed using the Vevo 2,100 high-resolution ultrasound scanner using a 30–70 MHz linear transducer (Visual Sonics Inc., Toronto, Canada) as described previously (20). Mice were kept under 1.5%–2% isoflurane anesthesia, the transducer was placed using a stereotactic holder and adjusted manually to visualise the external iliac artery. A vascular occluder (8 mm diameter, Harvard Apparatus, Boston, MA, USA) was placed around the lower limb (20). Baseline images of the vessel were first recorded, the cuff was inflated to 200 mmHg, and pressure was kept constant for 5 min (Druckkalibriergerät KAL 84, Halstrup Walcher, Kirchzarten, Germany); then the cuff was released to assess FMD. The upstream diameter of the vessel was determined every 30 s both during inflation and deflation of the cuff. Changes in vessel diameter were quantified as the percentage of baseline (%) = [diameter (max)/diameter (baseline)] × 100.

In vitro studies with isolated aortic rings

Solutions and drugs

Krebs-Ringer bicarbonate-buffered salt solution (KRB) contained (in mM): 118.5 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3 and 5.5 glucose. The KRB solution was continuously aerated with 95% O2/5% CO2 and maintained at 37°C. Indomethacin (INDO;

Sigma Aldrich) was dissolved in ethanol. Acetylcholine (ACH), phenylephrine (PHE), NG-nitro-arginine methyl ester (L-NAME) and sodium nitroprusside (SNP; all Sigma Aldrich) were dissolved in KRB solution. Myeloperoxidase inhibitor (AZD5904) was purchased from PromoCell and dissolved in dimethyl sulfoxide (DMSO).

Organ chamber experiments

Animals were euthanized under deep isoflurane anesthesia (4.5%). The thoracic aorta was dissected free from perivascular adipose tissue, and 2 mm size aortic rings were mounted in an organ bath equipped with force transducers (Hugo Sachs Electronic, HARVARD apparatus GmbH, March-Hugstetten Germany) or wire-myograph system (Danish Myotechnology, Aarhus, Denmark). Arterial segments were distended stepwise to 1 gr force or 9.8 mN. All aortic segments were allowed 40 min incubation prior to experiments and stimulated with depolarizing 60 mM KCl as described previously (21). Prior to experiment, endothelial integrity was checked by assessing relaxation responses to ACH (10 μM) in PHE (10 μM) pre-contracted arteries. The aortic segments were pre-contracted and segments which showed less than 80% relaxation responses were excluded from experiments.

Contributions of NO, cyclo-oxygenase products to endothelium-dependent relaxation Initially, a concentration-response curve (CRC) for PHE (0.001–10 μM) was constructed. During the contraction induced by 10 μM PHE, an ACH CRC (1 nM–10 μM) was generated. Next, experiments were repeated in the presence of the cyclooxygenase inhibitor INDO (10 μM) and in the presence of both INDO and the NOS inhibitor L-NAME (100 μM) to assess the contribution of NO to arterial relaxation.

Sensitivity of vascular smooth muscle to NO During contraction of the aortic rings with PHE (10 μM) in the presence of INDO (10 μM) and L-NAME (100 μM), the relaxing effects of the NO donor SNP (10 nM–10 μM) were recorded.

Co-incubation studies with human RBCs The protocol is based on previous studies (22) with adjustments for murine aortic rings. Briefly, thoracic aortas from 10 to 11 week-old mice were dissected and placed in cold KRB buffer. Anemic and non-anemic patient blood was collected in heparin-coated tubes, and 40% hematocrit (Hct) was prepared with KRB buffer. Aortic segments were transferred to the 40% Hct containing KRB buffer and placed in an incubator for 6 h at 37°C. We previously (unpublished data) assessed the best suitable time for co-incubation experiments based on the viability and maximum responses to agonists (PHE, ACH) of aortic rings. After 6 h of incubation, aortic segments were mounted in the wire-myograph system and endothelial-dependent relaxation responses were assessed. Approval numbers for patient

samples collection are 5481R, 2018–14, and 2018–47 issued by clinical ethics committee, Universitätsklinikum Düsseldorf, Germany. All patients were given their written consent according to Helsinki ethical principles.

Western blot

The thoracic aortas were thawed and homogenized using a mixer mill (MM400 Retsch Haan, Germany) and a brief sonification in a radioimmunoprecipitation assay (RIPA) buffer. For further processing, the protein concentration was determined with the DC™ Protein Assay Kit (Bio-Rad, Feldkirchen, Germany). The samples were denatured and loaded on a 4%–12% Bis-Tris gradient gel QPAGE™ (Smobio, Hsinchu City, Taiwan). Proteins were then transferred onto nitrocellulose membranes and a total protein staining was performed by using Revert Total Protein Stains (LI-COR Biosciences GmbH, Bad Homburg, Germany). The unspecific sites were blocked using intercept TBS Protein-Free Blocking Buffer (LI-COR) for 60 min at RT. Next, the membrane was incubated with either iNOS polyclonal antibody (1:1000 in blocking buffer; ThermoFisher Scientific, Schwerte, Germany) or 3-Nitrotyrosine monoclonal antibody (1:1000 in blocking buffer; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) for overnight at 4°C. Afterwards, the membranes were incubated with corresponding secondary antibodies (IRDye® 700/800CW secondary antibodies; LI-COR) for 1 h at room temperature. The membranes were visualized with the Odyssey® Fc Imaging System (LI-COR).

Immunohistochemistry

Thoracic aortas from anemic and respective sham mice were collected, fixed in formaldehyde (4%) for 2 h, and treated with sucrose (30%) overnight. Next, the aortic segments were embedded in Tissue-Tek O.C.T and frozen until further use. Tissue sections (4 µm) were incubated overnight at 4°C with rat anti-mouse CD31 [1:100 in blocking solution (2.5% BSA); BD Biosciences, Heidelberg, Germany] and either with mouse anti-eNOS/NOS Type III (1:100 in 2.5% BSA; BD Biosciences), mouse anti-iNOS (1:100 in 2,5% BSA; Abcam), rabbit anti-4HNE (1:200 in 2,5% BSA; Abcam) or rabbit anti-myeloperoxidase (1:200 in 2,5% BSA; antibody.com) antibodies. Followed by incubating with respective anti-Mouse Alexa Fluor 555 (1:1,000 in 2.5% BSA; Thermo Fisher), goat anti-rat Alexa Fluor 488 (1:1000 in 2.5% BSA; ThermoFisher) or anti-Rabbit Alexa Fluor 555 (1:1000 in 2.5% BSA; ThermoFisher) secondary antibodies. Additionally, autofluorescence was quenched using Vector® TrueVIEW® Autofluorescence Quenching Kit (BIOZOL Vectorlabs, Eching, Germany). The stained tissue sections were mounted with the ProLong™ Diamond Antifade Mountant mounting medium (ThermoFisher) and imaged with a Leica DM6 M microscope.

ELISA

The blood was collected from different experimental groups, plasma was prepared and snap-frozen. Plasma samples were stored at −80°C until further use. Mouse ICAM-1/CD54, VCAM-1/CD106, ELISA kits were purchased from R&D Systems (Bio-Techne GmbH, Wiesbaden Germany). Additionally, mouse haptoglobin, free hemoglobin, and transferrin plasma levels were assessed by using ELISA kits from Abcam. Mouse soluble transferrin receptor, ferritin and erythropoietin ELISA kits were obtained from MyBioSource (BIOZOL, Eching, Germany). All assays were performed according to the manufacturer's instructions. The plasma inflammatory markers were analyzed using a multiplex system (Bioplex, BioLegend).

qRT-PCR

After 6 weeks of anemia induction, the thoracic aorta was isolated from anemic and respective sham mice, and snap-frozen until further use. The aortas were thawed and homogenized in a lysis buffer using a mixer mill (MM400 Retsch). RNA was isolated from tissue lysates using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Reverse transcription was performed following the manufacturer's instructions using Superscript™ IV VILO™ Master Mix (Invitrogen). The real-time PCR was performed using the QuantStudio-7 Flex (ThermoFisher). The TaqMan probes (ThermoFisher) used for real-time PCR were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm03302249_g1), MPO (Mm00447886_m1) and iNOS (Mm00440502_m1,). Relative expression levels were obtained by normalization with GAPDH.

Statistical analysis

All concentration response curves (CRCs) for contractile stimuli are expressed as absolute values. Relaxing responses were expressed as a percentage reduction of the level of contraction. Individual CRCs were fitted to a non-linear sigmoid regression curve (Graphpad Prism 8.0). Data normality was checked with D'Agostino Person test. The normally distributed data for multiple groups were analysed with two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test and significance was calculated for all the concentration–response curves. Comparisons between two groups were performed with unpaired t-test or Mann–Whitney test depending on the normality. Sensitivity (pEC50), and maximal effect (Emax) are shown as means ± SEM.

Results

Chronic anemia is associated with progressive endothelial dysfunction

To assess the effect of CA on endothelial function in vivo, we determined flow-mediated dilation (FMD) responses before induction of anemia (basal) and after 2-, 4-, 6- weeks of anemia. The average FMD responses significantly abrogated after 4- and 6-weeks (6 ± 1% and 5 ± 2%, respectively) of anemia induction compared to before induction of anemia (13 ± 2%) (Figures 1A, B) indicating the progressive ED in CA. Furthermore, we assessed the endothelium-dependent and independent relaxation responses in the isolated aortic rings using an organ bath. The CA mice showed a reduced relaxation (Emax 68 ± 4%) response to acetylcholine (ACH) compared to sham mice (Emax 84 ± 4%) (Figure 2A; Table 1). Furthermore, in the presence of NOS inhibitor (L-NAME, 100 µM), relaxation responses were completely inhibited in both anemic and sham groups (Figure 2B; Table 1), which shows that the relaxation responses are entirely mediated by NO. In addition, the relaxation responses to exogenous NO donor [Sodium nitroprusside (SNP)] are similar in CA mice compared to the sham group suggesting that smooth muscle sensitivity to NO is unaltered in CA mice compared to sham mice (Figure 2C; Table 1). Taken together, CA mice showed reduced endothelium-dependent relaxation responses. These results demonstrate the altered endothelial function in CA.

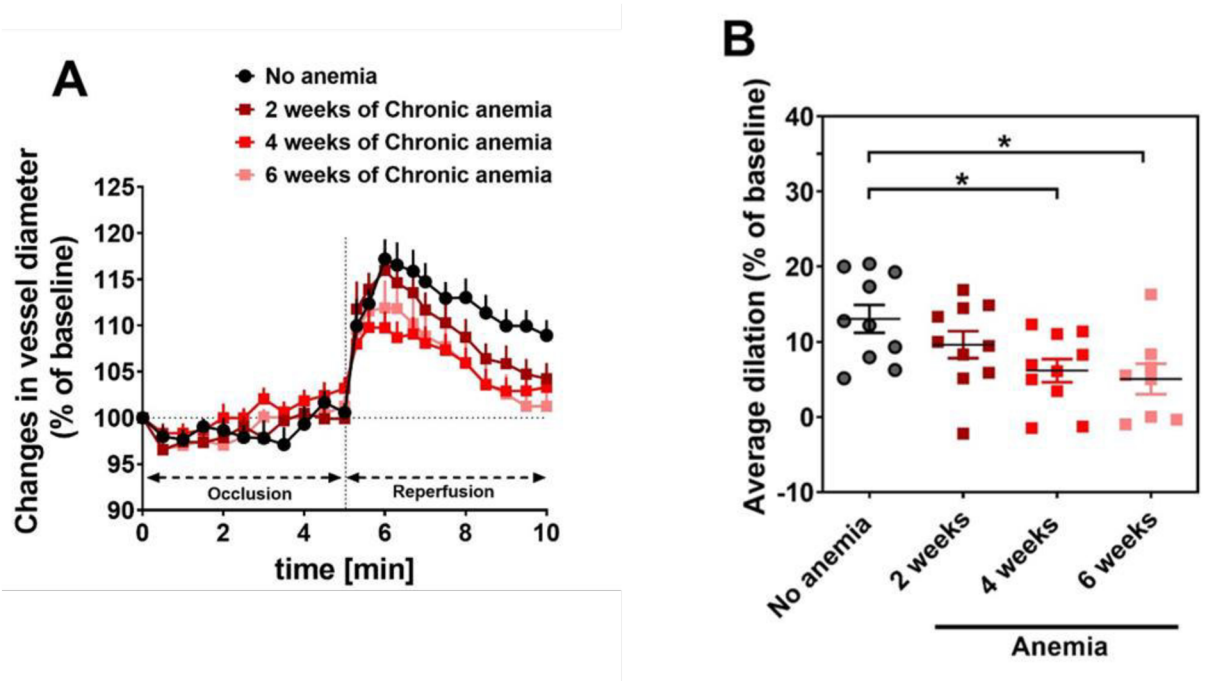

Figure 1. Chronic anemia is associated with altered flow-mediated dilation (FMD) responses. (A) Changes in vessel diameter (% ratio of baseline diameter) before (black circles), 2 (dark red squares), 4 (red squares), and 6 weeks (light red squares) after anemia induction. The occlusion and releasing phase of the cuff (reperfusion) are indicated in the panel. **(B)** Average FMD in the reperfusion phase (6–10 min) was normalized to baseline. Values are shown as the means ± SEM (n = 10 per group).*, $p \leq 0.05$. One-Way ANOVA with Sidak's post-hoc test was used to compare average FMD between baseline and respective week.

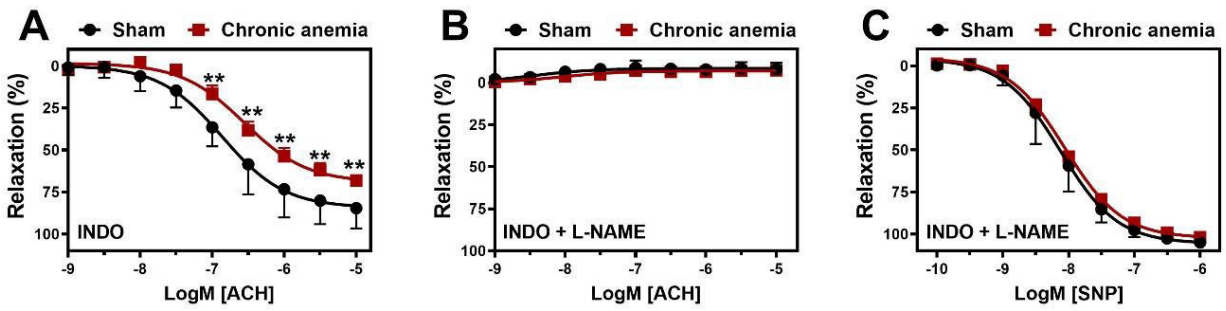

Figure 2. **Chronic anemia is associated with reduced endothelial-dependent relaxation responses.** Aortic rings were isolated from sham (black circles) and chronic anemic (dark red squares) mice. Aortic segments were pre-contracted with phenylephrine (10 µM) and relaxation responses to acetylcholine (ACH, 1 nM–10 µM) were measured using organ bath**. (A)** relaxation in the presence of indomethacin (10 µM, COX inhibitor). **(B)** relaxation in the presence of indomethacin and L-NAME (100 µM, NOS inhibitor). **(C)** Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 µM) in the presence of indomethacin and L-NAME. All values are mean values ± SEM (n = 8–10 per group). **, $p \leq 0.01$. CRCs were analysed by Two-Way ANOVA and Bonferroni 's post-hoc test to compare sham and chronic anemic groups.

Arginases do not contribute to the altered NO release in chronic anemia

Arginase 1 activity is known to be enhanced in various pathological states and is also known to limit NO bioavailability (4, 23). We assessed the relaxation responses after arginase inhibition in anemic mice. The aortic segments from anemic mice treated with Nor-NOHA (non-specific inhibitor of Arginase 1 & 2) did not show any improvement in the relaxation responses (Emax 53 ± 3%) compared to untreated (Emax 68 ± 5%) aortic segments (Figure 3A; Table 1). In addition, endothelial-dependent relaxation responses are not improved in anemic EC-Arg1-KO (Emax 74 ± 6%) and respective anemic control mice (Emax 77 ± 4%) compared to the sham mice (Emax 91 ± 5%) (Figure 3B).

Table 1. Effect of anemia on endothelial-dependent and -independent relaxation responses

	pEC50	E_{max} (%)	n
ACH-induced relaxation			
INDO			
Sham	6.86 ± 0.08	84 ± 4	10
Anemia	6.55 ± 0.09†	68 ± 4††	8
SNP-induced relaxation			
Control	8.10 ± 0.04	104 ± 1	10
Anemia	8.00 ± 0.03	102 ± 1	8
Arginase inhibition			
ACH-induced relaxation			
INDO			
Sham	6.86 ± 0.08	85 ± 3	10
Anemia	6.59 ± 0.08	68 ± 5	6
Anemia+Nor-NOHA	6.51 ± 0.07†	53 ± 3††	6
Arginase deletion			
ACH-induced relaxation			
INDO			
Control+Anemia	6.91 ± 0.11	77 ± 4	5
EC-Arg1-KO +Anemia	6.72 ± 0.11	74 ± 7	5
SNP-induced relaxtion			
Control + Anemia	7.76 ± 0.03	107 ± 2	5
EC-Arg1-KO +Anemia	7.68 ± 0.04	109 ± 3	5
NAC Supplementation			
ACH-induced relaxation			
INDO			
Sham + NAC	6.55 ± 0.09	68 ± 4	8
Anemia + NAC	6.76 ± 0.06	81 ± 3†	7
SNP-induced relaxation			
Control + NAC	8.06 ± 0.03	102 ± 1	8
Anemia + NAC	7.94 ± 0.04	104 ± 2	7
MPO inhibition			
ACH-induced relaxation			
INDO			
Anemia	6.65 ± 0.11	58 ± 4	6
Anemia	7.04 ± 0.13	79 ± 6	7
SNP-induced relaxation			
Anemia	7.57 ± 0.09	100 ± 2	6
Anemia	7.54 ± 0.03	101 ± 3	7

Table 1. The maximal relaxation response (E_{max}) $_{are}$ expressed as % reduction of the maximal contractile response to 10 µM PHE. In all experiments, aortic rings did not show any relaxation in the presence of L-NAME so the data is excluded from table. ACH: acetyl choline; INDO: indomethacin; SNP: sodium nitroprusside; MPO: myeloperoxidase; NAC: N-acetyl cysteine. All values are shown as mean ± SEM. †: P<0.05 compared to arteries of sham mice under the same condition. n/a. not applicable. ††: P<0.01 compared to arteries of sham mice under the same condition. n/a. not applicable.

These relaxation responses are mediated by NO (Figure 3C; Table 1). The endothelium-independent relaxation to SNP was comparable in both groups (Figure 3D; Table 1). Taken together, these results exclude the potential role of arginases in ED associated with anemia.

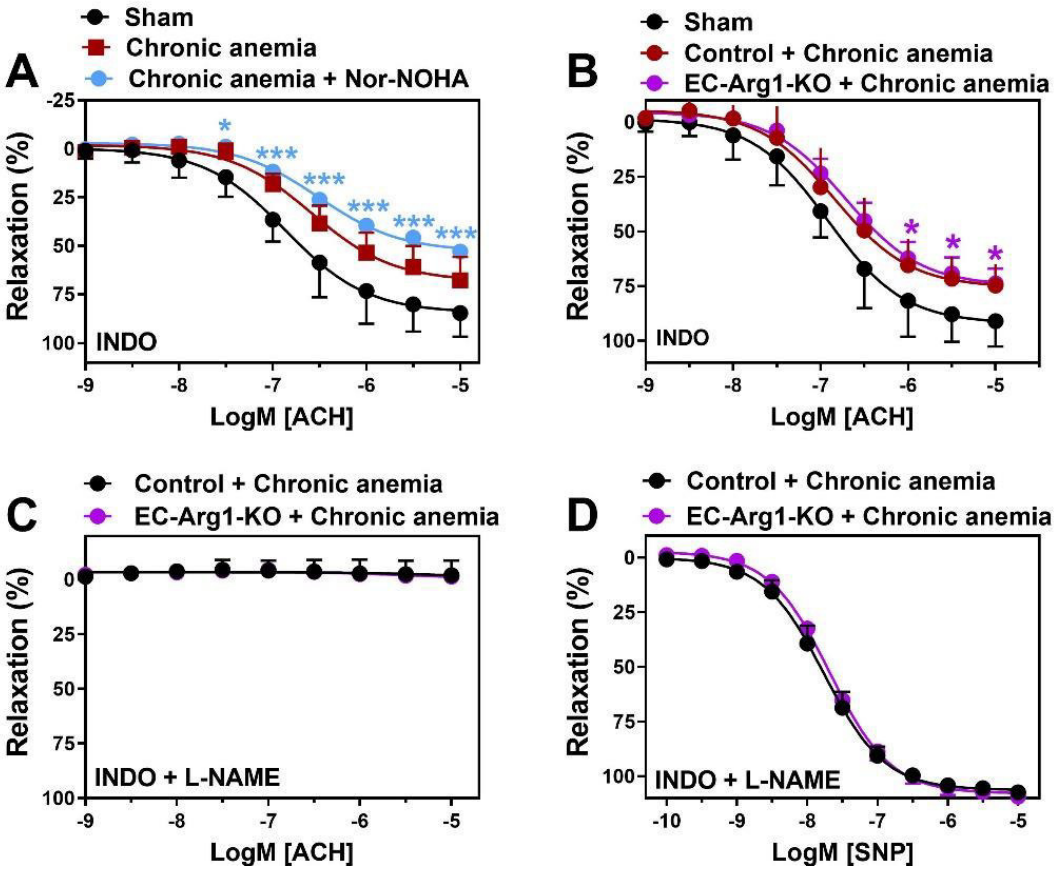

Figure 3. Inhibition of arginases or arginase 1 ablation does not improve endothelium- dependent relaxation responses in anemic mice. (A) Aortic rings were isolated from sham (black circles) and chronic anemic mice and incubated in the presence (blue circles) or absence (red squares) of Nor-NOHA (arginase inhibitor, 1 μM). Relaxation responses in the presence of indomethacin (10 μM, COX inhibitor) were measured in phenylephrine (10 μM) pre-contracted arteries. **(B–D)** Aortic rings were isolated from chronic anemic control (dark red circles) and EC-Arg1-KO mice (purple circles). All aortic segments were pre-contracted with phenylephrine (10 μM) and relaxation responses to acetylcholine (1 nM–10 μM) were measured using organ bath. (B) Relaxation in the presence of indomethacin (10 μM). **(C)** Relaxation in the presence of indomethacin and L-NAME (100 μM, NOS inhibitor). (D) Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 μM) in the presence of indomethacin and L-NAME. All values are mean values ± SEM (n = 6–10 per group). *, $p \leq 0.05$; ***, $p \leq 0.001$. CRCs were analysed by Two-Way ANOVA and Bonferroni's post-hoc test to compare anemic Nor-NOHA treated or anemic EC-Arg1-KO with sham mice.

Chronic anemia is associated with enhanced iNOS expression in vascular smooth muscle cells

To evaluate whether the reduced relaxation responses in CA are also associated with decreased eNOS abundance, we analyzed the protein expression of eNOS in aortic lysates from anemic and respective sham mice. Interestingly, the eNOS levels are similar in CA mice and sham mice (Figure 4A, Supplementary Figure S5). In addition, immunohistochemical analyses also demonstrated similar patterns of eNOS expression in CA and sham mice (Figure 4B). We further assessed the levels of iNOS, a signature molecule for vascular inflammation. The aortic lysates from CA mice showed enhanced iNOS expression compared to sham mice (Figure 4C, Supplementary Figure S5). The immunohistochemistry analysis showed that iNOS expression is increased in vascular smooth muscle cells of CA mice compared to sham mice (Figure 4D).

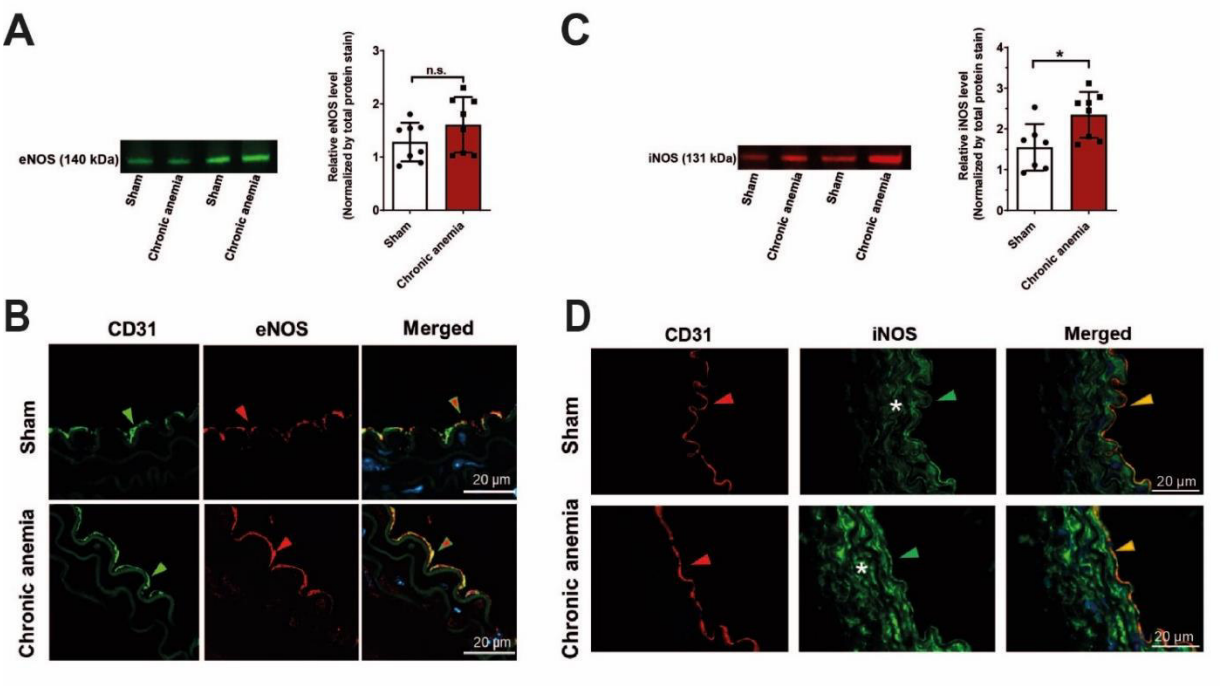

Figure 4. **Expression of iNOS is increased in chronic anemic mice**. (**A, C**) Representative Western blot images showing protein expression levels and quantification of eNOS (**A**) and iNOS (**C**) in the thoracic aortas isolated from sham and chronic anemic mice. Values are shown as means ± SEM (n = 7–8 per group). *, $p \leq 0.05$. Unpaired t-test (**A**) or Mann–Whitney test (**C**) was used to compare between two groups. (**B, D**) Representative immunohistochemistry images showing the expression of CD31, eNOS, or iNOS. DAPI (blue) staining is used to detect the nucleus. Arrowheads are in the luminal side pointing towards the respective staining. Asterisk represents the smooth muscle area. Results are representative pictures of 3 (sham) and 3 (chronic anemic) independent experiments.

Chronic anemia is associated with enhanced inflammation and oxidative stress

To investigate whether CA is associated with enhanced endothelial activation, we assessed plasma ICAM-1 and VCAM-1 level. The results showed that both ICAM-1 and VCAM-1 levels were increased in CA mice compared to sham mice (Figure 5A). Strikingly, we also observed increased plasma ICAM-1 and VCAM-1 levels in chronic coronary syndrome (CCS) patients with anemia compared to non-anemic patients (Figure 5B). Additionally, assessment of the systemic inflammatory markers demonstrated that IL6 and IL17A were progressively increased with duration of anemia (Supplementary Figure S2) demonstrating systemic inflammation in CA mice. To assess whether anemia leads to increased ROS generation in the aorta, we quantified generation of ROS-derived tyrosine adducts in the aortic lysates from anemic mice and control mice. Indeed, CA mice showed an enhanced 3-Nitrotyrosine levels in the aorta (Figure 5C). These results demonstrate that anemia results in the activation of endothelium and increased ROS production. Immunohistochemistry assessment of 4-hydroxynonenal (product of lipid peroxidation) in the aorta revealed an enhanced ROS activity in the CA mice (Figure 5D).

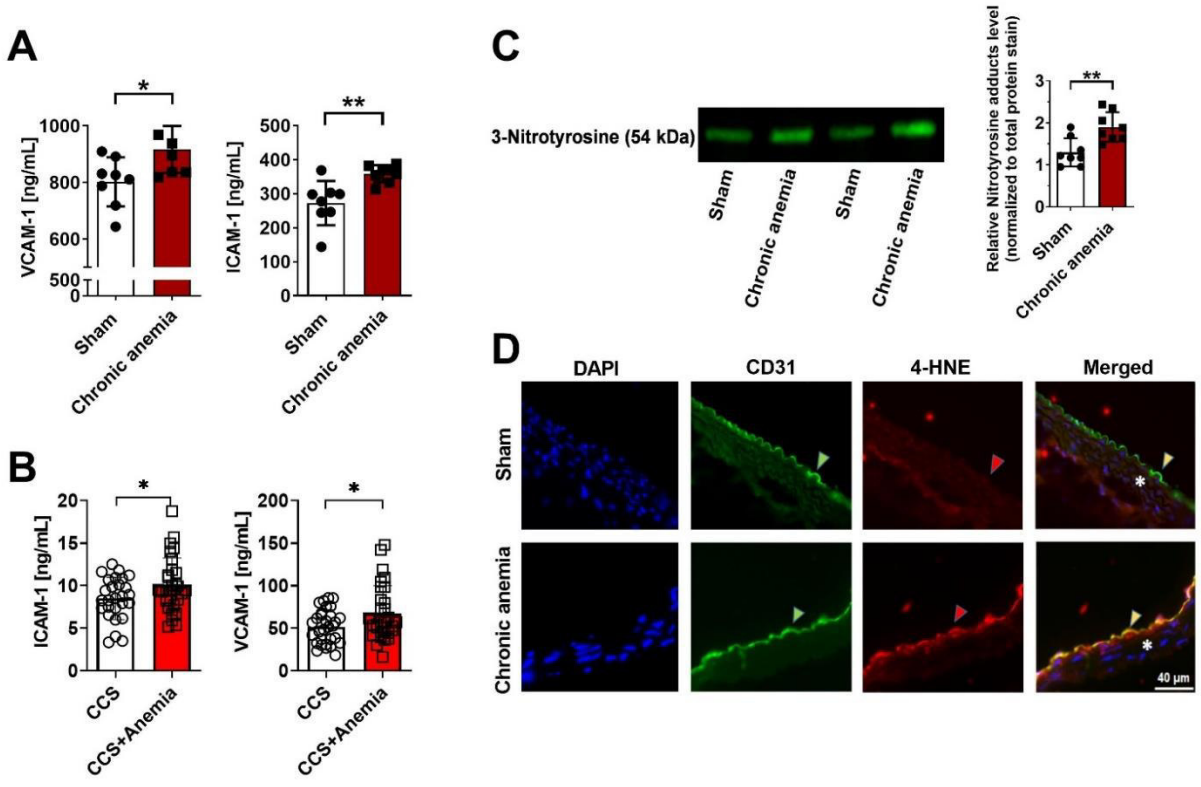

Figure 5. **Chronic anemia is associated with increased inflammation and ROS formation**. (**A**) Plasma VCAM-1 and ICAM-1 levels in sham (white bars), and chronic (dark red bar) anemic mice. (**B**) Plasma VCAM-1 and ICAM-1 levels in CCS patients without anemia (white bars) and with anemia (red bar). (**C**) Expression of nitrotyrosine protein adducts in the aortic lysates from sham (white bar), and anemic mice (red bar). Values are shown as means ± SEM. *, $p \leq 0.05$; **, $p \leq 0.01$. Unpaired *t*-test was used to compare between two groups. (**D**) Representative immunohistochemistry images of aortic sections stained with endothelial cell marker CD-31 (anti-CD31), and also for 4-HNE (anti-4-HNE). DAPI (blue) staining is used to detect the nucleus. Arrowheads are in the luminal side

pointing towards the respective staining. Asterisk represents the smooth muscle area. Results are representative pictures of 5 (sham) and 5 (chronic anemic) independent experiments.

ROS scavenging or MPO inhibition improves the endothelial dysfunction in chronic anemia

We assessed whether the ROS scavenger (NAC) improves the endothelial function and thus ED in CA mice. Aortic rings isolated from CA mice which were supplemented with NAC showed significantly improved endothelium-dependent relaxation responses (E_{max} 81 ± 3%) compared to untreated CA mice (E_{max} 68 ± 4%) (Figure 6A; Table 1). These relaxation responses are entirely attributed to NO (Figure 6B). The endothelium-independent relaxation to SNP was preserved in both groups (Figure 6C). A recent study showed that MPO expression is enhanced in ED as a consequence of vascular inflammation (15). Interestingly, the aortic lysates from CA mice also showed an increased expression of MPO compared to sham mice (Figure 6D, Supplementary Figure S5). MPO expression was also enhanced in the aortic endothelium of chronic anemic mice (Supplementary Figure S3). In addition, aortic rings treated with MPO inhibitor (AZD5904, 10 µM) showed significantly improved the relaxation responses in CA mice (Figures 6E–G; Table 1). These results conclude the potential role of ROS in anemia-associated ED.

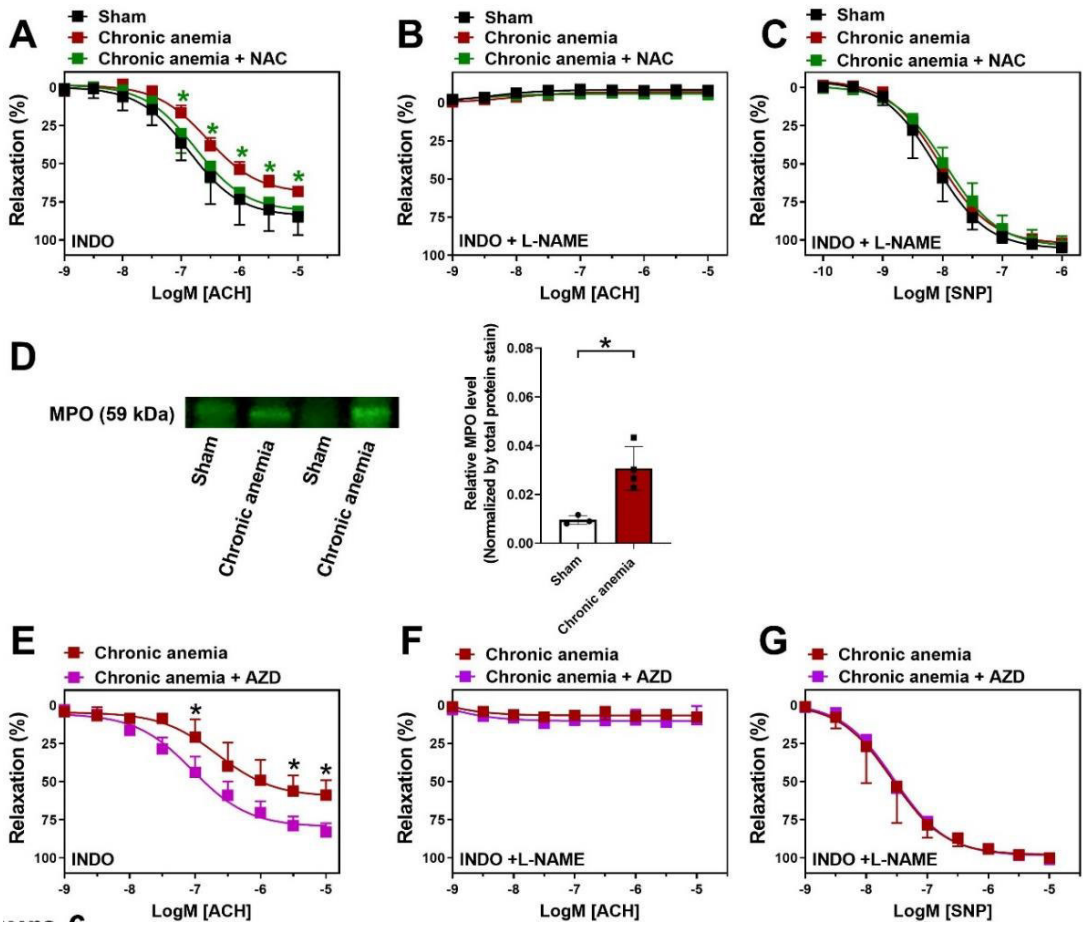

Figure 6. N-Acetyl cysteine (NAC) treatment or MPO inhibition improves the endothelium-dependent relaxation responses in chronic anemic mice. Aortic rings were isolated from sham (black circles), and chronic anemic mice with (green squares) and without (dark red squares) NAC supplementation. Aortic segments were pre-contracted with phenylephrine (10 μM) and relaxation responses to acetylcholine (1 nM–10 μM) were measured using an organ bath. (**A**) Relaxation in the presence of indomethacin (10 μM, COX inhibitor). (**B**) Relaxation in the presence of indomethacin and L-NAME (100 μM, NOS inhibitor). (**C**) Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 μM) in the presence of indomethacin and L-NAME. (**D**) Protein level expression of MPO in the aortic lysates isolated from sham (white bar), and chronic anemic mice (red bar). In a different experimental setup (**E–G**), aortic rings from chronic anemic mice were incubated in the presence (pink squares) or absence (dark red squares) of myeloperoxidase inhibitor (AZD5904, 10 μM) and the relaxation responses were assessed to acetylcholine (**E, F**) and SNP. (**G**) All values are mean values ± SEM (n = 7–8 per group). *, $p \leq 0.05$. CRCs were analysed by Two-Way ANOVA and Bonferroni's post-hoc test to compare treated (NAC or AZD) anemic group of mice with non-treated anemic mice. To compare MPO expression (**D**) Mann–Whitney test was used.

RBCs from anemic patients induce endothelial dysfunction

We further evaluated whether the dysfunctional RBCs impair the endothelial-dependent relaxation responses in anemia. Aortic rings from WT mice were incubated with RBCs (Hct 40%) isolated from CCS patients with and without anemia for 6 h at 37°C. The respective patients' characteristics are summarized in Supplementary Table S1. In a separate set of experiments, aortic rings from WT mice were incubated with RBCs (Hct 40%) from anemic mice and sham mice. Evaluation of vascular function using wire-myograph demonstrated that aortic segments incubated with RBCs from anemic CCS patients show a reduced ACH-induced relaxation response (Emax 65 ± 5%; Figure 7A) compared to aortic segments incubated with RBCs of non-anemic CCS patients (Emax 82 ± 5%; Figure 7A). Inhibition of NOS by L-NAME demonstrated that these relaxation responses are mediated by NO (Figure 7B). Endothelium-independent relaxation responses to SNP were similar in both groups (Figure 7C). We also observed altered endothelium-dependent and independent relaxation responses in the aortic rings incubated with anemic mice aortic RBCs (Supplementary Figure S4). In addition, elevated expression of 4-HNE was observed in the aortic rings incubated with RBCs from anemic patients compared to aortic rings incubated with non-anemic patients (Figure 7D). Taken altogether, these results demonstrate that RBCs from anemic patients induce ROS production in the endothelium resulting in endothelial dysfunction due to altered NO-mediated relaxation responses.

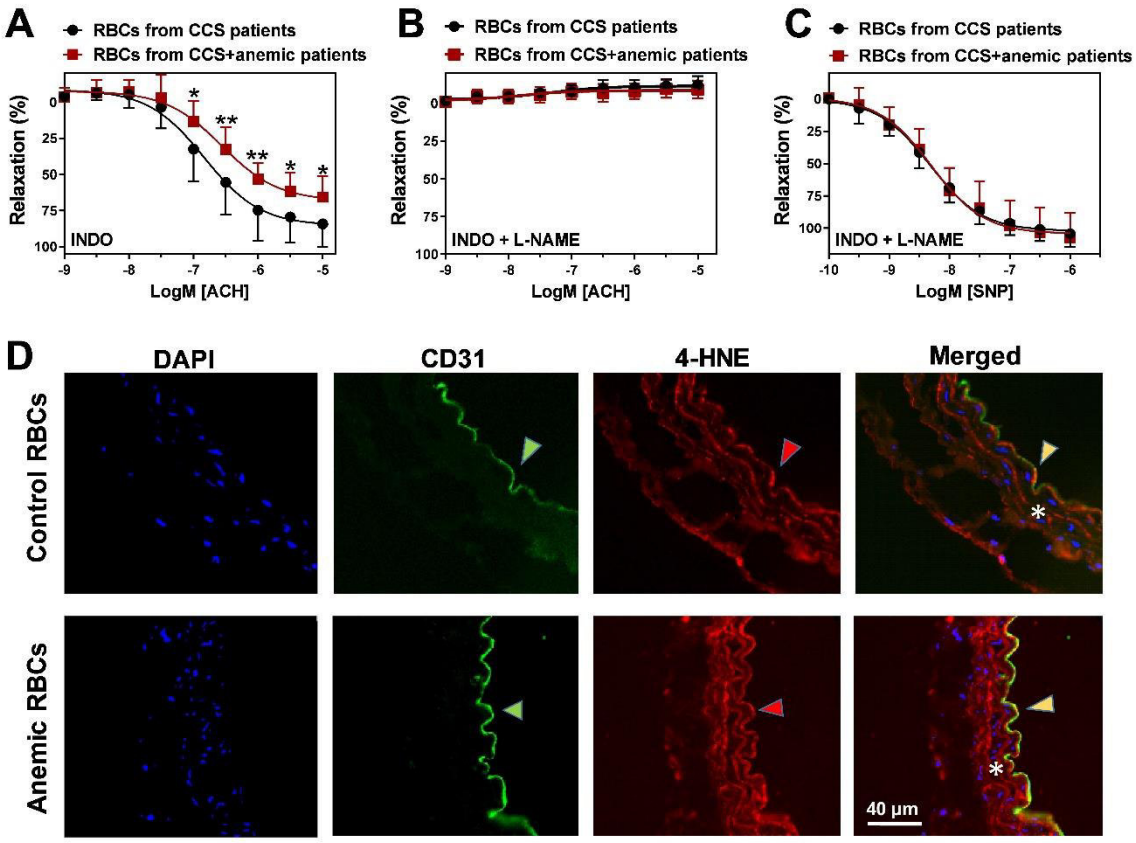

Figure 7. **RBCs from anemic patients induce endothelial dysfunction in murine aortic rings.** RBCs were isolated from chronic coronary syndrome (CCS) patients with anemia (red squares) and without anemia (black circles). Haematocrit (40%) was prepared in a KRB buffer. Aortic rings were incubated with haematocrit for 6 h at 37°C, mounted in a wire-myograph and pre-contracted with phenylephrine (10 µM). (**A**) Relaxation responses to acetylcholine (1 nM–10 µM) in the presence of indomethacin (10 µM, COX inhibitor). (**B**) Relaxation responses to acetylcholine in the presence of L-NAME (100 µM, NOS inhibitor) and indomethacin. (**C**) Endothelium-independent relaxation responses to (SNP, 10 nM-10 µM, NO donor). Values are shown as means ± SEM. *, $p \leq 0.05$; **, $p \leq 0.01$. CRCs were analysed by Two-Way ANOVA and Bonferroni's post-hoc test. Control group (stable CCS), $n = 10$; anemic group (CCS + Anemia), $n = 12$. (**D**) Thoracic aortas isolated from WT mice and incubated for 6 h at 37°C with RBCs from anemic or control patients. Representative immunohistochemistry images of aortic sections stained with endothelial cell marker CD-31 (anti-CD31), and 4-HNE (anti- 4-HNE). DAPI (blue) staining is used to detect the nucleus. Arrowheads are in the luminal side pointing towards the respective staining. Asterisk represents the smooth muscle area. Results are representative pictures of 5 (sham) and 5 (chronic anemic) independent experiments.

Discussion

In this study, we evaluated the effects of chronic anemia on ED in an established blood loss anemia mouse model with a hypothesis that CA alters endothelial function and lead to ED. The major findings of the study are that (1) anemic mice show impaired in vivo FMD responses, and abrogated endothelial NO-dependent relaxation responses in vitro, (2) the abrogated endothelial-dependent relaxation responses in CA are not attributed to enhanced arginase activity, (3) eNOS expression is preserved whereas enhanced iNOS expression is observed in vascular smooth muscle cells of CA mice, (4) CA is associated with increased endothelial activation and ROS production, (5) scavenging of ROS improved the endothelial function in CA mice, (6) RBCs from anemic patients induce ED in murine aortic rings.

We used a blood-loss CA mouse model to assess ED. In this model, we draw blood < 15% of the circulating volume/day on every third day for six weeks. We replaced the blood loss volume with 350 µl NaCl (0.9%) or saline to avoid any volume changes. This resulted in moderate anemia with increased plasma erythropoietin and soluble transferrin receptor levels. We previously demonstrated that arterial lactate concentration and the distribution of Evans blue dye remained within the normal range in this model indicating no signs of hemorrhage or rheological volume changes (19).

Decreased NO-dependent relaxation responses are a hallmark of ED (24, 25). Severe anemia in hemoglobinopathies such as sickle cell disease or β-thalassemia is associated with ED in both humans (26) and in respective mouse models (27, 28). Our moderate blood loss CA mouse model showed decreased NO-dependent relaxation responses similar to mouse models of hemoglobinopathies. However, the ED in hemoglobinopathies is majorly attributed to increased inflammation and hemolysis, which are mildly elevated in our CA model (Supplementary Figure S2). To prove that RBCs from anemic patients induce ED, we performed co-incubation experiments by incubating RBCs from anemic patients with mouse thoracic aortic rings. We used an adapted incubation protocol as described previously (22). In line with our hypothesis, the aortic rings incubated with RBCs of anemic patients showed reduced NO-dependent relaxation responses, which indicate that RBCs induce ED. The exact signalling mechanism of how anemic RBCs induce ED is not known. We believe that the altered secretome of RBCs might contribute to this. In addition, a recent study showed the possible role of RBC-derived extracellular vesicles in anemia-associated ED (29). We recently showed that acute (3 days) blood loss anemia is associated with a transient increase in FMD responses because of compensatory increases in cardiac output associated with increased shear stress

at the endothelial surface (19). Here we demonstrate unequivocally that the acute response to anemia is compromised by progressive decrease in FMD responses in CA mice correlating with the duration of anemia. In addition, inflammatory markers such as IL6 and IL-17A were unchanged in a subacute anemic mouse model, however, these markers are significantly increased in chronic anemia. Based on our previous publication (19) and the present research work, we summarized the vascular phenotypic differences between acute and chronic anemic mouse models in Supplementary Figure S6. The differences between the two models might be explained by acute and chronic adaptation of the vascular system to anemia. We believe that in acute anemia the vascular system undergoes compensatory changes to improve the endothelial function, especially at peripheral level. However, these compensatory effects are compromised over the time.

Enhanced arginase expression/activity was known to be one of the detrimental mechanisms for ED (30). In our study, endothelial-specific deletion of arginase 1 did not improve the relaxation responses in CA. It has been shown that arginase 2 is also highly expressed in endothelium and contributes to ED in different disease states (8). We used a classic pharmacological approach to inhibit both arginases to further evaluate the possible role of arginase 2 in CA. The aortic rings from anemic mice which were treated with Nor-NOHA did not show any improvement in the relaxation responses. These results largely exclude the potential roles of arginases in ED associated with CA.

We determined whether the reduced relaxation responses in CA are also associated with decreased eNOS expression in endothelial cells. However, eNOS expression at mRNA and protein levels was preserved in CA mice. These results are also in line with previous studies on β-thalassemia anemic mouse model (27). Of note, like in other pathologies (31, 32), the inflammatory iNOS is significantly increased in the vascular smooth muscle cells (VSMC) of CA mice. The enhanced expression of iNOS in inflammatory disease state such as sepsis is known to be detrimental. Additionally, our preliminary findings showed that interleukin IL-6 and interleukin IL-17A levels are significantly elevated in CA mice which might contribute to the enhanced iNOS expression in VSMC. The mechanistic aspects of IL-6 and IL-17A -mediated iNOS is beyond the current research focus but will be studied in the future. The increased circulatory VCAM-1 and ICAM-1 levels in CA mice further underline these findings implicating the role of anemia in inflammation and associated changes in the endothelium.

It is very well known that inflammation and oxidative stress concur with each other in vascular diseases (33). Taking this into consideration, we assessed 3-Nitrotyrosine levels in CA mice. In line with the recent study on iron deficiency mouse model (34), we also observed increased 3-Nitrotyrosine levels in the aortic lysates of CA mice indicating enhanced oxidative stress. NAC is known to be a potential ROS scavenger, which has beneficial effects on endothelial function in various CVDs (35). In our CA mouse model, supplementation of NAC completely reversed the impaired NO-dependent relaxation responses, concluding the potential role of ROS in mediating oxidative stress in CA. We also believe that the preserved eNOS expression and impaired NO-dependent relaxation responses in CA are associated with oxidative stress-mediated eNOS-uncoupling, which was also reported in other disease models (27, 36). Noteworthy, we cannot rule out some limitations of this study. Herein, we mainly focused on the effects of anemia on the endothelium. However, the exact mechanism how anemic RBCs induces ED requires further investigation. Our in vitro studies showed that MPO inhibition with AZD5904 improves endothelial function in CA mice aortic rings. However, further in vivo studies are needed to evaluate the beneficial effects of this MPO inhibitor in improving ED.

Conclusions

Our data suggest that chronic anemia is associated with decreased NO production due to increased inflammation and ROS production. Additionally, we also showed that RBCs from anemic patients attenuates the endothelial relaxation responses concluding the potential role of anemic RBCs in ED. We demonstrated that increased ROS production in the aortic endothelium of anemic mice, and treating the anemic mice with ROS scavenger (NAC) improved the relaxation responses in CA. In addition, MPO expression in endothelium is elevated in CA mice, and the MPO inhibitor AZD5904 also improved the relaxation responses in CA mice. Taken together, NAC supplementation and/or MPO inhibition improves endothelial function in anemia.

Ethics statement

The studies involving human participants were reviewed and approved by Clinical ethics committee, Universitätsklinikum Düsseldorf, Germany. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Landesamt für Natur, Umwelt- und Verbraucherschutz Nordrhein-Westfalen, Germany.

Author contributions

RC: contributed to conception, design, collection and analysis of the data. IS contributed to qRT-PCR, biochemical assays, Western Blot analysis. PD, FC, RC, VY: contributed to myograph and organ bath experiments. PW: contributed to FMD analysis. DP, IS, AS: contributed to immunohistochemistry. SB: contributed to anemia induction. CJ and RC: interpreted the data and drafted the manuscript. CJ, MK: obtained the funding. MH, CJ, NG, MK: critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary data

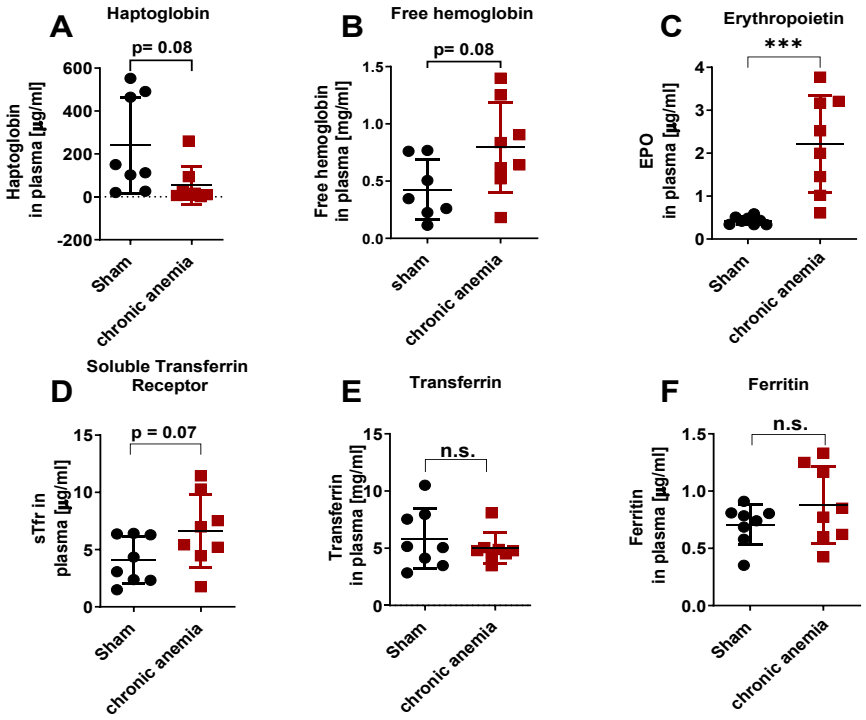

Figure S1. Chronic anemia is associated with increased haematological parameters for RBC synthesis. The blood is collected and plasma is prepared from control (black circles) and chronic (dark red squares) anemic mice. **(A)** Plasma haptoglobin, **(B)** free haemoglobin, **(C)** erythropoietin (EPO), **(D)** soluble transferrin receptor (sTfr), **(E)** transferrin, **(F)** ferritin levels were analysed. The increased serum free haemoglobin, erythropoietin and subsequent decreased haptoglobin levels hint that RBC synthesis is enhanced in CA mice. The Values are shown as means ± SEM. ***, p≤0.001 n=8 per group. Student t-test was used to compare between two groups.

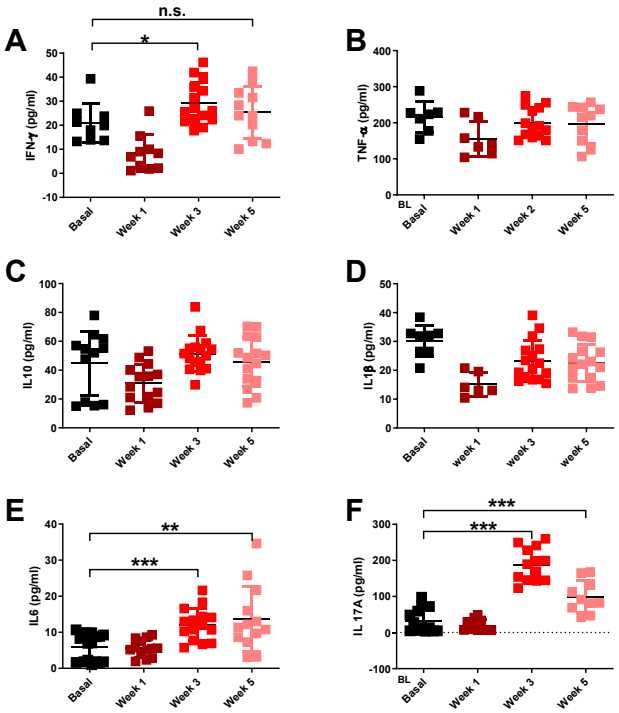

Figure S2. Chronic anemia is associated with increased inflammation. The blood is collected and plasma is prepared from basal, 1 week, 3 week, and 5 weeks after anemia induction. **(A)** Interferon gamma (IFN-γ), **(B)** Tumour Necrosis Factor alpha (TNF-α), **(C)** Interleukin 10 (IL-10), **(D)** Interleukin-1β (IL-1β), **(E)** Interleukin 6 (IL-6), **(F)** Interleukin 17A (IL-17A) were analysed using multiplex assay. Values are shown as means ± SEM. *, p≤0.05; **, p≤0.01 ; ***, p≤0.001 . n=8 per group. One-Way ANOVA and Bonferroni's post-hoc test was used compare baseline and respective week values.

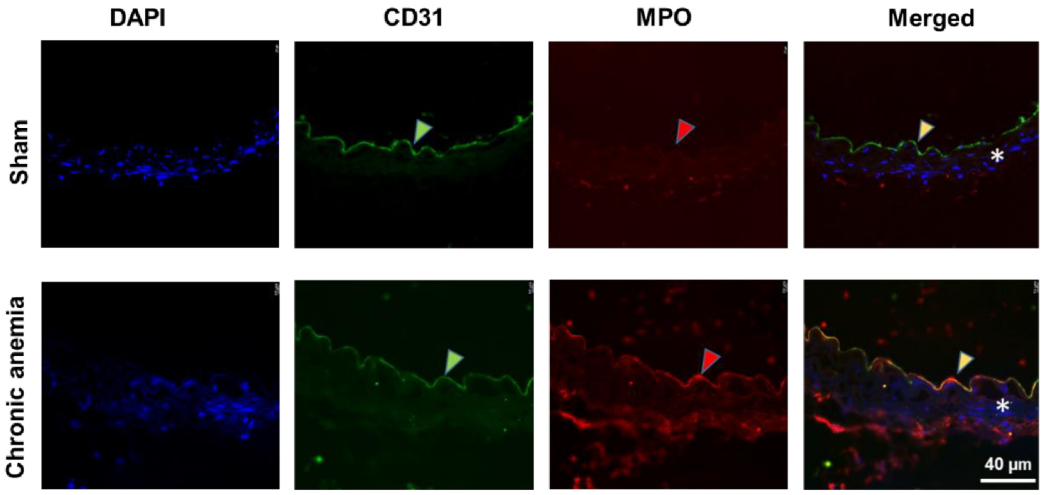

Figure S3. Expression of MPO in endothelium is increased in chronic anemic mice. Thoracic aortas isolated from sham and chronic anemic mice. Representative immunohistochemistry images of aortic sections stained with endothelial cell marker CD-31 (anti-CD31), and also for MPO (anti- MPO). DAPI (blue) staining is used to detect the nucleus. Arrowheads are in the luminal side pointing towards the respective staining. Results are representative pictures of 5 (sham) and 5 (chronic anemic) independent experiments. Adventitial layer showed some unspecific staining in both groups.

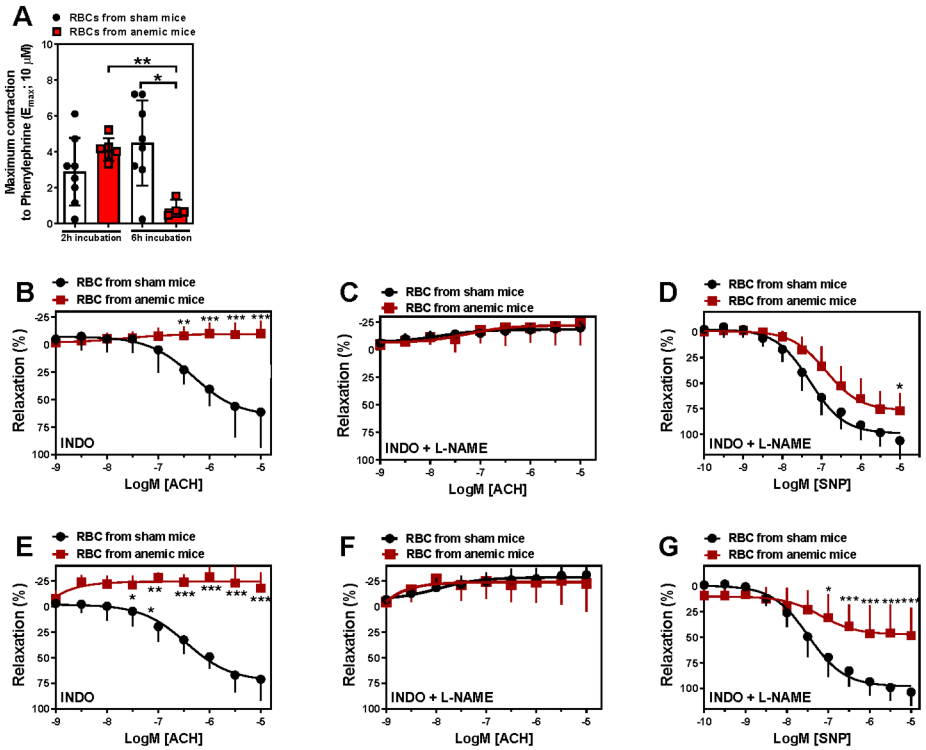

Figure S4. **RBCs from anemic mice induce endothelial dysfunction in murine WT mice aortic rings.** RBCs were isolated from chronic anemic mice (red squares) and sham mice (black circles). Haematocrit (40%) was prepared in a KREBS buffer. Aortic rings were incubated with haematocrit for 2 **(A-C)** or 6 **(D-F)** h at 37°C, mounted in a wire-myograph and pre-contracted with phenylephrine (10 µM, A). **(B, E)** Relaxation responses to acetylcholine (1 nM -10 µM) in the presence of indomethacin (10 µM, COX inhibitor). **(C, F)** Relaxation responses to acetylcholine in the presence of L-NAME (100 µM, NOS inhibitor) and indomethacin. **(D, G)** Endothelium-independent relaxation responses to SNP (10 nM -10 µM, NO donor). Values are shown as means ± SEM. *, p≤0.05. CRCs were analysed by Two-Way ANOVA and Bonferroni 's post-hoc test to compare sham and chronic anemic groups. Mann–Whitney test was used to compare the maximum contraction (E_{max}) responses. N=4-8 per group. Some of the anemic RBCs incubated aortic rings did not constrict to phenylephrine so we excluded them from experiments.

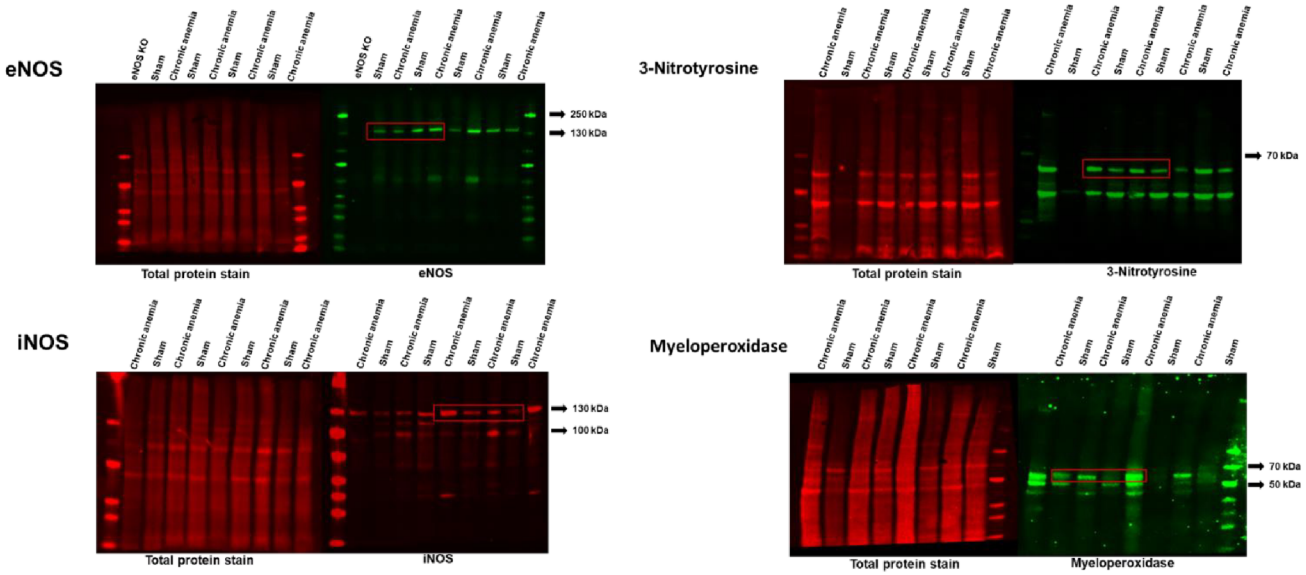

Figure S5. Western blot pictures (uncut) of eNOS, iNOS, 3-Nitrotyrosine and Myeloproxidase

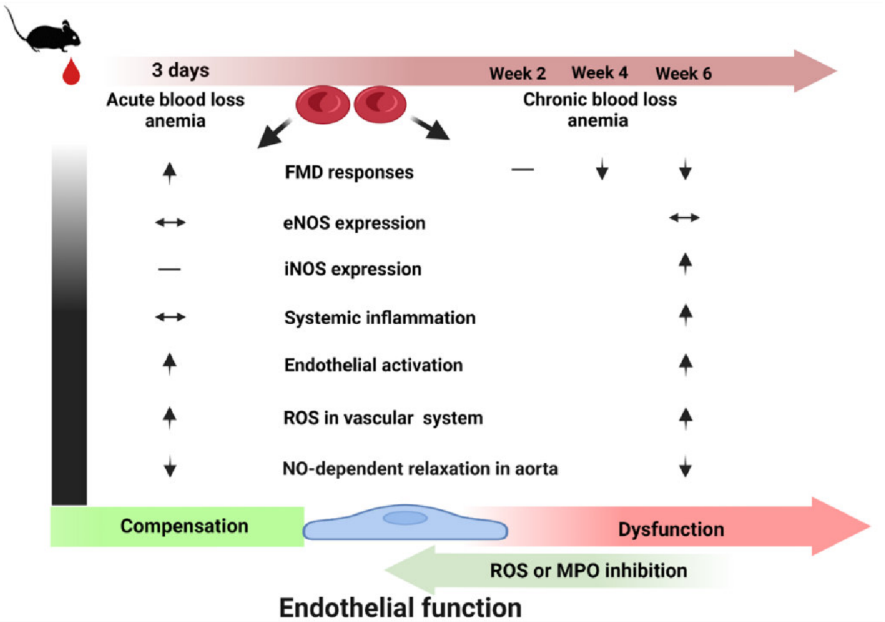

Figure S6. Illustration of acute and chronic blood loss anemia effects on vascular function. The comparison is performed based on present study results and previously published data (Ref 19). In contrary to acute anemia, chronic anemia is associated with progressive loss of FMD responses. ↑: increased; ↓: decrease; ↔: unchanged; -: not known. Inhibition of ROS or myeloperoxidase improved the endothelial dysfunction in CA.

Table S1. Patient characteristics of Chronic Coronary Syndrome patients (CCS) with anemia and without anemia.

	No anemia (n=11)	Anemia (n=11)	P-value
Age, years, median (IQR)	74 (65-77)	76 (53-80)	0.881
Male sex, n (%)	9 (82)	5 (45)	0.169
Hypertension, n (%)	9 (82)	5 (45)	0.076
Diabetes mellitus, n (%)	0	0	NA
Active smoking, n (%)	3 (18)	0 (0)	0.062
GFR, ml/min/1.73m^2 , median (IQR)	65 (54-90)	88 (58-102)	0.412
Hemoglobin, g/dl, median (IQR)	14.3 (13.4-15.2)	11.0 (8.0-12.0)	0.0001

The above patient samples were used to perform experiments mentioned in the main figure 7A-C.

IQR: Interquartile range; GFR: Glomerular filtration rate; NA: Not applicable

Table S2. Patient characteristics of Chronic Coronary Syndrome patients (CCS) with anemia and without anemia.

	No anemia (n=30)	Anemia (n=30)	P-value
Age, years, median (IQR)	76 (69-84)	80 (76-85)	0.043
Male sex, n (%)	18 (60)	20 (66.6)	0.789
Hypertension, n (%)	17 (56.6)	22 (73.3)	0.278
Diabetes mellitus, n (%)	0	0	NA
Active smoking, n (%)	3 (10)	10 (33.3)	0.232
GFR, ml/min/1.73m^2 , median (IQR)	60.5 (51-78)	65 (56-85)	0.293
Hemoglobin, g/dl, median (IQR)	13.6 (13.2-14.4)	9.1 (9.1-10.7)	0.0001

The above patient samples were used to perform experiments mentioned in the main Figures 5B, 5D, 7D, and Figure S3.

IQR: Interquartile range; GFR: Glomerular filtration rate; NA: Not applicable.

Chapter 3

Distinct effects of acute and chronic blood loss anemia on vascular function after acute myocardial infarction

Isabella Solga, Aslihan Sahin, Vithya Yogathasan, Lina Hofer, Feyza Gül Celik, Amira El Rai, Mohammed Rabiul Hosen, Patricia Wischmann, Stefanie Becher, Amin Polzin, Norbert Gerdes, Christian Jung, Malte Kelm, Ramesh Chennupati[1]

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(Adapted version)

Abstract

Background:

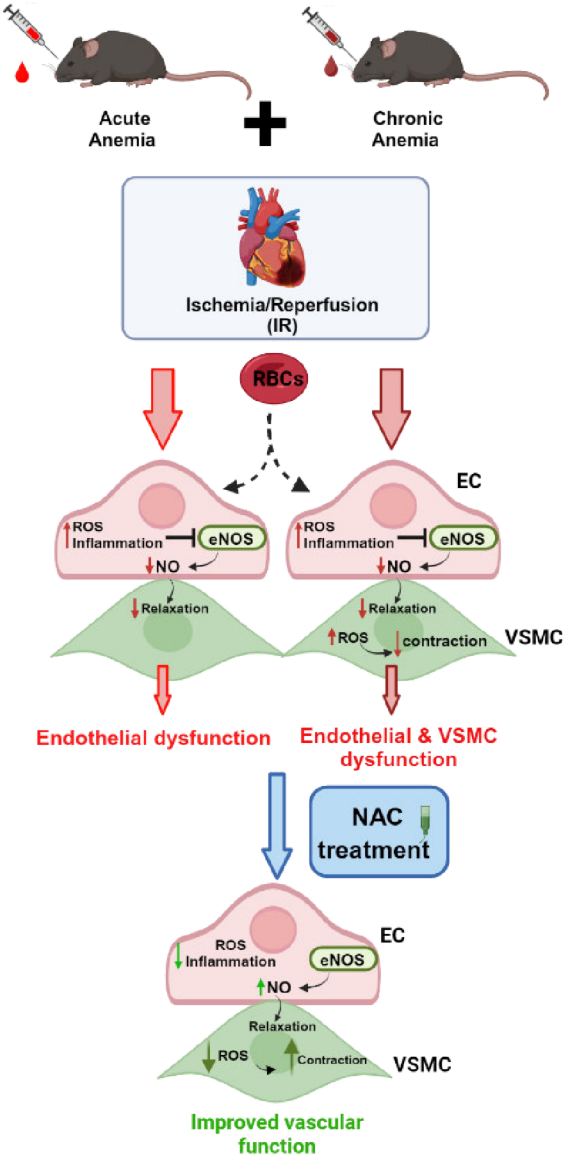
Anemia is frequently observed in patients with cardiovascular diseases (CVD). Anemia alone or in combination with other morbid conditions leads to poor prognosis in acute myocardial infarction (AMI). We recently showed that moderate blood loss anemia is associated with red blood cell (RBC) dysfunction and a compensatory increase in flow-mediated dilation (FMD) responses which are compromised in chronic blood loss anemia However, the effects of acute anemia (AA) and chronic anemia (CA) on endothelial function after AMI are unclear. In this study, we evaluated systemic endothelial function following AMI in established murine models of blood loss acute and chronic anemia. We hypothesize that both AA and CA aggravate systemic endothelial dysfunction (ED) after AMI.

Methods and results:

AA or CA was induced in male C57BL/6J mice by repeated blood withdrawal for three consecutive days or six weeks, respectively. Separate groups of anemic and non-anemic mice underwent AMI via left anterior descending artery (LAD) ligation (45 min), followed by reperfusion. Endothelial function was assessed using both *in vivo* and *in vitro* methods 24 h post-AMI. Impaired flow-mediated dilation (FMD, *in vivo*) and endothelium-dependent relaxation (EDR) responses were observed in the aorta, femoral, and saphenous arteries of AA mice compared to their respective control groups 24 h post AMI. The aorta and saphenous arteries from CA mice showed significantly reduced vascular smooth muscle (VSM) contractile responses after AMI. Analysis of oxidative products of nitric oxide (NO) in plasma revealed reduced nitrite and nitrate levels in both AA and CA mice compared to controls 24 h post- AMI. Immunohistochemistry of aortic tissues from both anemic groups showed increased reactive oxygen species (ROS) product 4-Hydroxynonenal (4-HNE). Co-incubation of RBCs from anemic mice or anemic ST-elevation myocardial infarction (STEMI) patients with aortic rings from wild type mice demonstrated attenuated VSM contractile and EDR responses. Supplementation with the ROS scavenger N-acetyl cysteine (NAC) for four weeks improved both *in vivo* and *ex vivo* EDR in AA and CA mice 24 h post-AMI.

Conclusion:

After AMI, both AA and CA are associated with severe ED, while VSM contractile responses specifically reduced in CA mice. These effects are accompanied by increased ROS and partly mediated by RBCs. Antioxidant supplementation with NAC is a potential therapeutic option to reverse the severe vascular dysfunction in anemia following AMI.



Graphical Abstract: Distinct effects of acute and chronic anemia on vascular function 24 h post- AMI. After acute myocardial infarction, acute and chronic anemia are associated with increased reactive oxygen species (ROS) and inflammation in endothelial cells (EC), leading to the inhibition of endothelial nitric oxide synthase (eNOS) and subsequent endothelial dysfunction by limiting NO bioavailability. Chronic anemia is additionally associated with decreased vascular smooth muscle cell (VSMC) function due to increased oxidative stress, leading to SMC dysfunction. After N-Acetyl-L-Cysteine (NAC) treatment, vascular function is improved in both anemic groups.

Introduction

Acute myocardial infarction (AMI) is one of the leading causes of sudden death worldwide due to a blockage in the coronary arteries resulting in necrosis of the myocardium (*1-3*). Anemia is a frequently diagnosed co-morbidity in patients with AMI, characterized by reduced hemoglobin levels, hematocrit, and a reduction in circulating red blood cells (RBCs). Many elderly patients with cardiovascular disease develop anemia during hospitalization due to blood loss during interventions or diagnoses, which is defined as hospital-acquired anemia which might have consequences on the vascular system and thus on prognosis during cardiovascular events (*4*). Anemia in association with acute coronary syndrome, stroke, or heart failure leads to poor cardiovascular disease prognosis (*5*). Furthermore, anemic patients have a higher risk of major bleeding, arrhythmias, and heart failure after AMI, leading to morbidity and mortality (*6, 7*). However, the underlying mechanisms of how hospital-acquired anemia influences vascular function is largely unknown.

The endothelium in arteries plays a fundamental role in regulating vascular tone by synthesizing and releasing an array of endothelium-derived relaxing factors, such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). NO is generated from L-arginine by endothelial NO synthase (eNOS), which diffuses into vascular smooth muscle cells (VSMCs) and activates guanylate cyclase, resulting in cyclic guanosine monophosphate (cGMP)-mediated vasodilation (*8*). Reduced production of endothelium-dependent NO and increased levels of reactive oxygen species (ROS) and inflammation are associated with many forms of cardiovascular diseases, including hypertension, coronary artery disease, and chronic heart failure, due to increased oxidative stress (*9–11*). It is well known that increased ROS in the endothelium uncouple eNOS, leading to reduced NO production and thus endothelial dysfunction (ED). ED is a hallmark of vascular dysfunction in AMI. Clinically, the assessment of flow-mediated dilation (FMD) is the gold standard for evaluating endothelial function *in vivo*, which is primarily mediated by NO (*12*). Decreased FMD responses are associated with various cardiometabolic diseases, such as myocardial infarction, diabetes, and coronary artery disease (*13*). Previous studies have shown that anemia is associated with increased FMD responses in humans without comorbidities (*14*). Anemia-related hemoglobinopathies, or anemia in combination with diabetes or chronic kidney disease (CKD), are associated with reduced FMD responses (*15–17*). These studies demonstrate that endothelial function is altered based on the type of anemia and associated co-morbid conditions. Additionally, we recently showed that acute blood loss anemia is associated with increased compensatory FMD responses, which are compromised in cases of chronic anemia (CA) due to increased oxidative stress (*18, 19*). In the same study, we demonstrated that CA is associated with progressive ED in large arteries due to increased oxidative stress in the endothelium (*19*). However, the effect of anemia on endothelial function after AMI remains largely unknown.

RBCs are known to induce ED in different disease states such as diabetes, hypocholesteremia, preeclampsia, and anemia (*19*–*22*). We have recently shown that RBCs in anemia lose their cardioprotective properties in ST-elevation myocardial infarction (STEMI) patients (*23*), demonstrating the potential role of RBCs in cardiometabolic diseases. It is not clear how acute and chronic blood loss anemia affects vascular function after myocardial infarction, and the potential role of RBCs in this has never been investigated. Therefore, in this study, we used two well-established acute and chronic blood-loss mouse models to study the effect of blood loss anemia on vascular function, namely, endothelial and VSMCs after Ischemia-Reperfusion (IR) injury. Additionally, we investigated whether RBCs from anemic mice with AMI and anemic STEMI patients affect endothelial function using co-incubation with aortic rings followed by VSCM and endothelial function analysis. To our knowledge, this is the first study to evaluate systemic ED after AMI in acute and chronic blood-loss anemia murine models.

Materials and Methods

Animals

All animal procedures used in the study were approved and performed in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) II guidelines and authorized by LANUV (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection) in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The approval numbers for the animal experiments are 84-02.04.2020.A073 and 84-02.04.2018.A234. The C57Bl/6J (wildtype, WT) mice were obtained from Janvier Labs (Saint-Berthevin Cedex, France). Mice were housed in standard cages (constant room temperature and humidity, with 12h light/dark cycles) and had free access to standard pelleted food and tap water.

Anemia induction

For the experimental approach, we used four different groups of mice. The mice were divided into AA, CA, and their respective non-anemic groups. AA was induced in 10- to 12-week-old male C57BL/6J wildtype (WT) mice by repetitive mild blood withdrawal on three consecutive days, resulting in <20 g/l changes in hemoglobin (Hb). The blood was withdrawn from the facial vein under isoflurane anesthesia (3%). The amount of daily blood loss per mouse was adjusted to <15% of the total blood volume and was replaced by saline administration. CA was induced by mild blood withdrawal every third day for a period of six weeks, also resulting in <20 g/l changes in Hb. At the end of the sixth week, mice with hemoglobin levels <10 g/l were considered for experiments. The two non-anemic groups were age-matched to the anemic groups

and underwent the same handling as the anemic mice (including puncture of the facial venin) without blood withdrawal.

Induction of AMI

In a separate set of anemic and non-anemic groups of mice, acute myocardial infarction (AMI) was induced. Briefly, mice were anesthetized with isoflurane, intubated, and ventilated with a tidal volume of 0.2-0.25 mL and a respiratory rate of 140 breaths per minute, using isoflurane (3%) and 30% O_2 with a rodent ventilator. Body temperature was controlled and maintained at 37°C throughout the surgical procedure. A left lateral thoracotomy was performed between the third and fourth rib, and the pericardium was exposed and dissected. Ischemia was induced by gently tightening a 7-0 surgical suture placed under the left anterior descending (LAD) artery. To ensure that the procedure was carried out correctly, in addition to the visible blanching of the apex, changes in the electrocardiogram (ECG; ST-segment elevation) were monitored. After 45 minutes, the ligation was removed, and the myocardium was reperfused for 24 h. Animals received buprenorphine (0.5 mg/kg BW) subcutaneously every 8 h until euthanasia.

Supplementation of NAC

To investigate the potential role of reactive oxygen species (ROS) in mediating endothelial dysfunction, additional groups of mice were supplemented with 1% N-acetylcysteine (NAC) (Sigma) through drinking water for 4 weeks. After 4 weeks, acute or chronic anemia was induced, and ischemia-reperfusion (IR) surgery was performed to induce AMI.

Collection of Blood from STEMI Patients

Blood was collected from STEMI patients, with and without anemia, within 24 h post-infarction in EDTA tubes. The patients were diagnosed based on changes in the ECG. According to the World Health Organization (WHO) guidelines, adult male patients with hemoglobin (Hb) levels below 13.0 g/dL and adult female patients with Hb levels below 12.0 g/dL are considered anemic (*24*). All patients included in this study provided written consent and were recruited from the Department of Cardiology, Pulmonology, and Angiology at Düsseldorf University. Permission numbers for blood sample collections are 5481R, 2018-14, and 2018-47, as approved by the ethics committee of Düsseldorf University Hospital.

In vitro studies with isolated aortic rings

Solutions and drugs

Krebs-Ringer bicarbonate-buffered salt solution (KRB) contained (in mmol/L): 118.5 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 5.5 glucose. The KRB solution was continuously aerated with 95% O_2/5% CO_2 and maintained at 37°C. Indomethacin (INDO; Sigma Aldrich,) was dissolved in ethanol. Acetylcholine (ACh), phenylephrine (PHE), N^{ω}-nitro-arginine methyl ester (L-NAME) and sodium nitroprusside (SNP; all Sigma Aldrich) were dissolved in KRB solution.

Organ chamber experiments

Mice were euthanized under deep isoflurane anesthesia (4.5%). The thoracic aorta was dissected free from perivascular adipose tissue and cut into 2 mm aortic rings. The segments were then mounted in a wire myograph system (Danish Myo Technology (DMT), Aarhus, Denmark) and stretched to a force of 9.8 mN. The segments were allowed to normalize for 45 minutes with a periodic buffer change three times. Saphenous and femoral arteries were dissected free from surrounding fat and connective tissue and mounted in a wire myograph (DMT). Arterial segments (2 mm) were stretched to the optimal diameter at which maximal contractile responses to 10 µM norepinephrine (NA) could be obtained (*25*).

Contributions of NO, cyclo-oxygenase products to endothelium-dependent relaxation

In the first step, a concentration-response curve (CRC) for PHE (0.001-10 µM) and ACh (0.001-10 µM) was generated in presence of the cyclooxygenase inhibitor indomethacin (INDO, 10 µM). Next, to evaluate the contribution of NO to the relaxation response in the arteries the CRC was repeated in the presence of both INDO and L-NAME (100 µM) a NOS inhibitor.

Sensitivity of vascular smooth muscle to NO

Additionally, the SMC sensitivity to NO was evaluated by performing a CRC in presence of INDO and L-NAME (100 µM), with the NO donor SNP (0.01-10 µM).

Co-incubation experiments with murine and human RBCs

For further investigations of dysfunctional RBCs on endothelial-dependent relaxation responses in anemia, we performed co-incubation studies with murine and human RBCs. Blood was collected in EDTA tubes from non-anemic and anemic mice 24 h post-AMI, and additionally from STEMI patients with and without anemia. The RBCs were isolated by centrifugation at 800 xg for 10 minutes at 4°C, and 40% hematocrit (hct) was prepared using KRB buffer. Aortic rings (2 mm) isolated from WT mice were co-incubated with the isolated RBCs for 2 h

(murine RBCs) or 6 h (human RBCs) at 37°C. After the incubation, the aortic segments were mounted in the wire myograph system, and vascular function was evaluated.

Flow-mediated dilation assessment

The C57BL/6J mice of week-old mice with and without anemia were used for the assessment of flow-mediated dilation (FMD) responses. The measurements were performed 24 h post - AMI. FMD responses were determined by using a Vevo 2100 high-resolution ultrasound scanner using a 30–70 MHz linear transducer (Visual Sonics Inc., Toronto, Canada) as described previously (*26*). During the whole procedure/measurement mice were kept under 2-2.5% isoflurane anaesthesia and a body temperature of 37°C was maintained. To visualise the femoral artery, the transducer was placed at the lower limb and a vascular occluder (8 mm diameter, Harvard Apparatus, Harvard, Boston, MA, USA) was used to perform the dilation measurement (*26*). Initially baseline images of the vessel were recorded. In order to measure changes in the vessel diameter, first an occlusion phase was performed by inflating the cuff to 250 mmHg. During the occlusion the pressure was kept constant for 5 min and an image was taken every 30 s (Druckkalibriergerät KAL 84, Halstrup Walcher, Kirchzarten, Germany). Afterwards the cuff was released (reperfusion phase) for 5 min and again every 30 s an image was taken to determinate the FMD. Changes in vessel diameter were quantified as percent of baseline (%) = [diameter (max)/diameter (baseline)] × 100.

Immunohistochemistry

Thoracic aortas from the anemic mice 24 h post AMI and respective non-anemic mice 24 h post AMI were fixed in formaldehyde (4%) for 2 h and stored in sucrose (30%) overnight. Afterwards the tissues were embedded in Tissue-Tek®, O.C.T and frozen until further use. Tissue sections (5 µm) were incubated overnight at 4 ℃ with rat anti-mouse CD31 (1:200 in blocking solution ((0.1% Saponin, 0.5% BSA, 0.2% fish gelatin in 1 x PBS) BD Biosciences) and goat anti-rabbit 4-HNE (1:200 in blocking solution; Abcam) antibodies. Next, the sections were incubated with respective goat anti-rat Alexa Fluor 488 (1:1000 in blocking solution; ThermoFisher) and goat anti-rabbit Alexa Fluor 555 (1:1000 in blocking solution; ThermoFisher) secondary antibodies. Additionally, autofluorescnece was quenched using Vector® TrueVIEW® Autofluorescence Quenching Kit (BIOZOL Vectorlabs). Prior to covering, a DAPI staining (1mg/ml) was performed for 5 min and then the sections were mounted with VectaMount® Aqueous Mounting Medium (ThermoFisher) and imaged with Leica DM6 M microscope (Leica Microsystems).

Assessment of plasma inflammatory markers

Blood was collected from all experimental groups in EDTA tubes. RBC and plasma samples were prepared and snap-frozen. The samples were stored at −80°C until further use. Plasma samples were used to assess inflammatory parameters in the different mice groups by using ELISA kits for VCAM-1 and ICAM-1 measurements (R&D Systems).

Results

Acute anemia is associated with altered endothelial function after AMI

To assess the effect of AA on vascular function 24 h post-AMI, contractile responses, and endothelium-dependent and -independent relaxation responses were measured in isolated aortic rings (large artery) using wire myograph. The contractile responses to phenylephrine did not significantly differ between AA mice and non-anemic mice 24 h post-AMI (Suppl. Table 1). However, the endothelium-dependent relaxation responses to acetylcholine in the presence of indomethacin were significantly reduced in AA mice (E_{max}: 25.22 ± 2.88%; p=0.0009) compared to the corresponding non-anemic group (E_{max}: 52.45 ± 6.02%) of mice 24 h post-AMI (Fig. 1A; Suppl. Fig. 1A; Suppl. Table 1). In the presence of the NOS inhibitor (L-NAME, 100 µM), relaxation responses were completely inhibited in both AA and non-anemic groups of mice (Fig. 1B), proving that the relaxation responses are mediated by NO. SMC sensitivity to NO and endothelium-independent relaxation were assessed using an exogenous NO donor, SNP. All groups showed similar relaxation responses, indicating that SMC sensitivity to NO was similar in both groups (Fig. 1C; Suppl. Table 1).

Vascular function was also assessed in femoral (medium sized) and saphenous arteries (small sized) to investigate possible effects of anemia on different vascular beds. In both femoral and saphenous arteries, the contractile responses to phenylephrine did not significantly differ between AA mice and their respective control group 24 h post-AMI (Suppl. Table 2-3). In the presence of indomethacin, femoral arteries showed reduced sensitivity to acetylcholine-mediated relaxation responses in AA mice (pEC_{50}: 6.86 ± 0.24; p=0.04) compared to non-anemic mice (pEC_{50}: 7.55 ± 0.20) 24 h post-AMI (Fig. 1D; Suppl. Fig. 1B; Suppl. Table 2). In the small resistance saphenous artery, the endothelium-dependent relaxation responses to acetylcholine were significantly reduced in AA mice (E_{max}: 50.36 ± 12.41%; p=0.03) compared to the non-anemic group of mice (E_{max}: 85.44 ± 7.59%) 24 h post-AMI (Fig. 1G; Suppl. Fig. 1C; Suppl. Table 3). In both femoral and saphenous arteries, in the presence of L-NAME, the relaxation responses were similar between anemic and non-anemic groups (Fig. 1E & H; Suppl. Tables 2-3). Similarly, endothelium-independent relaxation responses to SNP did not differ between AA mice and their respective control groups 24 h post-AMI (Fig. 1F & I; Suppl. Tables 2-3). These results conclude that AA is associated with reduced endothelium-dependent relaxation

responses, which are mainly mediated by NO, despite the smooth muscle sensitivity to NO remains unchanged.

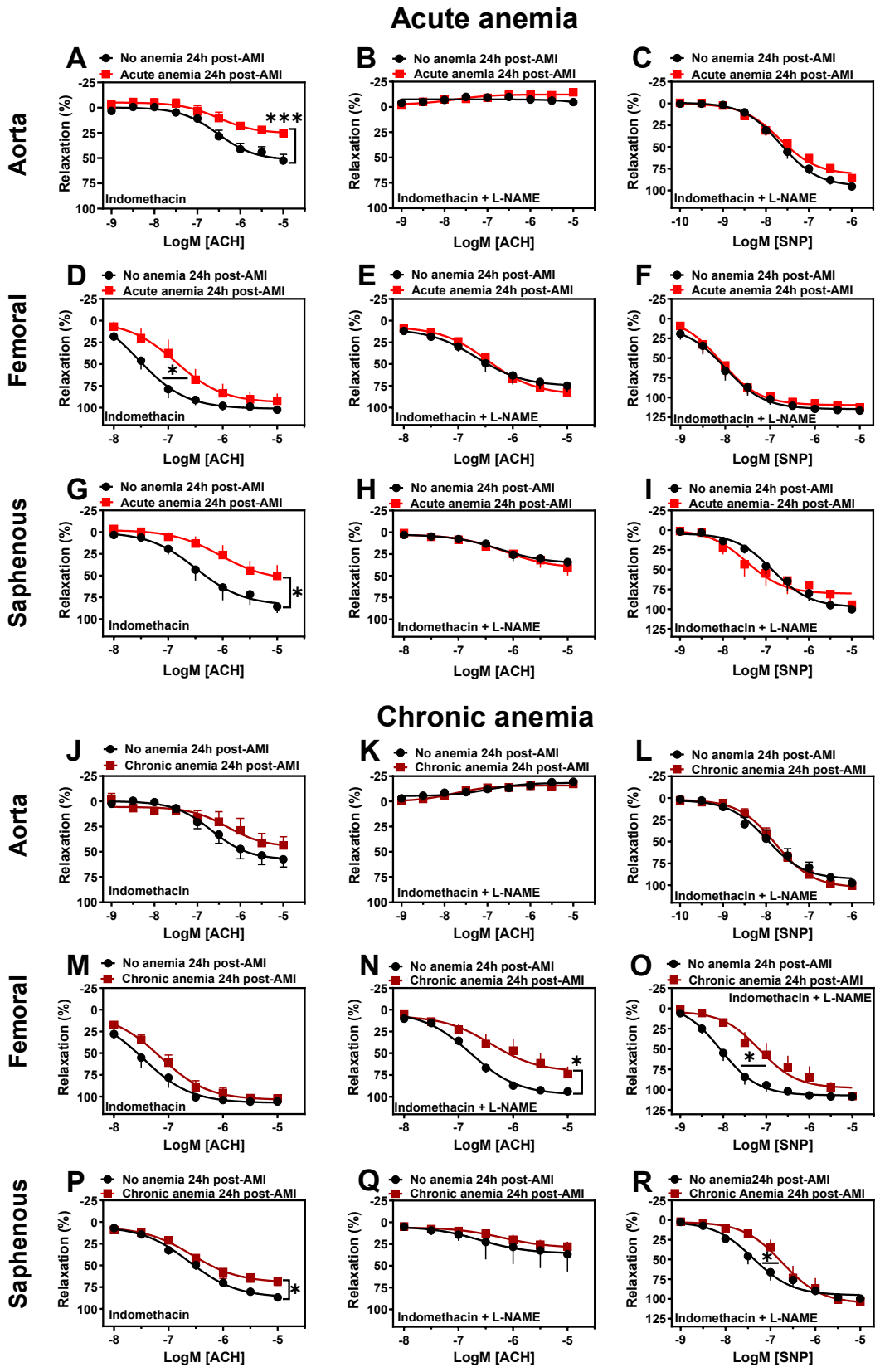


Figure 1: Both acute and chronic anemia are associated with vascular dysfunction 24 h post-AMI. Arterial segments of aorta **(A-C, J-L)**, femoral **(D-F, M-O)** and saphenous arteries **(G-I, P-R)** were isolated from acute (red

squares) and chronic (dark red squares) anemic mice and respective non-anemic mice (black circles) 24 h post IR. Arterial segments were pre-contracted using phenylephrine (PHE; 10 µM), and their relaxation responses to acetylcholine (ACh; 1 nM- 10 µM) were assessed using wire myography. **(A, D, G, J, M, P)** Relaxation (%) in the presence of indomethacin (10 µM, a COX inhibitor). **(B, E, H, K, N, Q)** Relaxation in the presence of indomethacin and L-NAME (100 µM, NOS inhibitor). Maximal endothelial-dependent relaxation response ($E_{max, \%}$). **(C, F, I, L, O, R)** Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 µM) in the presence of indomethacin and L-NAME. All values are mean values ± SEM (n = 8–10 per group). *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001. Concentration-response curves (CRCs) were analysed by Two-Way ANOVA and Bonferroni 's post-hoc test to compare acute, chronic and respective non-anemic group of mice.

Chronic anemia is associated with endothelial and vascular smooth muscle dysfunction after AMI

We next investigated the effect of CA on vascular function 24 h post-AMI. Contractile responses, and endothelium-dependent and -independent relaxation responses in the isolated aortic rings using a wire myograph. Interestingly, aortic rings isolated from CA mice showed significantly reduced contractile response to phenylephrine (E_{max}: 0.62 ± 0.08 mN, p=0.0002) compared to respective non-anemic mice (E_{max}: 3.56 ± 0.60 mN) 24 h post-AMI (Suppl. Fig. 2, Suppl. Fig. 3A-B; Suppl. Table 4). In contrast to the AA group, the relaxation responses to acetylcholine in the presence of indomethacin were not altered in the aorta of CA mice compared to the corresponding non-anemic group (Fig. 1J; Suppl. Table 4). In the presence of L-NAME, the relaxation responses were inhibited and to a similar extent in both groups (Fig. 1K). Additionally, smooth muscle sensitivity to NO was similar in both groups (Fig. 1L; Suppl. Table 4).

We also assessed the vascular function in the femoral and saphenous arteries of the CA mice 24 h post-AMI. In contrast to the aorta, the contractile responses of femoral arteries were unchanged in CA mice compared to the respective non-anemic group (Suppl. Fig. 3C-D; Suppl. Table 5). The relaxation responses to acetylcholine in the presence of indomethacin did not differ between CA mice and non-anemic mice 24 h post-AMI (Fig. 1M; Suppl. Table 5). In the presence of L-NAME, the CA mice showed significantly reduced relaxation responses (E_{max}: 73.84 ± 8.60%; p=0.04) compared to non-anemic mice (E_{max}: 93.81 ± 1.48%) 24 h post-AMI (Fig. 1N; Suppl. Table 5). However, in the small resistance saphenous artery, similar to the aorta, the contractile responses to phenylephrine were significantly reduced in CA mice (E_{max}: 6.71 ± 1.65 mN; p=0.01) compared to non-anemic mice (E_{max}: 11.75 ± 0.88 mN) 24 h post-AMI (Suppl. Fig. 3E-F; Suppl. Table 6). Furthermore, endothelial-dependent relaxation responses to acetylcholine in the presence of indomethacin were significantly reduced in CA mice (E_{max}: 67.93 ± 5.16%; p=0.004) compared to non-anemic mice (E_{max}: 86.70 ± 2.95%) 24 h post-AMI (Fig. 1P; Suppl. Table 6). The SMC sensitivity to NO was preserved in the aorta of both groups

(Fig. 1O & R; Suppl. Tables 5-6). However, the smooth muscle sensitivity to SNP were significantly reduced in femoral and saphenous arteries (Fig. 1O & R; Suppl. Tables 5-6).

These results demonstrate that contractile responses are significantly decreased in large and small resistance arteries. Additionally, small resistance arteries also show impaired endothelial-dependent and -independent NO-mediated relaxation responses.

Flow-mediated dilation (FMD) responses are impaired in both acute and chronic anemic mice after AMI

In addition to the detailed *ex vivo* assessment of vascular function in isolated arteries, we also assessed *in vivo* endothelial function by measuring FMD. AA mice showed significantly reduced FMD responses (5.702 ± 0.845%) compared to the respective non-anemic control group (9.270 ± 0.938%) 24 h post-AMI (Fig. 2A-B). Interestingly, similar to AA mice, CA mice also showed significantly reduced FMD responses (5.231 ± 0.547%) compared to the respective non-anemic group of mice (8.553 ± 0.767%) 24 h post-AMI (Fig. 2C-D).

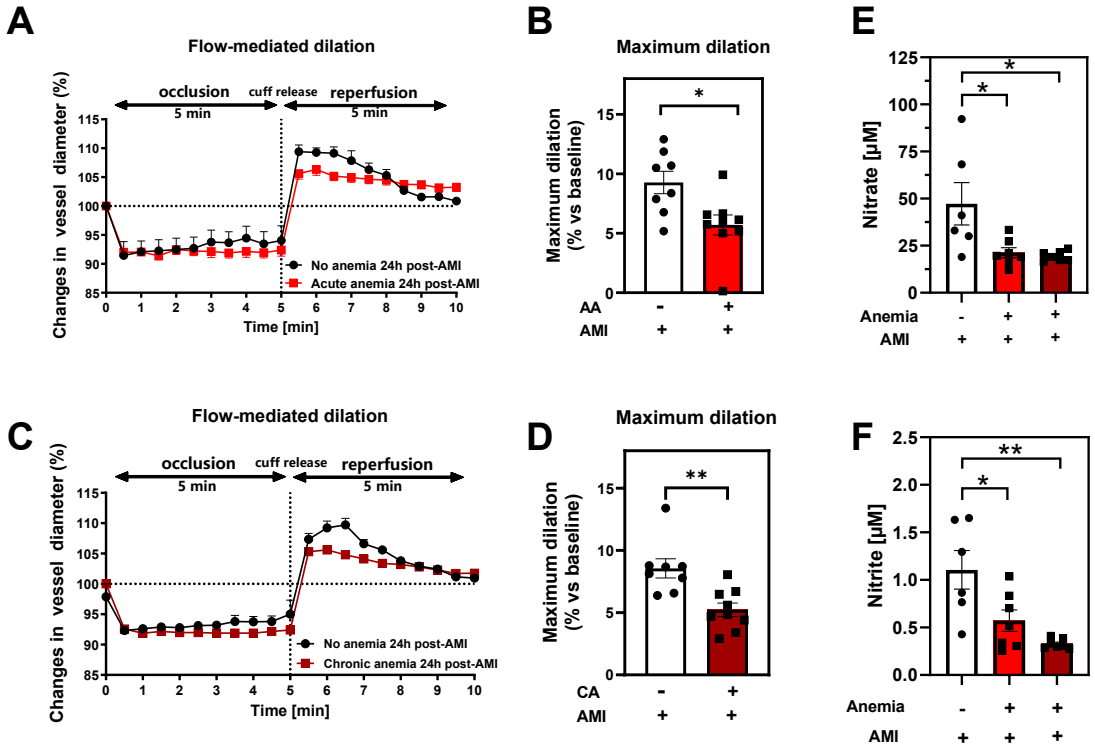


Figure 2: Acute and chronic anemia are associated with reduced flow-mediated dilation responses and reduced oxidative NO products 24 h post-AMI. (A, C) Changes in vessel diameter in acute (red squares), chronic (dark red squares), and respective non-anemic group (black circles) of mice 24 h post IR. **(B, D)** Maximal FMD response (% ratio vs. baseline). **(E, F)** Plasma nitrate and nitrite levels in the in acute (red bar), chronic (dark red bar), and respective non-anemic group (white bar). The values are presented as means ± SEM (n=8-9 per group). *, $p \leq 0.05$; **, $p \leq 0.01$. The average FMD in reperfusion phase was compared with the student t-test between the groups.

Endothelial dysfunction is often associated with reduced plasma oxidative nitric oxide (NOx) products. We assessed the NOx products nitrite and nitrate in the plasma. Both AA and CA mice showed significantly reduced plasma nitrate (Fig. 2E) and nitrite (Fig. 2F) levels compared to the respective non-anemic groups.

These results clearly demonstrate that, in line with the reduced *ex vivo* endothelial-dependent relaxation responses and *in vivo* FMD responses, NO bioavailability is significantly reduced in both AA and CA mice compared to respective non-anemic mice.

Both acute and chronic anemia are associated with increased oxidative stress in the vessels

In our recent study, we showed that CA is associated with increased oxidative stress and endothelial activation (*19*). We further investigated the potential role of inflammation and reactive oxygen species (ROS) in the observed vascular dysfunction in both acute and chronic blood-loss anemia 24 h post-AMI. To detect ROS in the vessels, we performed immunofluorescence staining for the ROS product 4-HNE. The aortic sections from both AA and CA mice 24 h post-AMI showed significantly higher 4-HNE levels compared to the non-anemic mice (Fig. 3A-B). It is well known that ROS and inflammation concur with each other, so we also measured endothelial activation markers ICAM-1 and VCAM-1 in plasma. VCAM-1 was significantly increased in both AA and CA mice compared to their respective non-anemic mice 24 h post-AMI (Fig. 3C-D). These results suggest that AA and CA are associated with increased oxidative stress and endothelial inflammation after AMI.

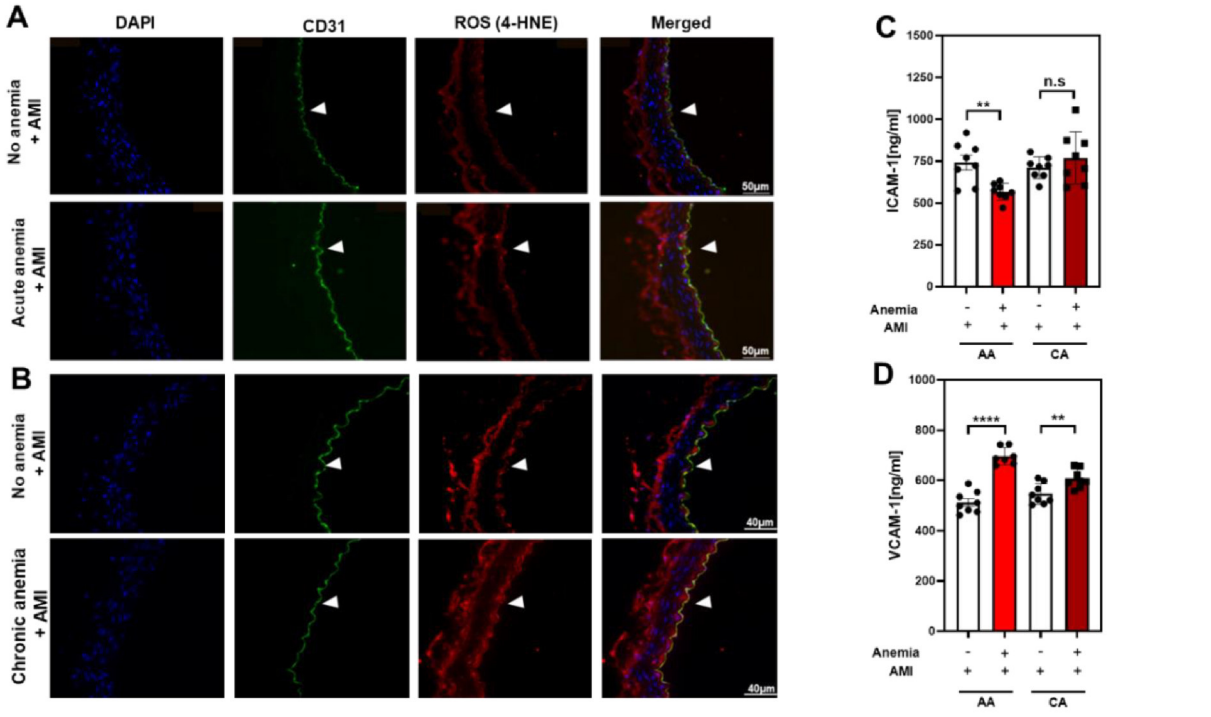


Figure 3: Anemia 24 h post-AMI is associated with increased oxidative stress and inflammation. Thoracic aortas isolated from acute, chronic and respective non-anemic mice 24 h post-AMI**. (A, B)** Representative images of sections that are stained with endothelial cell marker CD31 (green) and ROS-marker 4-HNE (red). DAPI (blue) staining is used to detect the nucleus. Arrowheads are in the luminal side pointing towards the respective staining. Results are representative pictures of 3 (non-anemic), 3 (acute anemic), 5 (chronic anemic) independent experiments **(C-D).** Plasma ICAM-1 and VCAM-1 levels in acute (AA, red bar), chronic (CA, dark red bar), and respective non-anemic group (white bar) of mice 24 h post-AMI. All values are presented as means ± SEM. **, $p \leq 0.01$; ****, $p \leq 0.0001$; ns, not significant.

NAC treatment reversed the vascular dysfunction in both acute and chronic anemic mice

From our data, it is evident that ROS mediates vascular dysfunction in both AA and CA mice. To further confirm this, we supplemented mice with NAC and assessed vascular function after AMI. The contractile responses to phenylephrine were not altered between AA mice and the respective non-anemic group of mice (Suppl. Table 1). Interestingly, after NAC supplementation, the abrogated contractile responses in CA mice were significantly improved in the aorta (E_{max}: 3.14 ± 0.64 mN; p=0.004) compared to CA mice without NAC treatment (E_{max}: 0.62 ± 0.08 mN) 24 h post-AMI (Suppl. Fig. 3G-H; Suppl. Table 4). The contractile responses were similar between CA mice and non-anemic mice 24 h post-AMI. Similar improvements in contractile responses were also observed in saphenous arteries (Suppl. Fig. 3K-L). Next, in the same mice, we assessed endothelium-dependent and -independent relaxation responses in both AA and CA mice and the respective control group 24 h post-AMI. As expected, in both anemic groups and all types of arteries, the endothelium-dependent relaxation responses were improved with NAC treatment (Fig. 4A, D, G, J, M&P Suppl. Fig. 4A-F; Suppl. Tables 1-6). The endothelium-independent relaxation to SNP was preserved in both anemic groups and all three types of arteries (Fig. 4C, F, I, L, O&R; Suppl. Tables 1-6).

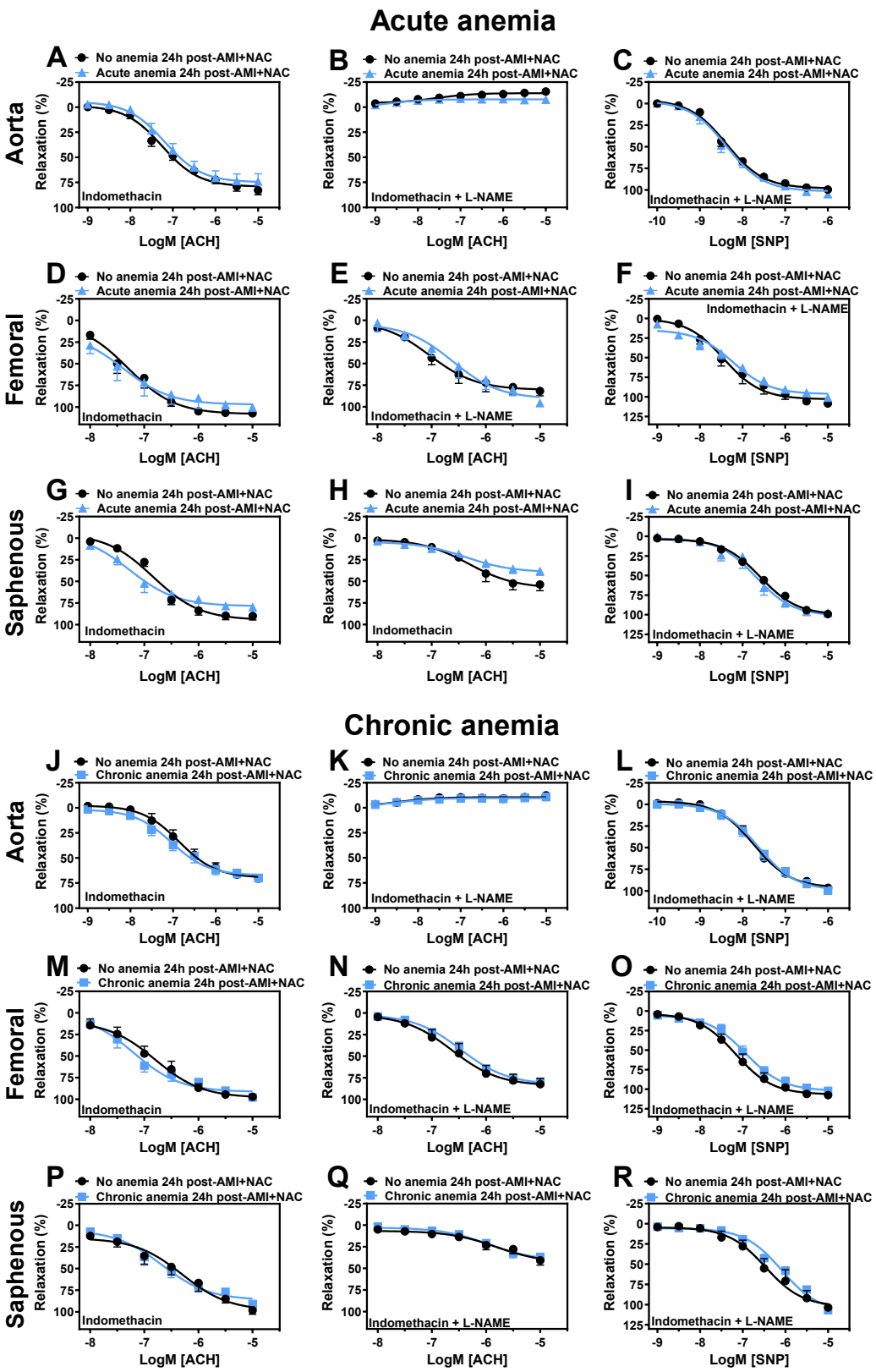


Figure 4: NAC supplementation improves endothelial-dependent relaxation response in anemia 24 h post-AMI. Arterial segments of aorta **(A-C, J-L)**, femoral **(D-F, M-O)** and saphenous arteries **(G-I, P-R)** were isolated from acute (light blue triangles) and chronic (light blue squares) anemic mice and respective non-anemic mice (black circles) 24h post-AMI. Arterial segments were pre-contracted using phenylephrine (PHE; 10 μM), and their

relaxation responses to acetylcholine (ACH; 1 nM- 10 µM) were assessed using wire myography. **(A, D, G, J, M, P)** Relaxation (%) in the presence of indomethacin (10 µM, a COX inhibitor). **(B, E, H, K, N, Q)** Relaxation in the presence of indomethacin and L-NAME (100 µM, NOS inhibitor). Maximal endothelial-dependent relaxation response ($E_{max, \%}$). **(C, F, I, L, O, R)** Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 µM) in the presence of indomethacin and L-NAME. All values are mean values ± SEM (n = 8–10 per group). Concentration-response curves (CRCs) were analysed by Two-Way ANOVA and Bonferroni 's post-hoc test to compare acute, chronic and respective sham group of mice.

These results demonstrate that NAC treatment effectively reverses vascular dysfunction in both acute and CA mice by improving both contractile and endothelium-dependent relaxation responses, indicating the critical role of ROS in mediating vascular dysfunction.

NAC supplementation improved flow-mediated dilation (FMD) responses in both acute and chronic anemic mice after AMI.

We further assessed the FMD responses in both AA and CA mice and their respective non-anemic groups 24 h post-AMI. In line with *ex vivo* data, after NAC supplementation, both AA and CA mice showed improved FMD responses (Fig. 5A-D; Suppl. Fig. 5A-D). This further confirms that ROS mediates vascular dysfunction in both anemic groups after AMI. Additionally, after NAC supplementation, both nitrite and nitrate levels improved in AA and CA mice compared to the respective non-anemic groups 24 h post-AMI (Fig. 5E-F). These results conclude that NAC supplementation improves endothelial dysfunction and the bioavailability of NO.

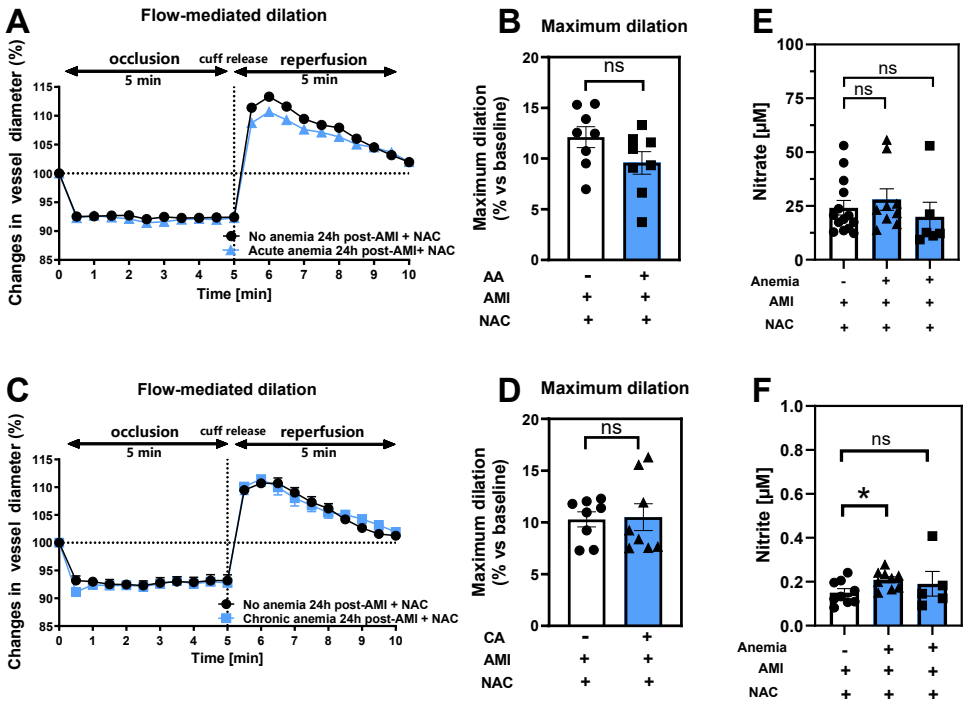


Figure 5: NAC treatment improves flow-mediated dilation (FMD) response in acute and chronic anemia 24 h post-AMI. **(A, C)** Changes in vessel diameter in acute (light blue triangles), chronic (light blue, squares), and respective non-anemic groups (black, circles) of mice 24 h post-AMI. **(B, D)** Maximal FMD response (% ratio vs. baseline). **(E, F)** Plasma nitrate and nitrite levels in the in acute (light blue bar, triangles), chronic (light blue bar, squares), and respective non-anemic group (white bar, circles). The values are presented as means ± SEM (n=8-9 per group). *, $p \leq 0.05$; **, $p \leq 0.01$; ns, not significant. The average FMD in the reperfusion phase was compared with the Student's t-test between the groups.

By performing immunofluorescence staining, we demonstrated that NAC-treated mice had reduced ROS formation in the aortic segments (Fig. 6A-B). Additionally, the increased plasma levels of VCAM-1 were normalized in the CA mice after NAC treatment (Fig. 3D and 6C) whereas they remained high in AA mice (Fig. 3D and 6C). These findings support a potential role for ROS and inflammation in anemia-associated endothelial dysfunction post-AMI.

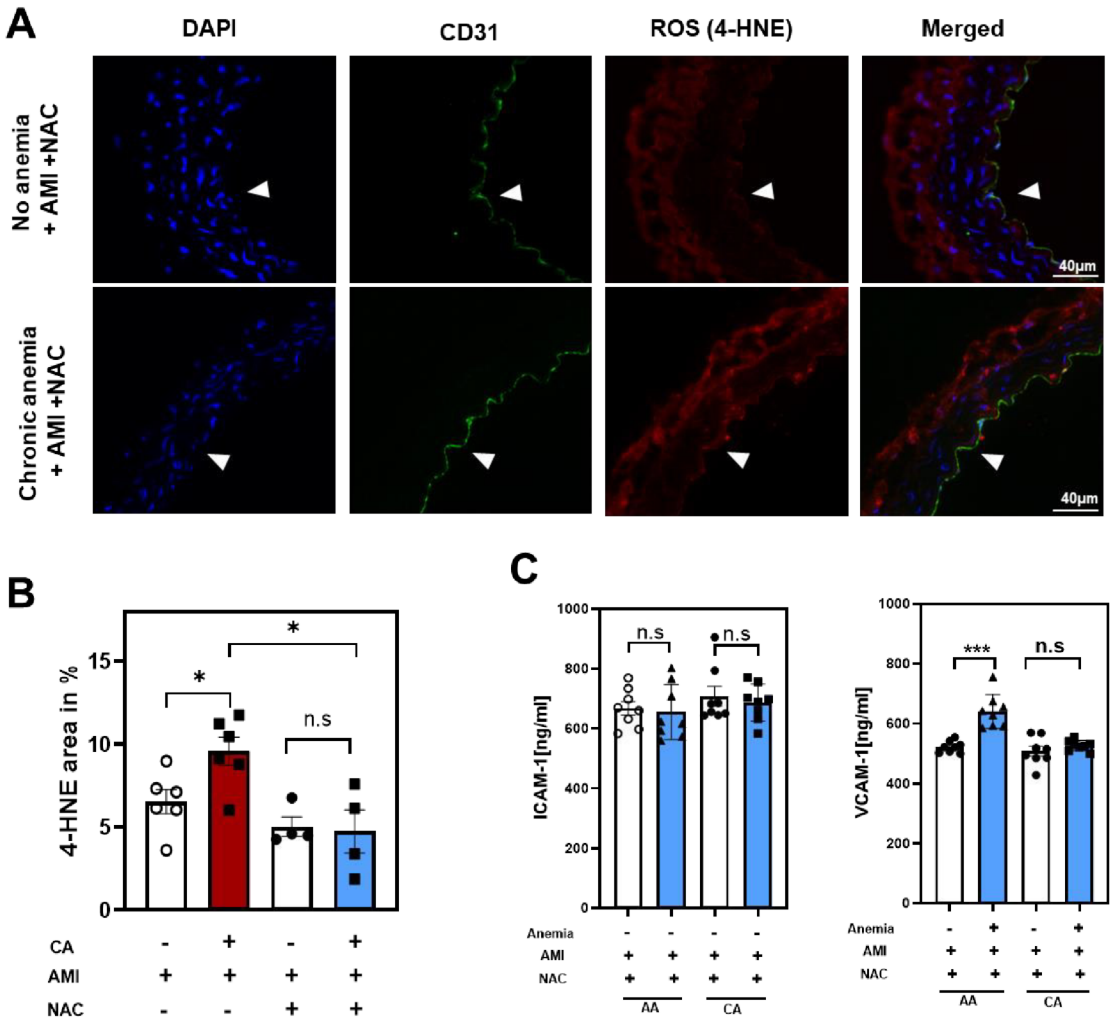


Figure 6: NAC treatment reduces ROS formation and endothelial inflammation in anemic mice 24 h post-AMI. (A-B) Thoracic aortas isolated from chronic anemic mice and respective non-anemic mice 24 h post IR. **(A)** Representative images of sections that are stained with endothelial cell marker CD31 (green) and ROS-marker 4-HNE (red). DAPI (blue) staining is used to detect the nucleus. Arrowheads are in the luminal side pointing towards the respective staining. For statistical analysis of the co-staining six images were evaluated and were compared using a student t-test between sham and anemic groups. Scale bar=40 µm. **(B)** Quantification of 4-HNE in chronic (dark red bar, squares), non-anemic (white bar, open circles), NAC treated chronic anemic (blue bar, squares) and non-anemic (white bar, closed circles) group of mice 24 h post-AMI. **(C)** Plasma ICAM-1 and VCAM-1 levels in acute (light blue, triangles), chronic (light blue, squares), and respective non-anemic groups (white bar, open and closed circles) of mice 24 h post-AMI. All values are presented as means ± SEM. *, $p \leq 0.05$; ***, $p \leq 0.001$; ns, not significant.

Anemic RBCs promote endothelial dysfunction

Previous studies have demonstrated that RBCs induce endothelial dysfunction in various cardiometabolic diseases (*20*). Given this, we investigated the potential role of anemic RBCs in mediating ED after AMI. First, we incubated RBCs from anemic and non-anemic mice 24 h post-AMI, co-incubated (2 h at 37°C) with WT aortic rings, and assessed the vascular responses. The endothelium-dependent relaxation responses to acetylcholine in the presence

of indomethacin were completely inhibited in the aortic rings incubated with anemic RBCs compared to non-anemic mice (Figure 7A). The relaxation responses in the presence of L-NAME were completely inhibited in both groups (Figure 7B), indicating that relaxation responses are entirely mediated by NO in these vessels. In addition, the relaxation responses to SNP were mildly abrogated in anemic RBCs-incubated aortic rings compared to the non-anemic group (Figure 7C).

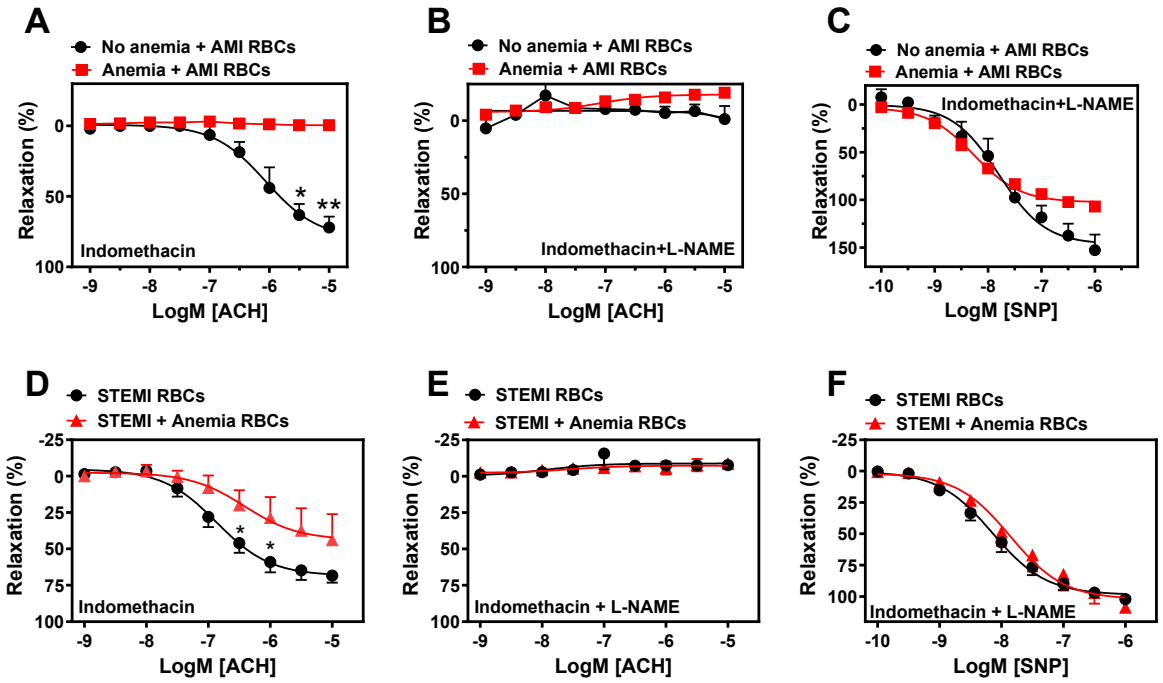


Figure 7: RBCs from anemic mice 24h post-AMI and anemic STEMI patients induce endothelial dysfunction. **(A-D)** RBCs were isolated from non-anemic (black circles) and anemic mice (red squares) 24 h post-AMI. **(E-H)** RBCs were isolated from STEMI patients without anemia (black circles) and with anemia (red triangles). Haematocrit (40%) was prepared in a KREBS buffer. Aortic rings from WT mice were incubated with haematocrit either 2 (mice) or 6 (humans) h and mounted in wire myograph. **(B, F)** Relaxation responses (%) to acetylcholine in the presence of indomethacin (10 µM, COX inhibitor) in aortic rings incubated with mice RBCs. **(C, G)** Relaxation responses to acetylcholine in the presence of indomethacin (10 µM, COX inhibitor). **(D, H)** Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 µM) in the presence of indomethacin and L-NAME. Values are shown as means ± SEM. n= 4 for mice (per group). STEMI group: n=8, STEMI + anemia: n=4. *, $p \leq 0.05$; **, $p \leq 0.01$. CRCs were analysed by Two-Way ANOVA and Bonferroni ‘s post-hoc test.

We also investigated whether RBCs from anemic STEMI patients induce ED. Our results showed that aortic rings incubated with RBCs from anemic STEMI patients showed reduced endothelial-dependent relaxation responses compared to STEMI patients without anemia (Figure 7D). These relaxation responses are endothelial-dependent and mediated by NO (Figure

7E). Endothelium-independent relaxation responses to SNP were similar in both groups (Figure 7F). These results further demonstrate that RBCs from anemic STEMI patients induce ED by altering the NO-mediated relaxation responses.

Discussion

In this study, we examined the effect of acute and chronic anemia on vascular function after AMI. The key findings of the study are that (1) AA and CA are associated with reduced endothelial NO-dependent relaxation responses *ex vivo* and impaired *in vivo* flow-mediated dilation responses 24 h post-AMI, (2) Plasma nitrate and nitrite levels were significantly reduced in AA and CA mice 24 h post-AMI, (3) VSMC contractile responses were specifically abrogated in large and small resistance arteries of CA mice 24 h post-AMI, (4) AA and CA are associated with increased production of reactive oxygen species (ROS) in the vessels, (5) NAC treatment improved vascular function (both contractile and relaxation responses) in AA and CA mice 24 h post-AMI, (6) RBCs from anemic mice 24 h post-AMI and STEMI patients with anemia induced vascular dysfunction in murine aortic rings, highlighting the potential role of RBCs in endothelial dysfunction.

Several comorbidities lead to adverse outcomes after AMI, particularly anemia, which is clinically presented in elderly patients during admission, hospitalization, or post-AMI (*27, 4*). Understanding the effects of blood loss anemia on vascular function is clinically relevant, as it is the main cause of reduced Hb during hospitalization due to blood sampling for laboratory tests or clinical interventions such as the insertion of catheters, endotracheal tubes, etc. (*28*). These interventions cannot be avoided in the hospital setting. Therefore, in this study, we examined both acute and chronic blood loss anemic mouse models, which reflect blood loss anemia during hospitalization in patients. Of note, we previously showed that these mouse models are mildly anemic, and we did not observe any severe consequences due to blood loss, such as oedema (*18, 19*). Additionally, AMI was induced in both mouse models to investigate the possible reasons for the poor prognosis of anemic patients after AMI in relation to overall vascular function. Interestingly, our findings demonstrate that AA results in a worsening of endothelial function in different types of vessels and also *in vivo* FMD responses. In our previous study we demonstrated that AA (without AMI) is associated with compensatory increased FMD responses (*18*). However, after AMI, the compensatory improved FMD responses are compromised, which is explained by increased oxidative stress and endothelial activation/inflammation. In addition, previous clinical studies demonstrated that anemia without comorbidities results in increased FMD responses in healthy volunteers, whereas anemia in combination with CKD or diabetes worsens endothelial function and thus FMD responses (*14–16*). Altogether,

co-morbidities and acute stress condition such as AMI worsen the endothelial function in anemia. To our knowledge, this is the first study to demonstrate how endothelial function is affected after AMI in anemia.

Studies have shown that uncoupling of eNOS leads to ED in several pathologies (*29*). Additionally, in different disease states, it has been shown under oxidative stress conditions, O_2 reacts with NO to form $ONOO^-$, resulting in eNOS uncoupling, lipid peroxidation, and vascular damage (*30, 31*). Moreover, eNOS uncoupling leads to generation superoxide instead of NO, further becoming a source of detrimental free radicals, impairing endothelial function (*32*). In this study, we observed an increase in the ROS oxidative product 4-HNE in the vessels of both AA and CA mice, indicating the potential role of ROS in mediating detrimental effects on the endothelium and, consequently, on NO bioavailability. In our recent study, we demonstrated that endothelium-dependent relaxation responses are impaired in aortic rings from CA mice (*19*). However, 24 h post-AMI, we did not observe significantly impaired relaxation responses in CA mice compared to non-anemic mice. A possible explanation for this could be that the amplitude of contraction of VSMC was drastically reduced, which may have masked the changes in endothelium-dependent relaxation measurements to acetylcholine in the *ex vivo* setting. The relaxation and contractile responses in femoral arteries are preserved in CA mice compared to the respective sham group. However, in small resistance saphenous arteries, the contraction and relaxation responses are significantly impaired in CA mice after AMI. These results indicate that the effects of CA vary across different vascular beds following AMI.

The effect of anemia on VSMC dysfunction is not well studied. However, a recent study demonstrated that intracellular iron deficiency leads to VSMC dysfunction, resulting in pulmonary hypertension mediated by increased endothelin (*33*). In addition, sickle cell anemia is also associated with VSMC dysfunction in pulmonary circulation due to increased oxidative stress, severe haemolysis, and inflammation (*34*). In the current study, we demonstrated that AA does not affect SMC contractile responses, whereas these responses are abrogated in CA. We believe that these effects are caused by chronic mild hypoxia due to the diminished oxygen-carrying capacity of RBCs to vascular smooth muscle cells in CA mice (*35*). The other possible explanation is that chronic hypoxia results in increased ROS production, which might disrupt the contractile machinery in VSMCs (*36*). However, we do not know the exact mechanism of how anemic RBCs impair the contractile responses which will be evaluated in the future.

Recent studies from our group and others demonstrated that RBCs plays a crucial role in cardiovascular diseases. In addition, several studies demonstrated that RBCs mediate ED in various pathological conditions, such as diabetes mellitus and hypercholesterolemia, due to elevated arginase 1 levels and increased ROS formation in endothelial cells mediated by RBC (*21, 22*). Our results from the co-incubation experiments of RBCs from anemic mice 24 h post-

AMI and anemic STEMI patients with murine aortic rings show a clear induction of ED in the aortic rings. However, performing co-incubation studies with RBCs and Nω-Hydroxy-nor-L-arginine acetate (nor-NOHA), a specific arginase inhibitor, did not show any improvement in endothelial function after arginase inhibition in the murine aortas (data not shown). These results suggest a different underlying mechanism of inducing ED in anemia. Extracellular vesicles (EVs) are known to mediate intercellular communication by transferring different molecules and therefore playing an important role in cardio vascular diseases (*37, 38*). Previous studies in sickle cell anemia and diabetes mellitus type 2 have implicated RBC-derived extracellular vesicles (REVs) in mediating vascular dysfunction (*39, 40*). In the current study, we primarily focused on the assessment and comparison of vascular function in acute and chronic anemia after AMI. The investigation of the potential role of REVs in anemia-associated ED after AMI was beyond the scope of this study, but it needs further evaluation. Notably, we cannot overlook some limitations of this study. We strongly believe that the observations made here need to be re-evaluated in another type of anemic mouse model to determine whether the effects are specific to this particular type of anemia or apply to all types of anemia.

Conclusions

Our data suggest that both acute and chronic blood-loss anemia are associated with decreased NO production after AMI due to increased inflammation and ROS production. Additionally, chronic anemia specifically led to reduced smooth muscle contractile responses. We also demonstrated that RBCs from anemic STEMI patients attenuate endothelial-dependent relaxation responses as well as vascular smooth muscle contractile responses, highlighting the potential role of anemic RBCs in vascular dysfunction. Furthermore, we showed that treating anemic mice with a ROS scavenger (NAC) improved relaxation responses in both acute and chronic anemic mice. Taken together, NAC supplementation improves vascular function in blood-loss anemia after AMI.

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

IS and RC designed the work. IS contributed to qRT-PCR, biochemical assays, Western Blot analysis. IS, AS, FC, RC, VY contributed to myograph and organ bath experiments. PW contributed to arrangement of STEMI patient samples. AS, IS contributed to immunohistochemistry. SB contributed to anemia induction. IS and RC interpreted the data and drafted the manuscript. MK, CJ, NG, RC obtained the funding. MRH, AP, CJ, NG, MK, RC critically revised the manuscript. All authors contributed to the article and approved the data for submission.

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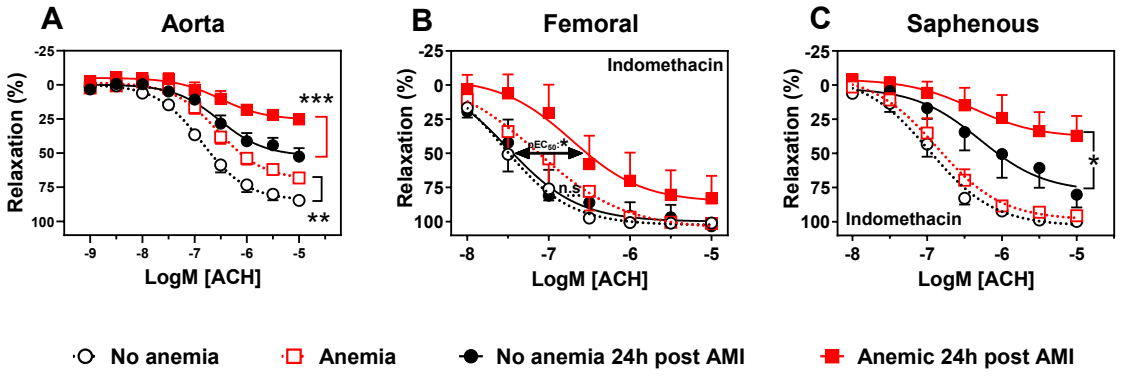
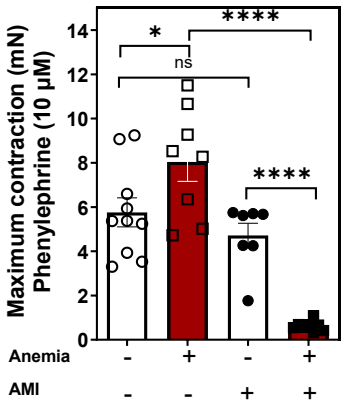
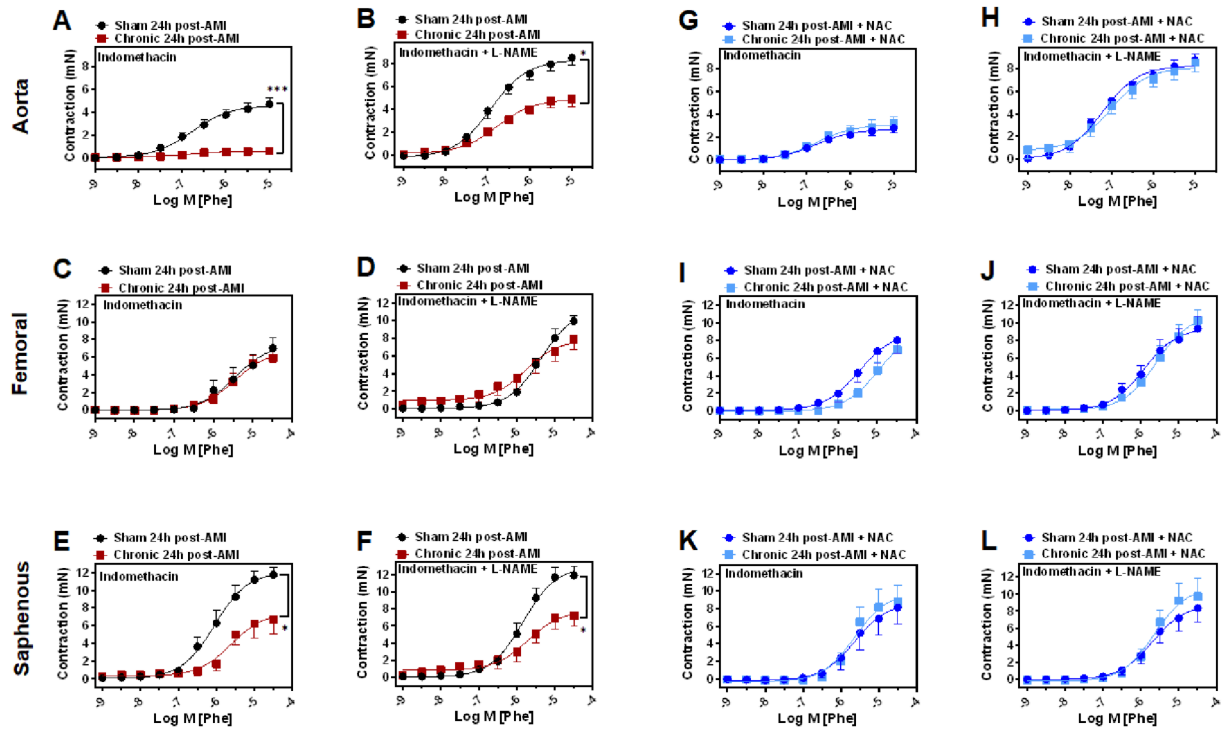
Supplementary data



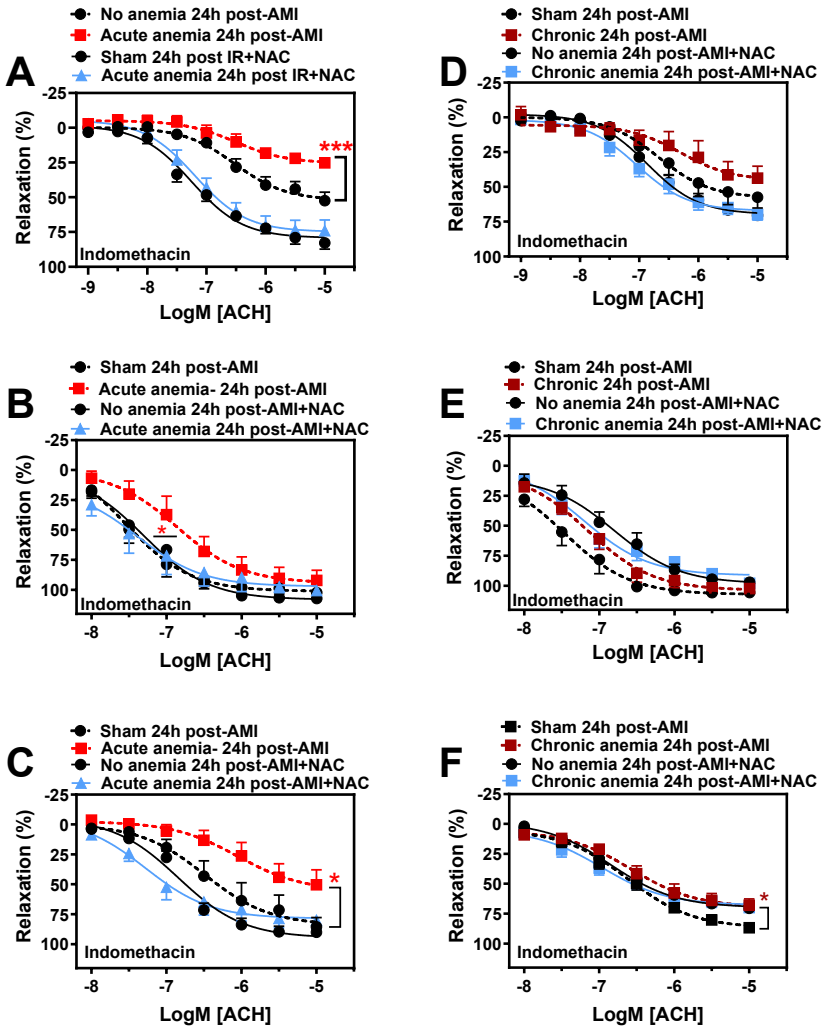
Supplementary Figure 1: Acute anemia is associated with aggravated endothelial dysfunction 24 h post-AMI. Aorta, femoral and saphenous arteries were isolated from acute anemic mice (red squares) and respective non-anemic mice (black circles) before (dashed line) and after (solid line) AMI. Arterial segments were pre-contracted using phenylephrine (PHE; 10 µM), and their relaxation responses to acetylcholine (ACH; 10 nM-10 µM) were assessed using wire myograph. **(A-C)** Relaxation (%) in the presence of indomethacin (10 µM, a COX inhibitor). All values are mean values ± SEM (n = 8–10 per group). *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001. Concentration-response curves (CRCs) were analysed by Two-Way ANOVA and Bonferroni 's post-hoc test to compare acute and respective non-anemic group of mice.



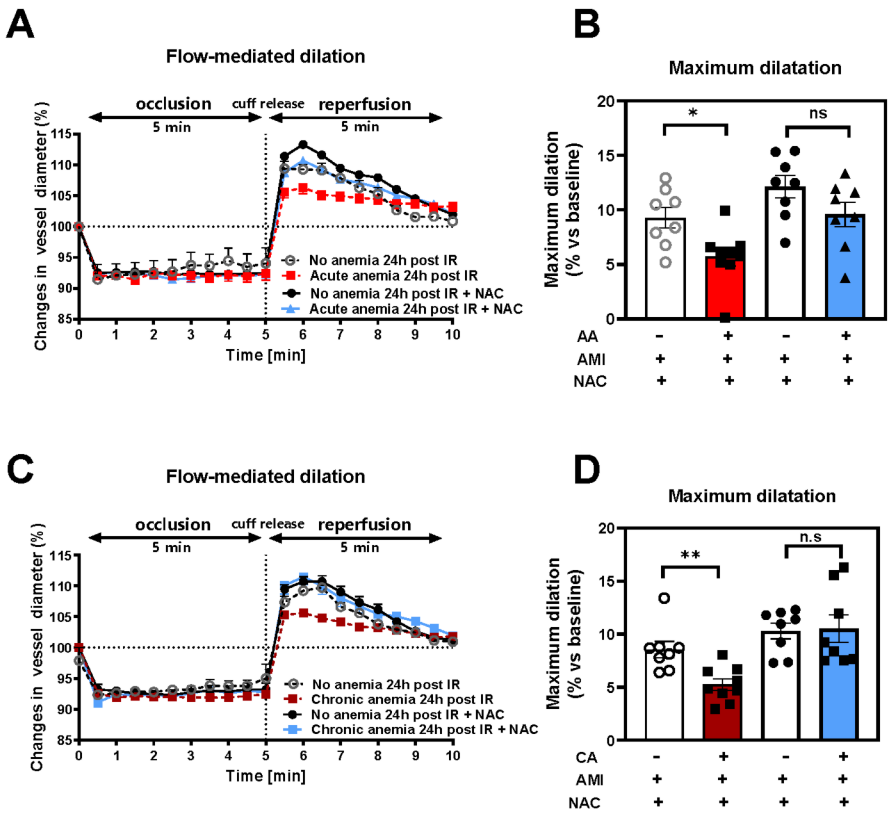
Supplementary Figure 2: Comparison of contractile responses to phenylephrine in chronic anemic mice before and after AMI in the aorta. Aortic segments were isolated from chronic anemic mice (dark red squares) and respective non-anemic mice (black circles) 24 h post-AMI. For comparison, aortic segments from chronic anemic (dark red, open squares) and respective non-anemic mice (black, open circles) without AMI were also included. Contraction was measured in the presence of indomethacin (10 µM, a COX inhibitor). A Student's t-test was used to compare the two groups, with p ≤ 0.01. All values are presented as means ± SEM (n = 8–10 per group). *, p ≤ 0.05; ****, p ≤ 0.0001.



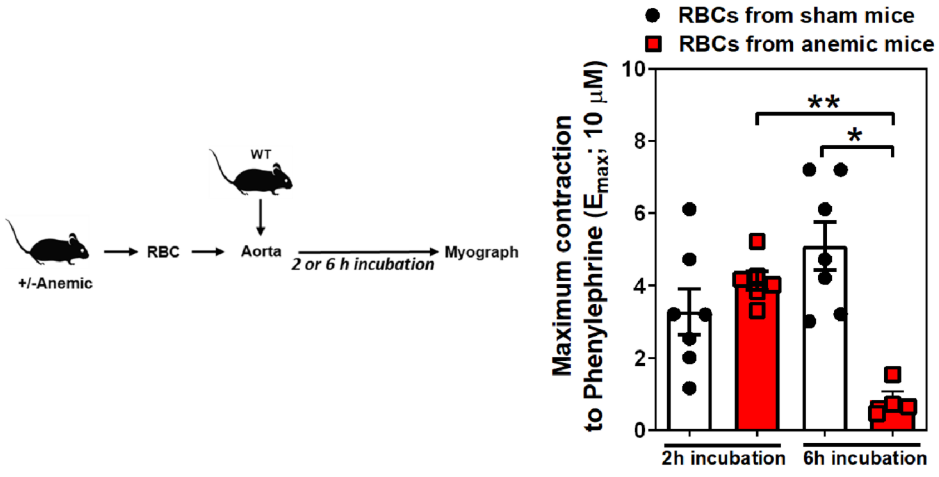
Supplementary Figure 3: Contractile responses are abrogated in chronic anemic mice 24 h post-AMI, and are improved after NAC supplementation. Arterial segments of the aorta **(A-B, G-H)**, femoral **(C-D, I-J)**, and saphenous arteries **(E-F, K-L)** were isolated from chronic anemic (dark red squares), respective non-anemic mice (black circles), chronic anemic mice + NAC (light blue squares), and non-anemic mice + NAC (dark blue circles) 24 h post-AMI. **(A, C, E, G, I, K)** Contraction in the presence of indomethacin (10 µM, a COX inhibitor). **(B, D, F, H, J, L)** Contraction in the presence of indomethacin and L-NAME (100 µM, a NOS inhibitor). All values are mean values ± SEM (n = 8–10 per group). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. CRCs were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test to compare acute, chronic, and respective sham groups of mice.



Supplementary Figure 4: Comparison of endothelium-dependent relaxation responses in acute and chronic anemic mice before and after NAC treatment, 24 h post-AMI. Arterial segments of the aorta **(A, D)**, femoral **(B, E)**, and saphenous arteries **(C, F)** were isolated from N-acetyl cysteine (NAC)-treated acute anemic (light blue triangles), chronic anemic (light blue squares), and respective non-anemic (black circles) mice, 24 h post-AMI. Additionally, segments from acute anemic (dashed line, red squares), chronic anemic (dashed line, dark red squares), and respective non-anemic (dashed line, black circles) mice, 24 h post-AMI, were included for comparison. Arterial segments were pre-contracted using phenylephrine (PHE; 10 µM), and their relaxation responses to acetylcholine (ACH; 10 nM–10 µM) were assessed using wire myography. **(A–F)** Relaxation was measured in the presence of indomethacin (10 µM, a COX inhibitor). All values are presented as means ± SEM (n = 8–10 per group). *, $p \leq 0.05$; ***, $p \leq 0.001$. CRCs were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test to compare acute, chronic, and respective sham groups of mice.

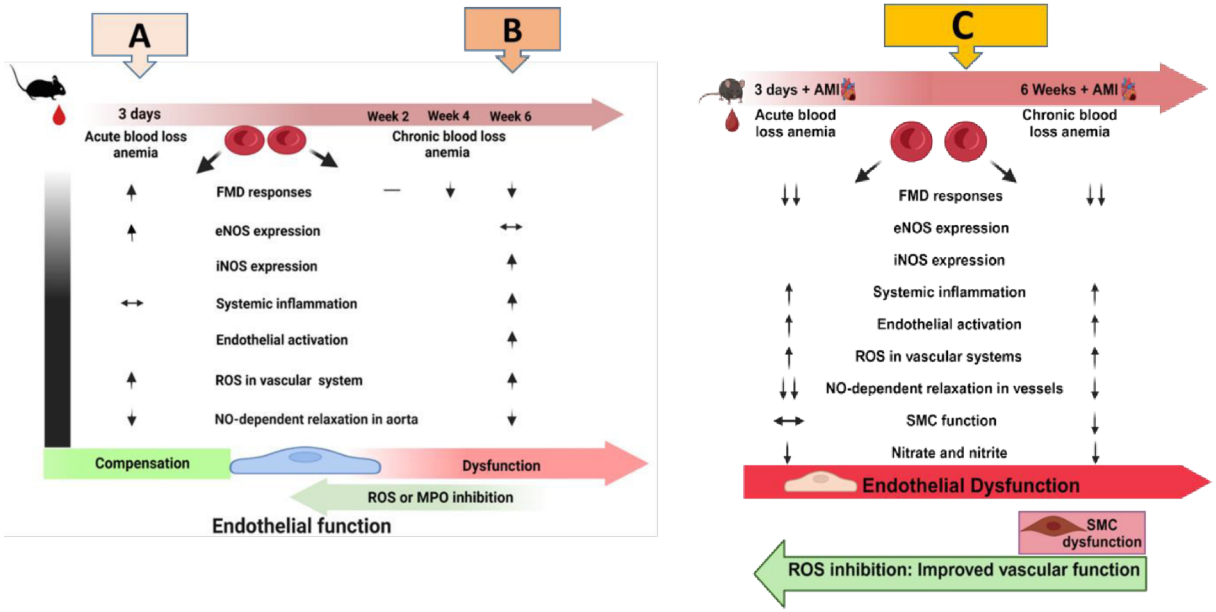


Supplementary Figure 5: Comparison of flow-mediated dilation (FMD) response in NAC treated acute and chronic anemic mice 24 h post-AMI. **(A, C)** Changes in vessel diameter in N-acetyl cysteine treated (NAC) acute (blue triangles), chronic (blue squares), and respective non-anemic groups (black circles) of mice 24 h post-AMI. Additionally, acute (red squares), chronic (dark red squares), and respective non-anemic (open or closed black circles) groups of mice 24 h post-AMI were used as comparison. **(B, D)** Maximal FMD response (% ratio vs. baseline). AA, Acute anemia; CA, Chronic anemia. The values are presented as means ± SEM (n=8-9 per group). *, $p \leq 0.05$; **, $p \leq 0.01$; ns, not significant. The average FMD in the reperfusion phase was compared with the Student's t-test between the groups.



Supplementary Figure 6: RBCs from anemic mice abrogate the contractile responses. RBCs were isolated from non-anemic (black circles) and anemic mice (red squares). Hematocrit (40%) was prepared in a KREBS buffer.

Aortic rings from WT mice were incubated with hematocrit for either 2 or 6 h and mounted in a wire myograph. Contractile responses to Phenylephrine (10 µM) were measured. All values are mean values ± SEM. *, $p \leq 0.05$; **, $p \leq 0.01$. A Student's t-test was used to compare between the two groups.



Supplementary Figure 7. Illustration of acute and chronic blood loss anemia effects on vascular function before **(A-B)** and after AMI **(C).** The comparison is based on *Wischmann et al. 2020* **(A),** *Chennupati et al. 2023*, and the current research by *Solga et al.* **(C). (A-B)** Contrary to acute anemia, chronic anemia without AMI is associated with a progressive loss of FMD responses. **(C)** After AMI, endothelial dysfunction is aggravated in acute anemic mice, whereas chronic anemic mice show both endothelial (reduced FMD) and vascular smooth muscle cell (SMC) dysfunction. Inhibition of ROS or myeloperoxidase improved endothelial dysfunction in chronic anemia, as demonstrated in *Chennupati et al*. In the current study, ROS inhibition with N-acetyl cysteine improved both endothelial and vascular smooth muscle function in both acute and chronic anemic mice after AMI. ↑: increased; ↓: decreased; ↔: unchanged; blank space: not investigated

	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n	p-value pEC_{50}	p-value E_{max}
PHE (Indomethacin)								
Sham 24h AMI vs. Acute 24h AMI	6.77 ± 0.14	4.71 ± 0.55	7	6.92 ± 0.19	5.58 ± 0.84	8	0.55	0.42
Sham 24h + NAC vs. Acute 24h+NAC	6.87 ± 0.14	5.23 ± 0.73	8	6.90 ± 0.11	5.74 ± 0.49	7	0.87	0.58
Sham 24h vs. Sham 24h+NAC	6.77 ± 0.14	4.71 ± 0.55	7	6.87 ± 0.14	5.23 ± 0.73	8	0.62	0.59
Acute 24h vs. Acute 24h+NAC	6.92 ± 0.19	5.58 ± 0.84	8	6.90 ± 0.11	5.74 ± 0.49	7	0.93	0.88
PHE (Indomethacin +L-NAME)								
Sham 24h vs. Acute 24h	6.92 ± 0.09	8.49 ± 0.59	7	7.23 ± 0.23	8.15 ± 1.50	8	0.26	0.84
Sham 24h + NAC vs. Acute 24h+NAC	7.48 ± 0.10	11.80 ± 0.80	8	7.46 ± 0.09	10.04 ± 0.68	7	0.89	0.12
Sham 24h vs. Sham 24h+NAC	6.92 ± 0.09	8.49 ± 0.59	7	7.48 ± 0.10	11.80 ± 0.80	8	**0.001**	**0.006**
Acute 24h vs. Acute 24h+NAC	7.23 ± 0.23	8.15 ± 1.50	8	7.46 ± 0.09	10.04 ± 0.68	7	0.39	0.29
ACH (Indomethacin)								
Sham 24h vs. Acute 24h	6.52 ± 0.15	52.45 ± 6.02	7	6.51 ± 0.22	25.22 ± 2.88	8	0.97	**0.0009**
Sham 24h + NAC vs. Acute 24h+NAC	7.25 ± 0.09	80.19 ± 4.57	8	7.19 ± 0.19	74.05 ± 7.62	7	0.78	0.49
Sham 24h vs. Sham 24h+NAC	6.52 ± 0.15	52.45 ± 6.02	7	7.25 ± 0.09	80.19 ± 4.57	8	**0.0009**	**0.003**
Acute 24h vs. Acute 24h+NAC	6.51 ± 0.22	25.22 ± 2.88	8	7.19 ± 0.19	74.05 ± 7.62	7	**0.04**	**0.0001**
SNP								
Sham 24h vs. Acute 24h	6.65 ± 0.08	95.44 ± 4.50	7	6.75 ± 0.08	85.75 ± 4.15	8	0.4	0.14
Sham 24h + NAC vs. Acute 24h+NAC	7.38 ± 0.05	99.47 ± 0.72	8	7.38 ± 0.09	104.75 ± 2.67	7	1	0.06
Sham 24h vs. Sham 24h+NAC	6.65 ± 0.08	95.44 ± 4.50	7	7.38 ± 0.05	99.47 ± 0.72	8	**0.0001**	0.36
Acute 24h vs. Acute 24h+NAC	6.75 ± 0.08	79.28 ± 2.11	8	7.38 ± 0.09	104.75 ± 2.67	7	**0.0002**	**0.0001**

Supplementary Table 1. The effect of acute anemia on contraction and endothelial-dependent and -independent relaxation responses 24h post-AMI in the aorta. The contraction responses to PHE are presented as maximal contraction (E_{max}) and pEC50 values. The maximal relaxation responses to ACH or SNP are expressed as a percentage reduction of the maximal contractile response to 10 μM PHE. As aortic rings did not show any relaxation in the presence of L-NAME in all experiments, the data is excluded from the table. All values are shown as mean ± SEM. E_{max} and pEC_{50} between the two groups are compared using Student's t-test, and the significant values are represented in the table. ACH, acetylcholine; INDO, indomethacin; SNP, sodium nitroprusside; NAC, N-acetyl cysteine.

	pEC50	E_{max}	n	pEC50	E_{max}	n	p-value pEC50	p-value Emax
PHE (Indomethacin)								
Sham 24h vs. Acute 24h	5.27 ± 0.19	6.56 ± 1.33	9	4.85 ± 0.20	5.12 ± 0.98	8	0.15	0.41
Sham 24h + NAC vs. Acute 24h+NAC	5.02 ± 0.10	7.91 ± 0.64	7	4.67 ± 0.18	5.47 ± 0.64	6	0.1	0.02
Sham 24h + NAC vs. Sham 24h+NAC	5.27 ± 0.19	6.56 ± 1.33	9	5.02 ± 0.1	7.91 ± 0.64	7	0.3	0.42
Acute 24h + NAC vs. Acute 24h+NAC	4.85 ± 0.20	5.12 ± 0.98	8	4.67 ± 0.18	5.47 ± 0.64	6	0.53	0.79
PHE (Indomethacin +L-NAME)								
Sham 24h vs. Acute 24h	5.99 ± 0.14	8.20 ± 1.0	9	5.56 ± 0.14	7.16 ± 0.85	8	**0.05**	0.45
Sham 24h + NAC vs. Acute 24h+NAC	5.49 ± 0.11	9.26 ± 0.63	7	4.65 ± 0.24	6.36 ± 1.07	6	**0.007**	**0.03**
Sham 24h + NAC vs. Sham 24h+NAC	5.99 ± 0.14	8.20 ± 1.0	9	5.49 ± 0.11	9.26 ± 0.63	7	**0.02**	0.42
Acute 24h + NAC vs. Acute 24h+NAC	5.56 ± 0.14	7.16 ± 0.85	8	4.65 ± 0.24	6.36 ± 1.07	6	**0.005**	0.56
ACH (Indomethacin)								
Sham 24h vs. Acute 24h	7.55 ± 0.20	102.26 ± 2.32	9	6.86 ± 0.24	92.12 ± 8.37	8	**0.04**	0.24
Sham 24h + NAC vs. Acute 24h+NAC	7.30 ± 0.17	107.20 ± 1.10	7	6.51 ± 0.28	99.81 ± 2.71	6	**0.03**	**0.03**
Sham 24h + NAC vs. Sham 24h+NAC	7.55 ± 0.20	102.26 ± 2.32	9	7.30 ± 0.17	107.20 ± 1.10	7	0.37	0.1
Acute 24h + NAC vs. Acute 24h+NAC	6.86 ± 0.24	92.12 ± 8.37	8	6.51 ± 0.28	99.81 ± 2.71	6	0.36	0.46
ACH (Indomethacin +L-NAME)								
Sham 24h vs. Acute 24h	6.66 ± 0.22	74.73 ± 6.73	9	6.46 ± 0.13	82.29 ± 5.55	8	0.46	0.41
Sham 24h + NAC vs. Acute 24h+NAC	7.09 ± 0.19	83.40 ± 5.30	7	6.47 ± 0.19	93.68 ± 2.65	6	**0.04**	0.13
Sham 24h + NAC vs. Sham 24h+NAC	6.66 ± 0.22	74.73 ± 6.73	9	7.09 ± 0.19	83.40 ± 5.30	7	0.18	0.35
Acute 24h + NAC vs. Acute 24h+NAC	6.46 ± 0.13	82.29 ± 5.55	8	6.47 ± 0.19	93.68 ± 2.65	6	0.96	0.12
SNP								
Sham 24h vs. Acute 24h	8.00 ± 0.15	116.52 ± 3.06	9	8.10 ± 0.14	112.43 ± 4.97	8	0.64	0.48
Sham 24h + NAC vs. Acute 24h+NAC	7.53 ± 0.11	108.14 ± 0.62	7	7.23 ± 0.10	100.92 ± 1.22	6	**0.07**	**0.0002**
Sham 24h + NAC vs. Sham 24h+NAC	8.00 ± 0.15	116.52 ± 3.06	9	7.53 ± 0.11	108.14 ± 0.62	7	**0.03**	**0.03**
Acute 24h + NAC vs. Acute 24h+NAC	8.10 ± 0.14	112.43 ± 4.97	8	7.23 ± 0.10	100.92 ± 1.22	6	**0.0005**	**0.07**

Supplementary Table 2. The effect of acute anemia on endothelial-dependent and -independent relaxation responses 24h post-AMI in the femoral artery. The contraction responses to PHE are presented as maximal contraction (Emax) and pEC50 values. The maximal relaxation responses to ACH or SNP are expressed as a percentage reduction of the maximal contractile response to 10 μM PHE. All values are shown as mean ± SEM. Emax and pEC50 between the two groups are compared using Student's t-test, and the significant values are represented in the table. ACH, acetyl choline; INDO, indomethacin; SNP, sodium nitroprusside; NAC, N-acetyl cysteine. All values are shown as mean ± SEM.

	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n	p-value pEC_{50}	p-value E_{max}
PHE (Indomethacin)								
Sham 24h vs. Acute 24h	5.50 ± 1.60	9.47 ± 1.81	7	4.82 ± 0.29	7.88 ± 2.45	6	0.71	0.61
Sham 24h + NAC vs. Acute 24h+NAC	5.29 ± 0.11	13.46 ±1.43	8	5.79 ± 0.19	9.70 ± 1.77	11	0.06	0.14
Sham 24h vs. Sham 24h+NAC	5.50 ± 1.60	9.47 ± 1.81	7	5.29 ± 0.11	13.46 ±1.43	8	0.86	0.10
Acute 24h vs. Acute 24h+NAC	4.82 ± 0.29	7.88 ± 2.45	6	5.79 ± 0.19	9.70 ± 1.77	11	**0.01**	0.55
PHE (Indomethacin +L-NAME)								
Sham 24h vs. Acute 24h	5.77 ± 0.16	9.62 ± 1.67	7	5.18 ± 0.21	8.03 ± 2.24	6	**0.04**	0.57
Sham 24h + NAC vs. Acute 24h+NAC	5.78 ± 0.11	13.96 ± 1.31	8	5.81 ± 0.17	10.93 ± 1.96	11	0.89	0.25
Sham 24h vs. Sham 24h+NAC	5.77 ± 0.16	9.62 ± 1.67	7	5.78 ± 0.11	13.96 ± 1.31	8	0.96	0.09
Acute 24h vs. Acute 24h+NAC	5.18 ± 0.21	8.03 ± 2.24	6	5.81 ± 0.17	10.93 ± 1.96	11	**0.04**	0.37
ACH (Indomethacin)								
Sham 24h vs. Acute 24h	6.49 ± 0.23	85.44 ± 7.59	7	6.05 ± 0.31	50.36 ± 12.41	6	0.27	**0.03**
Sham 24h + NAC vs. Acute 24h+NAC	6.84 ± 0.10	90.00 ± 4.59	8	7.27 ± 0.26	79.94 ± 7.57	11	0.19	0.32
Sham 24h vs. Sham 24h+NAC	6.49 ± 0.23	85.44 ± 7.59	7	6.84 ± 0.1	90.00 ± 4.59	8	0.17	0.61
Acute 24h vs. Acute 24h+NAC	6.05 ± 0.31	50.36 ± 12.41	6	7.27 ± 0.26	79.94 ± 7.57	11	**0.01**	**0.05**
ACH (Indomethacin +L-NAME)								
Sham 24h vs. Acute 24h	6.30 ± 0.28	37.67 ± 6.70	7	6.28 ± 0.42	36.64 ± 7.90	6	0.97	0.92
Sham 24h + NAC vs. Acute 24h+NAC	6.32 ± 0.19	53.59 ± 7.32	8	6.37 ± 0.22	38.25 ± 4.88	11	0.87	0.09
Sham 24h vs. Sham 24h+NAC	6.30 ± 0.28	37.67 ± 6.70	7	6.32 ± 0.19	53.59 ± 7.32	8	0.95	0.14
Acute 24h vs. Acute 24h+NAC	6.28 ± 0.42	36.64 ± 7.90	6	6.37 ± 0.22	38.25 ± 4.88	11	0.86	0.86
SNP								
Sham 24h vs. Acute 24h	6.84 ± 0.13	100.23 ± 2.19	7	7.50 ± 0.22	94.56 ± 3.51	6	**0.02**	0.18
Sham 24h + NAC vs. Acute 24h+NAC	6.58 ± 0.13	98.99 ± 3.11	8	6.69 ± 0.12	97.50 ± 1.60	11	0.55	0.65
Sham 24h vs. Sham 24h+NAC	6.84 ± 0.13	100.23 ± 2.19	7	6.58 ± 0.13	98.99 ± 3.11	8	0.18	0.76
Acute 24h vs. Acute 24h+NAC	7.50 ± 0.22	94.56 ± 3.51	6	6.69 ± 0.12	97.50 ± 1.60	11	**0.003**	0.39

Supplementary Table 3. The effect of acute anemia on endothelial-dependent and -independent relaxation responses 24h post-AMI in the saphenous artery. The contraction responses to PHE are presented as maximal contraction (Emax) and pEC50 values. The maximal relaxation responses to ACH or SNP are expressed as a percentage reduction of the maximal contractile response to 10 μM PHE. All values are shown as mean ± SEM. Emax and pEC50 between the two groups are compared using Student's t-test, and the significant values are represented in the table. ACH, acetyl choline; INDO, indomethacin; SNP, sodium nitroprusside; NAC, N-acetyl cysteine. All values are shown as mean ± SEM.

	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n	p-value pEC_{50}	p-value E_{max}
PHE (Indomethacin)								
Sham 24h vs. Chronic 24h	6.82 ± 0.19	3.56 ± 0.60	7	6.86 ± 0.17	0.62 ± 0.08	8	0.88	**0.0002**
Sham 24h + NAC vs. Chronic 24h+NAC	6.79 ± 0.14	2.77 ± 0.38	12	6.78 ± 0.21	3.14 ± 0.64	11	0.97	0.62
Sham 24h vs. Sham 24h+NAC	6.82 ± 0.19	3.56 ± 0.60	7	6.79 ± 0.14	2.77 ± 0.38	12	0.90	0.26
Chronic 24h vs. Chronic 24h+NAC	6.86 ± 0.17	0.62 ± 0.08	8	6.78 ± 0.21	3.14 ± 0.64	11	0.78	**0.004**
PHE (Indomethacin +L-NAME)								
Sham 24h vs. Chronic 24h	7.15 ± 0.18	7.57 ± 1.05	7	6.78 ± 0.14	4.90 ± 0.69	8	0.12	**0.05**
Sham 24h + NAC vs. Chronic 24h+NAC	7.22 ± 0.08	8.74 ± 0.54	12	7.01 ± 0.18	8.23 ± 0.87	11	0.28	0.62
Sham 24h vs. Sham 24h+NAC	7.15 ± 0.18	7.57 ± 1.05	7	7.22 ± 0.08	8.74 ± 0.54	12	0.69	0.29
Chronic 24h vs. Chronic 24h+NAC	6.78 ± 0.14	4.90 ± 0.69	8	7.01 ± 0.18	8.23 ± 0.87	11	0.36	**0.01**
ACH (Indomethacin)								
Sham 24h vs. Chronic 24h	6.67 ± 0.19	57.44 ± 7.82	7	6.30 ± 0.35	43.64 ± 8.61	8	0.39	0.26
Sham 24h + NAC vs. Chronic 24h+NAC	6.86 ± 0.11	70.45 ± 2.93	12	7.04 ± 0.13	70.25 ± 4.11	11	0.3	0.97
Sham 24h vs. Sham 24h+NAC	6.67 ± 0.19	57.44 ± 7.82	7	6.86 ± 0.11	70.45 ± 2.93	12	0.34	0.08
Chronic 24h vs. Chronic 24h+NAC	6.30 ± 0.35	43.64 ± 8.61	8	7.04 ± 0.13	70.25 ± 4.11	11	**0.04**	**0.007**
SNP								
Sham 24h vs. Chronic 24h	7.98 ± 0.11	97.18 ± 2.97	7	7.78 ± 0.06	100.58 ± 2.66	8	0.12	0.41
Sham 24h + NAC vs. Chronic 24h+NAC	7.74 ± 0.06	96.41 ± 1.54	12	7.63 ± 0.09	99.37 ± 2.77	11	0.31	0.35
Sham 24h vs. Sham 24h+NAC	7.98 ± 0.11	97.18 ± 2.97	7	7.74 ± 0.06	96.41 ± 1.54	12	**0.05**	0.80
Chronic 24h vs. Chronic 24h+NAC	7.78 ± 0.06	100.58 ± 2.66	8	8.07 ± 0.10	99.37 ± 2.77	11	**0.04**	0.76

Supplementary Table 4. The effect of chronic anemia on endothelial-dependent and -independent relaxation responses 24h post-AMI in the aorta. The contraction responses to PHE are presented as maximal contraction (Emax) and pEC50 values. The maximal relaxation responses to ACH or SNP are expressed as a percentage reduction of the maximal contractile response to 10 μM PHE. As aortic rings did not show any relaxation in the presence of L-NAME in all experiments, the data is excluded from table. All values are shown as mean ± SEM. Emax and pEC50 between the two groups are compared using Student's t-test, and the significant values are represented in the table. ACH, acetyl choline; INDO, indomethacin; SNP, sodium nitroprusside; NAC, N-acetyl cysteine. All values are shown as mean ± SEM.

	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n	p-value pEC_{50}	p-value E_{max}
PHE (Indomethacin)								
Sham 24h vs. Chronic 24h	5.46 ± 0.21	6.52 ± 1.17	9	5.46 ± 0.15	6.32 ± 0.84	9	1	0.38
Sham 24h + NAC vs. Chronic 24h+NAC	5.47 ± 0.18	8.61 ± 1.49	8	4.97 ± 0.18	7.27 ± 1.35	9	**0.07**	0.51
Sham 24h vs. Sham 24h+NAC	5.46 ± 0.21	6.52 ± 1.17	9	5.47 ± 0.18	8.61 ± 1.49	8	0.97	0.23
Chronic 24h vs. Chronic 24h+NAC	5.46 ± 0.15	6.32 ± 0.84	9	4.97 ± 0.18	7.27 ± 1.35	9	**0.05**	0.56
PHE (Indomethacin +L-NAME)								
Sham 24h vs. Chronic 24h	5.37 ± 0.09	9.92 ± 0.65	9	5.77 ± 0.25	7.84 ± 1.11	9	0.15	0.13
Sham 24h + NAC vs. Chronic 24h+NAC	5.92 ± 0.15	9.32 ± 1.30	8	5.59 ± 0.14	10.23 ± 1.28	9	0.13	0.63
Sham 24h vs. Sham 24h+NAC	5.37 ± 0.09	9.92 ± 0.65	9	5.92 ± 0.15	9.32 ± 1.30	8	**0.006**	0.68
Chronic 24h vs. Chronic 24h+NAC	5.77 ± 0.25	7.84 ± 1.11	9	5.59 ± 0.14	10.23 ± 1.28	9	0.54	0.18
ACH (Indomethacin)								
Sham 24h vs. Chronic 24h	7.46 ± 0.2	105.62 ± 1.53	9	7.15 ± 0.14	102.26 ± 4.43	9	0.22	0.48
Sham 24h + NAC vs. Chronic 24h+NAC	6.8 ± 0.15	96.93 ± 2.18	8	7.11 ± 0.18	97.80 ± 2.65	9	0.21	0.81
Sham 24h vs. Sham 24h+NAC	7.46 ± 0.2	105.62 ± 1.53	9	6.8 ± 0.15	96.93 ± 2.18	8	**0.02**	**0.005**
Chronic 24h vs. Chronic 24h+NAC	7.15 ± 0.14	102.26 ± 4.43	9	7.11 ± 0.18	97.80 ± 2.65	9	0.86	0.4
ACH (Indomethacin +L-NAME)								
Sham 24h vs. Chronic 24h	6.78 ± 0.09	93.81 ± 1.48	9	6.41 ± 0.29	73.84 ± 8.60	9	0.24	**0.04**
Sham 24h + NAC vs. Chronic 24h+NAC	6.63 ± 0.19	82.33 ± 6.39	8	6.47 ± 0.14	79.48 ± 4.74	9	0.5	0.72
Sham 24h vs. Sham 24h+NAC	6.78 ± 0.09	93.81 ± 1.48	9	6.63 ± 0.19	82.33 ± 6.39	8	0.47	0.08
Chronic 24h vs. Chronic 24h+NAC	6.41 ± 0.29	73.84 ± 8.60	9	6.47 ± 0.14	79.48 ± 4.74	9	0.85	0.57
SNP								
Sham 24h vs. Chronic 24h	8.06 ± 0.11	108.34 ± 1.05	9	7.15 ± 0.18	108.35 ± 2.78	9	**0.0005**	1
Sham 24h + NAC vs. Chronic 24h+NAC	7.21 ± 0.08	107.65 ± 1.24	8	6.94 ± 0.09	102.01 ± 2.22	9	**0.04**	**0.05**
Sham 24h vs. Sham 24h+NAC	8.06 ± 0.11	108.34 ± 1.05	9	7.21 ± 0.08	107.65 ± 1.24	8	**0.0001**	0.73
Chronic 24h vs. Chronic 24h+NAC	7.15 ± 0.18	108.35 ± 2.78	9	6.94 ± 0.09	102.01 ± 2.22	9	0.31	0.09

Supplementary Table 5. The effect of chronic anemia on endothelial-dependent and -independent relaxation responses 24h post-AMI in the femoral artery. The contraction responses to PHE are presented as maximal contraction (Emax) and pEC50 values. The maximal relaxation responses to ACH or SNP are expressed as a percentage reduction of the maximal contractile response to 10 μM PHE. All values are shown as mean ± SEM. Emax and pEC50 between the two groups are compared using Student's t-test, and the significant values are represented in the table. ACH, acetyl choline; INDO, indomethacin; SNP, sodium nitroprusside; NAC, N-acetyl cysteine. All values are shown as mean ± SEM.

	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n	p-value pEC_{50}	p-value E_{max}
PHE (Indomethacin)								
Sham 24h vs. Chronic 24h	6.05 ± 0.12	11.75 ± 0.88	10	5.61 ± 0.24	6.71 ± 1.65	8	0.1	**0.01**
Sham 24h + NAC vs. Chronic 24h+NAC	5.64 ± 0.23	8.13 ± 1.88	7	5.66 ± 0.17	8.74 ± 1.95	7	0.95	0.83
Sham 24h vs. Sham 24h+NAC	6.05 ± 0.12	11.75 ± 0.88	10	5.64 ± 0.23	8.13 ± 1.88	7	0.11	0.07
Acute 24h vs. Acute 24h+NAC	5.61 ± 0.24	6.71 ± 1.65	8	5.66 ± 0.17	8.74 ± 1.95	7	0.87	0.43
PHE (Indomethacin +L-NAME)								
Sham 24h vs. Chronic 24h	5.84 ± 0.10	11.94 ± 1.10	10	5.72 ± 0.24	7.25 ± 1.26	8	0.63	**0.01**
Sham 24h + NAC vs. Chronic 24h+NAC	5.70 ± 0.18	8.36 ± 1.63	7	5.63 ± 0.18	9.75 ± 2.07	7	0.79	0.61
Sham 24h vs. Sham 24h+NAC	5.84 ± 0.10	11.94 ± 1.10	10	5.70 ± 0.18	8.36 ± 1.63	7	0.48	0.08
Acute 24h vs. Acute 24h+NAC	5.72 ± 0.24	7.25 ± 1.26	8	5.63 ± 0.18	9.75 ± 2.07	7	0.77	0.30
ACH (Indomethacin)								
Sham 24h vs. Chronic 24h	6.62 ± 0.10	86.70 ± 2.95	10	6.58 ± 0.16	67.93 ± 5.16	8	0.83	**0.004**
Sham 24h + NAC vs. Chronic 24h+NAC	6.35 ± 0.17	98.83 ± 3.93	7	6.47 ± 0.19	89.88 ± 6.48	7	0.65	0.26
Sham 24h vs. Sham 24h+NAC	6.62 ± 0.10	86.70 ± 2.95	10	6.35 ± 0.17	98.83 ± 3.93	7	0.17	**0.02**
Acute 24h vs. Acute 24h+NAC	6.58 ± 0.16	67.93 ± 5.16	8	6.47 ± 0.19	89.88 ± 6.48	7	0.66	**0.02**
ACH (Indomethacin +L-NAME)								
Sham 24h vs. Chronic 24h	6.61 ± 0.32	36.70 ± 6.27	10	6.20 ± 0.36	27.89 ± 5.62	8	0.41	0.32
Sham 24h + NAC vs. Chronic 24h+NAC	5.61 ± 0.24	38.92 ± 5.60	7	6.08 ± 0.21	38.53 ± 5.50	7	0.17	0.96
Sham 24h vs. Sham 24h+NAC	6.61 ± 0.32	36.70 ± 6.27	10	5.61 ± 0.24	38.92 ± 5.60	7	**0.04**	0.81
Acute 24h vs. Acute 24h+NAC	6.20 ± 0.36	27.89 ± 5.62	8	6.08 ± 0.21	38.53 ± 5.50	7	0.79	0.20
SNP								
Sham 24h vs. Chronic 24h	7.38 ± 0.12	99.53 ± 1.05	10	6.88 ± 0.17	97.92 ± 1.67	8	**0.03**	0.41
Sham 24h + NAC vs. Chronic 24h+NAC	6.32 ± 0.17	104.03 ± 3.09	7	5.99 ± 0.11	102.39 ± 2.77	7	0.13	0.7
Sham 24h vs. Sham 24h+NAC	7.38 ± 0.12	99.53 ± 1.05	10	6.32 ± 0.17	104.03 ± 3.09	7	**0.0001**	0.13
Acute 24h vs. Acute 24h+NAC	6.88 ± 0.17	97.92 ± 1.67	8	5.99 ± 0.11	102.39 ± 2.77	7	**0.0009**	0.18

Supplementary Table 6. The effect of chronic anemia on endothelial-dependent and -independent relaxation responses 24h post-AMI in the saphenous artery. The contraction responses to PHE are presented as maximal contraction (Emax) and pEC50 values. The maximal relaxation responses to ACH or SNP are expressed as a percentage reduction of the maximal contractile response to 10 μM PHE. All values are shown as mean ± SEM. Emax and pEC50 between the two groups are compared using Student's t-test, and the significant values are represented in the table. ACH, acetyl choline; INDO, indomethacin; SNP, sodium nitroprusside; NAC, N-acetyl cysteine. All values are shown as mean ± SEM.

Chapter 4

Large extracellular vesicles derived from red blood cells promote endothelial dysfunction in patients with anemia

Isabella Solga, Vithya Yogathasan, Tin Yau Pang, Patricia Wischmann, Sebastian Temme, Lina Hofer, Anja Stefanski, Ann Kathrin Bergmann, Stefanie Becher, Norbert Gerdes, Mohammed Rabiul Hosen, Georg Nickenig, Christian Jung, Malte Kelm, Ramesh Chennupati

Scientific chapter

Abstract

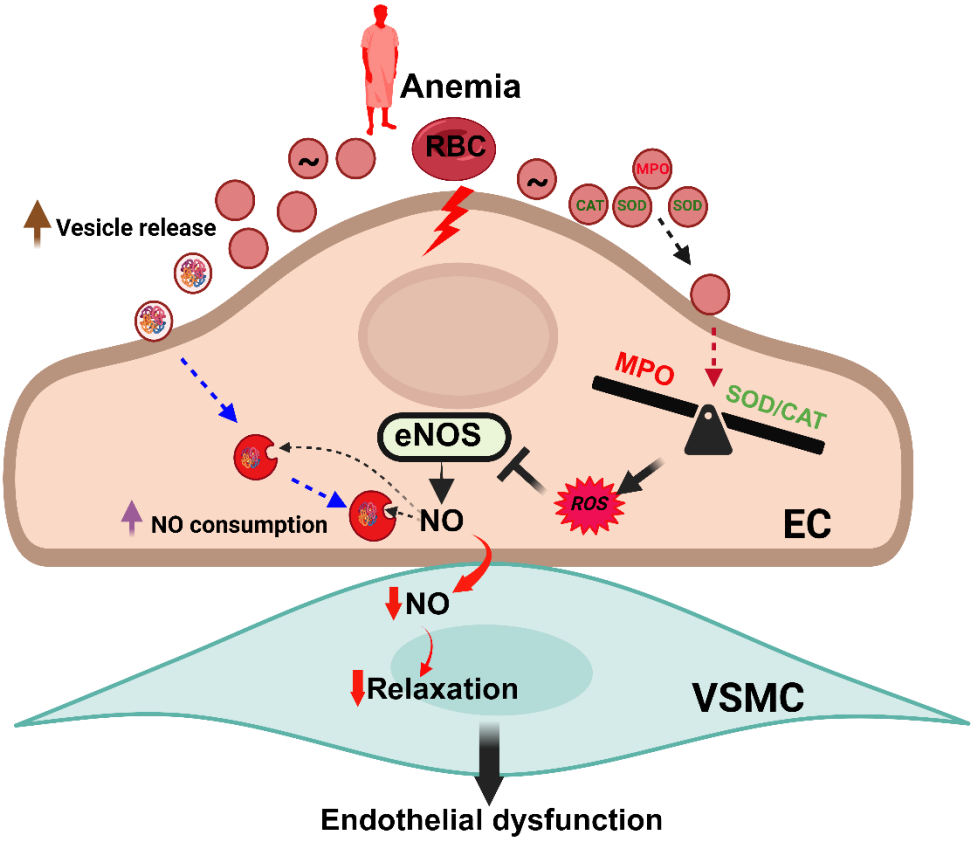
Background and purpose: Endothelial dysfunction (ED) is a hallmark of cardiovascular disease (CVD). We recently showed that anemia is associated with worsening of endothelial function after acute myocardial infarction (AMI). However, the exact mechanism by which dysfunctional red blood cells (RBCs) in anemia contribute to ED via cellular crosstalk remains unclear. Extracellular vesicles (EVs) being communicators between cells reported to play vital role in different CVD, including, AMI. However, their specific role in anemic stable coronary artery disease (CAD) conditions, particularly in relation to ED, has not yet been systematically investigated.

Experimental approach: RBC-derived EVs (REVs) and plasma-derived EVs (PLEVs) from all blood cells and endothelium were isolated from patients with stable coronary artery disease (CAD) and characterized according to current guidelines of International Society of Extracellular Vesicles (ISEV). The isolated large REVs and PLEVs were characterized using dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), fluorescence-activated cell sorting (FACS), and Western blotting. Uptake assays were performed by co-incubating labeled REVs and PLEVs with human umbilical vein endothelial cells (HUVECs). Nitric oxide (NO) consumption ability of REVs was analyzed using a chemiluminescence detector (CLD). After co-incubation of aortic rings explanted from wild-type (WT) mice with REVs and PLEVs from anemic and non-anemic CAD patients, endothelial function was assessed using a wire myograph system. To investigate differences in the content of REVs and PLEVs between anemic and non-anemic CAD patients, proteomic analysis was performed.

Key results: Both REVs and PLEVs were successfully isolated using the modified method. DLS analysis showed that both REVs and PLEVs were within the size distribution range of 100-1000 nm. NTA analysis revealed increased release of REVs in anemic patients compared to non-anemic patients. Co-incubation of labeled REVs and PLEVs with HUVECs demonstrated their uptake by HUVECs in vitro. REVs from anemic patients showed increased NO consumption compared to those from non-anemic patients. Aortic rings co-incubated with REVs from anemic patients showed attenuated endothelial NO-dependent relaxation responses compared to non-anemic patients. Proteomics analysis of REVs from anemic patients revealed numerous differentially expressed proteins, including decreased averaged abundance of antioxidant proteins such as catalase 1 (CAT1), superoxide dismutase 1 (SOD1) and increased oxidative stress promoting myeloperoxidase (MPO).

Conclusion: Anemia is associated with increased release of RBC-derived large vesicles and enhanced nitric oxide consumption, which promotes endothelial dysfunction. This is further

exacerbated by an altered redox balance by REVs, implicating therapeutic importance in anaemic patients with CAD.



Graphical Abstract: Red blood cell-derived large extracellular vesicles promote endothelial dysfunction in anemic patients. Red blood cells (RBCs) release RBC-derived extracellular vesicles (REVs), which are taken up by endothelial cells. Increased vesicle release, along with enhanced nitric oxide (NO) consumption, contributes to NO dysregulation. REVs carry various redox enzymes, including the oxidative stress-promoting enzyme myeloperoxidase (MPO), as well as antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). An imbalance in the content of these redox enzymes leads to increased oxidative stress and endothelial nitric oxide synthase (eNOS) uncoupling, resulting in endothelial dysfunction (ED). The patients included in the study are stable coronary artery disease (CAD) patients with and without anemia. VSMC: vascular smooth muscle cells; EC: endothelial cells; SOD: superoxide dismutase; CAT: catalase.

Introduction

Extracellular vesicles (EVs) are released by different cells and play a crucial role in intercellular communication (*1*). Over recent years, the study of EVs has gained significant attention due to their involvement in different physiological and pathological conditions in the cardiovascular system (*2*). EVs are heterogeneous membranous particles enclosed by a lipid bilayer (*3*). Based on their origin and size, they are typically classified into three distinct subtypes (*4*). Small particles, known as small EVs (formerly known as exosomes, 30-100 nm), large EVs or (formerly known microvesicles, 100-1000 nm) and apoptotic bodies (1-5 µm) (*4*). Large EVs are secreted by direct outward budding or shedding from the plasma membrane of the parental cell (*5*). The formation of EVs is triggered by different stimuli such as shear stress, cell injury, cytokines, adenosintriphosphate (ATP) depletion and calcium influx (*6*).

The content of EVs is highly variable and includes proteins such as surface receptors, signalling proteins, transcription factors, enzymes but also carry lipids and nucleic acids such as miRNA, mRNA and DNA (*7*). These bioactive cargoes are, recently reported as important regulator of cell function via EV-mediated horizontal transfer in cellular crosstalk (*8-10*). When released from donor cells, EVs interact with recipient cells, triggering intercellular signalling and altering molecular processes, potentially affecting their physiological or pathological states (*11*). Previous studies showed elevated EVs in patients with cardiovascular risk factors like diabetes mellitus, hypertension, hypercholesterolemia, as well as in those with cardiovascular diseases (CVD) or myocardial disorders (*12, 13*). Furthermore, they are also proposed to be used as clinical markers for inflammation and tissue/organ damage, thus may also contribute in therapeutic approaches such as cardiovascular regeneration and protection (*14*).

Red blood cells (RBCs) are primarily known for their function of transport of gases, but they are also involved in the maintenance of vascular homeostasis through the regulation of nitric oxide (NO) pool and the regulation of redox balance (*15, 16*). In our recent studies, we demonstrated that anemia is associated with RBC and endothelial dysfunction (ED) (*17, 18*). In the same study we showed that chronic anemia is associated with inflammation and increased ROS formation, which plays a crucial role in anemia associated ED (*18*). Additionally, co-incubation of anemic RBCs from both mice and humans with murine aortic segments revealed impaired endothelial function, suggesting that anemic RBCs play a crucial role in mediating ED in anemia. However, the exact mechanisms by which anemic RBCs contribute to ED is not fully understood. RBC-derived EVs (REVs) have been shown to play a crucial role in various pathological conditions, including sickle cell anemia, thrombosis, cardiovascular diseases, diabetes, cancer, and inflammation (*6, 19, 20*). They contain a wide range of different compounds, including enzymes involved in redox homeostasis such as glutathione S transferase

(GST), thioredoxin and peroxiredoxin but also myeloperoxidase (MPO), cholesterol and haemoglobin (Hb). These factors can affect vascular inflammation, reactive oxygen species (ROS) production, endothelial damage, and coronary heart disease. Additionally, the REVs are known to be potent NO scavengers, which reduce NO bioavailability and influence vasoregulation (*6*, *20*). The role of REVs in mediating ED is not clearly understood. We hypothesize that REVs play a central role in the communication between RBCs and endothelial cells in anemia, contributing to the development of ED.

In this study, we investigated the role of anemic RBC-derived extracellular vesicles (REVs) in mediating endothelial dysfunction (ED) using samples from stable coronary artery disease (CAD) patients, aiming to uncover the impact of intercellular communication in these patient cohorts. Furthermore, from the same patients, we will investigate the role of anemic plasma-derived extracellular vesicles (PLEVs) in ED, which are derived from various cell types including RBCs, endothelial cells, and platelets. To our knowledge, this is the first study to explore the specific role of anemic REVs and PLEVs in ED associated with anemia in CAD patients.

Materials and Methods

Collection of blood from human

Blood samples from anemic and non-anemic patients were collected in EDTA tubes. Male patients were classified as anemic if their hemoglobin (Hb) levels were below 13.0 g/dL, while female patients were considered anemic if their Hb levels were below 12.0 g/dL (according to WHO guidelines). All patients included in this study had given their consent and were recruited from the Department of Cardiology, Pulmonology, and Angiology at Düsseldorf University Hospital. The approval numbers for human blood samples collection are 5481R, 2018-14, and 2018-47, as approved by the ethics committee of Düsseldorf University Hospital.

Animals

All animal procedures used in the study were approved and performed in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) II guidelines and authorized by LANUV (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection) in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The approval numbers for the animal experiments are 84-02.04. 2020.A073 and 84-02.04.2018.A234. The C57Bl/6J (wildtype, WT) mice were obtained from Janvier Labs (Saint-Berthevin Cedex, France). Mice were housed in standard cages (constant room temperature and humidity, with 12 h light/dark cycles) and had free access to standard pelleted food and tap water.

REVs and PLEVs isolation

15 ml of whole blood from anemic and non-anemic stable coronary arteries disease (CAD) patients was collected in EDTA tubes. To isolate EVs from mice, blood was pooled from three mice yielding a total volume of 3 ml. The blood was centrifuged, and plasma was collected. The buffy coat was removed, and the red blood cells (RBCs) were washed three times with phosphate buffered saline (PBS, Sigma). The RBC suspension (40% haematocrit with PBS) was stored at 4°C for 48 h to facilitate the vesiculation. Next, the RBC suspension was centrifuged at 800 x g for 10 min, the supernatant was filtered using a 0,8 µm syringe filter (Corning) to remove large particles such as apoptotic bodies and debris. Next, filtered supernatant was centrifuged at 21,000 x g at 4°C for 45 min and washed with filtered PBS. This centrifugation step was repeated for two times. Plasma vesicles were also isolated following same centrifugation steps.

Characterization of REVs and PLEVs

Dynamic Light Scattering (DLS)

Brownian motion based DLS method was used to assess the size distribution of the isolated EVs. As described above, both REVs and PLEVs were isolated from anemic and non-anemic patients, the pellet was dissolved in 250 µl of filtered PBS. The particle size distribution was analysed using the NANOTRAC WAVE II/Q/ ZETA (Microtrac MRB, USA). Five measurements per sample were performed and then average values were collected using the DIMENSIONS LS software.

Nanoparticle tracking analysis (NTA)

NTA was used to determine the size and concentration based on a principle that a laser beam illuminates the particles resulting in scattered light, which is captured by a microscope. As described above, both REVs and PLEVs were isolated from both anemic and non-anemic patients, diluted in 1000 µl demineralized water, and injected into the chamber of NanoSight NS300 (Malvern Panalytical). For each sample, three recordings were made, each lasting 30 seconds. The particle size distribution and concentration were analysed with the Nanosight NTA 1.4 software.

Transmission electron microscopy (TEM)

As described above, both PLEVs and REVs were isolated from anemic human and mouse samples. The EVs were fixed by mixing the sample in a 1:4 ratio with fixative (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4) and incubated for 10 min at RT. Approximately, 5 µl of the mixture was placed on a grid (SP162, pioloform on copper, 200

mesh, Plano, Wetzlar, Germany). Following this, a negative contrast staining with uranyl acetate was performed, where a drop of uranyl acetate was applied and removed three times. Imaging was conducted using the H-7100 TEM (Hitachi, Tokyo, Japan) with a Morada camera (EMSIS GmbH, Münster, Germany) and iTEM software (Olympus, Münster, Germany).

Flow cytometry

Blood was collected and centrifuged at 800 x g for 10 min at 4 °C, the buffy coat was removed, and RBC pellet was washed 3 times with PBS. The washed RBCs were either stored for 48 h at 4°C or immediately used. For flow cytometric analysis RBCs were stained with following antibodies for 20 min at RT in the dark: CD41a-APC-Cy7 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD235a-APC (Biolegend) and CD71-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and acquired with the BD FACSVerse™ Cell Analyzer (BD, Heidelberg, Germany). The data were analyzed with the FlowJo software v10.5.3.

Western Blot

After REVs isolation, the pellet was lysed with 30 µl of radioimmunoprecipitation assay (RIPA) buffer. Afterwards, REVs concentrations of the samples were measured using the DC™ Protein Assay Kit (Bio-Rad, Feldkirchen, Germany) and samples were prepared for WB. The samples were denatured and loaded on a 4%–12% Bis-Tris gradient gel QPAGE™ (Smobio, Hsinchu City, Taiwan). Followed by a transfer onto nitroclellulose membrane and a total protein staining by using Total Protein Stains (LI-COR Biosciences GmbH, Bad Homburg, Germany). The total protein staining was detected at 700 nm on the Odyssey® Fc Imaging System. Membrane was blocked with intercept TBS Protein-Free Blocking Buffer (LI-COR) for 1 h at RT, followed by overnight incubation of the primary antibody at 4°C. Corresponding secondary antibody was incubated for 1 h at RT and detected at the the Odyssey® Fc Imaging System.

NO consumption assay

The extent to which REVs consume NO was assessed using a consumption assay, as previously described (*21*) with a chemiluminescence detector (CLD 88, Eco Medics, Duernten, Switzerland). Briefly, blood was collected in EDTA tubes and centrifuged at 800 x g for 10 min at 4 °C, the buffy coat was removed, and REVs were isolated from 7 ml of RBC suspension from both groups. The pellet was stored until further use.

First, the system was calibrated by injecting known amounts of nitrite into a reduction solution comprising 45 mmol/L potassium iodide (KI) and 10 mmol/L iodine (I2) in glacial acetic acid, kept in a septum-sealed reaction chamber at 60°C and continuously purged with helium. The reaction chamber was then cleaned with 0.1 M NaOH and ultrapure water. Ten microliters of a freshly prepared stock solution of DetaNONOate (Cayman Chemicals; 120 µM) in PBS (pH 7.4) was added to the reaction chamber containing 40 ml of PBS. After the signal for NO release from the NO donor stabilized, REVs resuspended in 50 µL ultrapure water were injected into the reaction chamber. The resulting in a transient decrease in the NO signal indicative of NO consumption. The extent of NO consumption by REVs was calculated by integrating the (negative) areas under the curve of the quenching reaction and comparing them to the (positive) areas for NO generated by the chemical reduction of known amounts of nitrite standards.

In vitro assessment of EVs uptake by endothelial cells

The uptake of EVs by endothelial cells were assessed using the PKH67 green Fluorescent Cell Linker Kit (Sigma Aldrich, USA) following the company's instructions. REVs and PLEVs were prepared as described before and labelled. Briefly, the EV pellet was resuspended in 1 % BSA containing PBS. After 30 min of incubation, 2 µl CD63-AF 646 antibody/TSG101-AF 646 antibody was added (1:5000 dilution). The suspension was incubated overnight at 4°C. Afterwards the EV suspension was centrifuged at 21000 x g for 30 min at 4°C. The pellet was resuspended in 1 ml PBS and the suspension was centrifuged again at 21000 x g for 30 min at 4°C. The supernatant was removed, 0,5 ml of diluent C was added to the EV pellet, and resuspended. A dye solution with 2 µl of PKH67 and 0,5 ml of diluent C was prepared, added to the EV suspension, and incubated for 5 min. The staining was stopped by adding 1 ml of 1 % BSA in PBS solution for 1 min. After that, the EV suspension was centrifuged for 30 min at 21000 x g and the supernatant was removed. The centrifugation step was repeated, and the supernatant was discarded. The pellet was then dissolved in 100 µl serum-free HUVEC medium and incubated with HUVECs. After 24 h incubation, the cells were fixed on microscope cover slides and imaged with Leica DM6 M microscope (Leica Microsystems).

To verify RBC vesiculation and the uptake of released REVs by endothelial cells, RBC membranes were labeled with the PKH67 Green Fluorescent Cell Linker Kit (Sigma Aldrich, USA), according to the manufacturer's protocol. Briefly, the RBC pellet was prepared by centrifuging whole blood at 800 x g for 10 minutes at 4°C. The buffy coat was then removed, and the pellet was washed three times with PBS. Then, 4 ml of diluent C was added to the RBC pellet and resuspended. A dye solution containing 16 µl of PKH67 and 4 ml of diluent C was prepared. Dye solution was added to the RBC suspension and incubated for 10 min. The staining was stopped by adding 8 ml of 1% BSA PBS solution for 1 minute. Next, the RBC suspension was

centrifuged at 800 x g for 30 minutes. The supernatant was removed, and the centrifugation step was repeated. The RBC pellet was dissolved in 2 ml of PBS and incubated at 4°C for 48 h to facilitate vesiculation. After incubation, REVs were isolated as mentioned before. The REV pellet was then dissolved in 100 µl of serum-free medium and incubated with HUVECs. After 24 h of incubation, the cells were fixed on slide and imaged with Leica DM6 M microscope (Leica Microsystems).

In vitro studies with isolated aortic rings

To assess the effect of anemic REVs and PLEVs on endothelial function, the EVs were co-incubated with wild-type (WT) mouse aortic rings, and vascular function was evaluated. Both REVs or PLEVs were isolated from anemic and non-anemic CAD patients. The pellet (12.5 µg) was dissolved in 100 µl of serum free HUVEC medium. Mouse thoracic aorta was carefully dissected and separated from surrounding perivascular adipose tissue. The aorta was gently perfused with prepared REVs or PLEVs and placed in a petri dish containing 1.9 ml of serum free HUVEC medium. The aorta was incubated for 18 h at 37°C. After the incubation, the aorta was cut into 2 mm segments, mounted on a wire myograph system (Danish Myo Technology, Aarhus, Denmark) and stretched to 9.8 mN. The segments were allowed to equilibrate for 45 minutes in Krebs-Ringer bicarbonate-buffered salt solution (KRB, 118.5 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 5.5 glucose in mmol/L).

After normalization, a concentration-response curve (CRC) for phenylephrine (PHE, 0.001-10 µM, Sigma Aldrich) and acetylcholine (Ach, 0.001-10 µM, Sigma Aldrich) was generated in presence of the cyclooxygenase inhibitor indomethacin (INDO, 10 µM). Next, to evaluate the contribution of NO to the relaxation response in the arteries the CRC was repeated in the presence of both INDO and Nω-nitro-arginine methyl ester (L-NAME,100 µM, Sigma Aldrich) a NOS inhibitor. Additionally, the SMC sensitivity to NO was evaluated by performing a CRC in presence of INDO and L-NAME (100 µM), with the NO donor sodium nitroprusside (SNP, 0.01-10 µM, Sigma Aldrich).

Mass spectrometry-based proteomics

Both REVs and PLEVs were isolated from anemic and non-anemic CAD patient samples as described before. The samples were lysed with 2% SDS in 300 mM Tris/HCl, (pH 8.0) soni-cated to facilitate the complete lysis for EVs. Then the samples were reduced with 20 mM DTT and alkylated with 300 mM IAA. Next, the lysed REVs were digested (SP3 bead digestion), the resulting peptides will be separated using nano-HPLC and analyzed with Orbitrap Fusion

Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific). Data was processed using Proteome Discoverer software (version 2.4.1.15, Thermo Fisher Scientific). RAW files were searched against the human SwissProt database and the MaxQuant contaminant database. A precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.5 Da were applied. The following modifications were considered: methionine oxidation, N-terminal acetylation, N-terminal methionine loss, and N-terminal methionine loss combined with acetylation as variable modifications, and carbamidomethylation as a static modification. Tryptic cleavage specificity was set with a maximum of two missed cleavage sites.

Statistical analysis

In *ex vivo* vascular function experiments, all CRCs for contractile stimuli are expressed as absolute values. Relaxing responses were expressed as percentage reduction of the level of contraction. Individual CRCs were fitted to a non-linear sigmoid regression curve (Graphpad Prism 8.0). Sensitivity (pEC_{50}), maximal effect (E_{max}) are shown as means ± SEM. In all experiments two groups were compared by unpaired t-tests. Two-way ANOVA followed by a Bonferroni post-hoc test was used to compare multiple groups.

Results

Characterization of RBC-derived large extracellular vesicles

To verify our hypothesis that anemic REVs play a crucial role in mediating endothelial dysfunction (ED), we first established a new method for isolating REVs. One of the major challenges in extracellular vesicle (EV) isolation is obtaining a sufficient quantity of EVs from a given volume of blood samples (whether human or mouse) for experimental purposes. To address this, we prepared a suspension of RBCs in PBS and incubated the suspension for 48 h at 4°C. The released REVs in the supernatant were collected and processed through filtration and centrifugation steps (Fig. 1A). We then characterized both plasma-derived extracellular vesicles (PLEVs) and REVs using various methods. Dynamic light scattering (DLS) analysis was used to assess the size distribution of the isolated vesicles. Both REVs and PLEVs ranged in size from 100 to 1000 nm (Fig. 1B), with REVs showing a peak at approximately 200 nm and PLEVs at around 150 nm. The size distribution was consistent between anemic and non-anemic patients, indicating that isolated REVs and PEVs were categorized to large EVs (Fig. 1B).

Transmission electron microscopy (TEM) images further supported these findings, showing a higher number of vesicles per visual field in anemic coronary artery disease (CAD) patients

compared to non-anemic controls (Fig. 1C), suggesting that anemia is associated with increased vesicle release. Furthermore, in our proteomic analysis we detected EV-specific markers, such as flotillin, CD9, and annexin in both, REVs and PLEVs (Fig. 1D), hinting towards another layer of evidence that isolated EVs are pure and contamination-free from both sources. Finally, we confirmed the presence of REVs using the RBC-specific marker, CD235a, which was enriched in REVs but not in PLEVs (Fig. 1E).

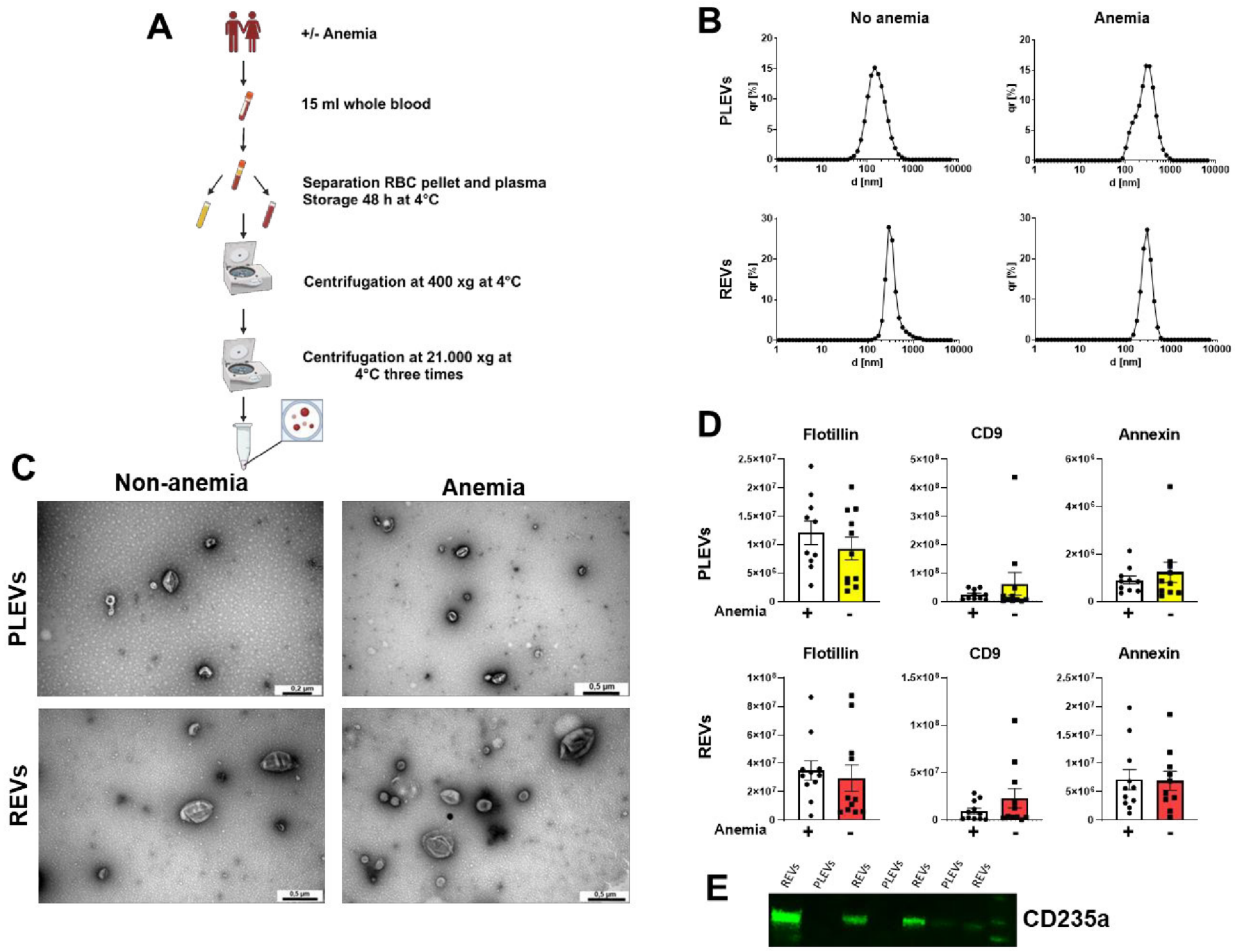


Figure 1: Characterization of RBC-derived (REVs) and plasma-derived extracellular vesicles (PLEVs) isolated from anemic and non-anemic CAD patients. **(A)** Isolation protocol for REVs and PLEVs from whole blood. **(B)** Representative size distribution of REVs and PLEVs in non-anemic and anemic CAD patients. **(C)** Representative TEM images of EVs from anemic and non-anemic patients. **(D)** Presence of EV markers: flotillin, CD9, and annexin in PLEVs and REVs isolated from anemic and non-anemic patients. The abundance is derived from proteomic data. **(E)** Protein expression of the RBC marker CD235a in lysed REVs and PLEVs. CAD: stable coronary artery disease.

Anemia triggers REVs release in patients

Nanoparticle tracking analysis (NTA) revealed increased vesicle release in anemic patients compared to non-anemic patients (Fig. 2A), while PLEVs concentrations remained unchanged (Fig. 2B). We also observed increased NO consumption in anemic REVs compared to non-anemic REVs (Fig. 2C) concluding that anemic REVs play a crucial role in regulating NO bio-availability.

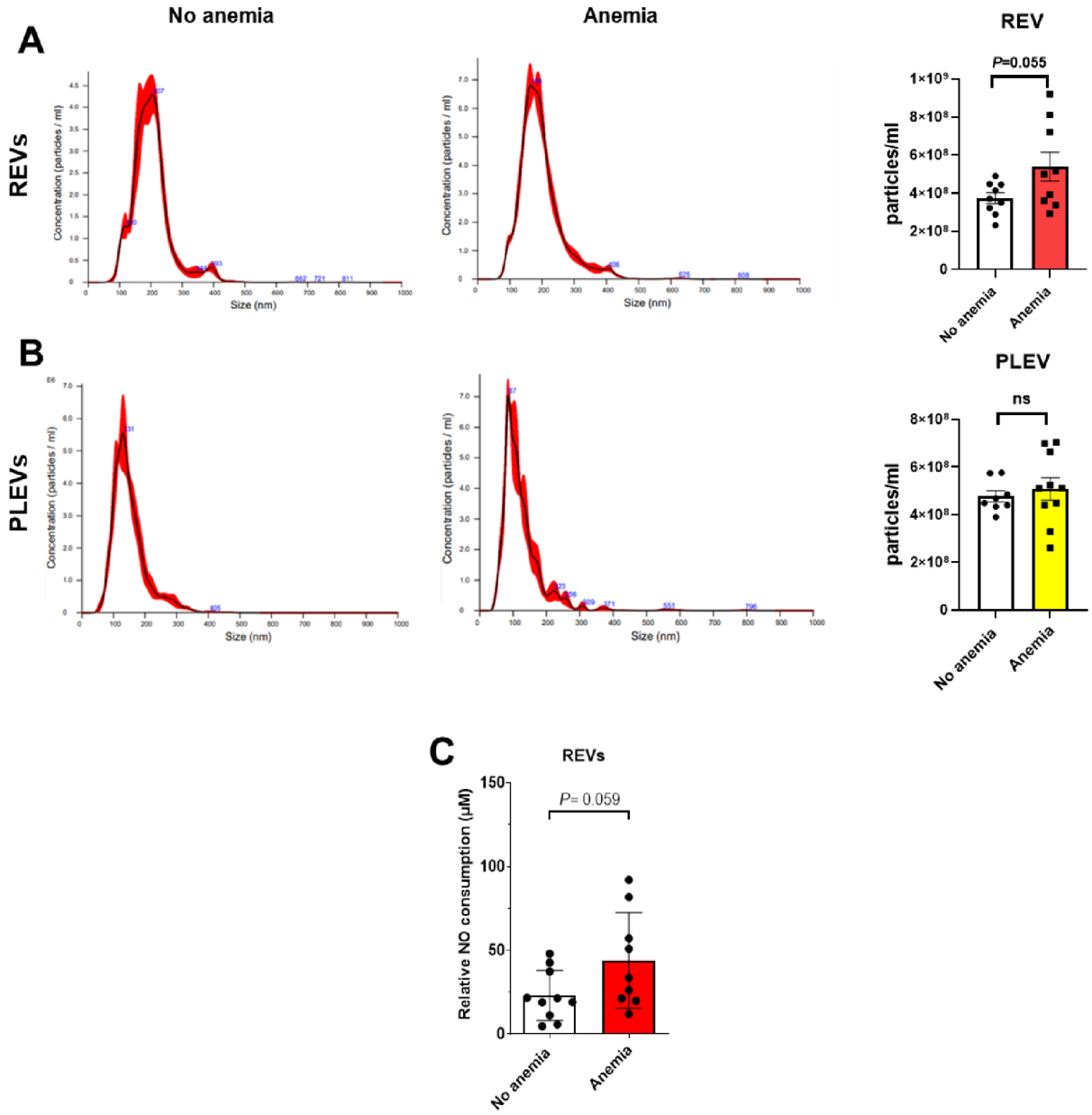


Figure 2: Anemic red blood cells (RBCs) show increased vesicle release and NO consumption. RBC-derived **(**REVs) and plasma-derived extracellular vesicles (PLEVs) were isolated from anemic (red bar) and non-anemic (white bar) CAD patients. Particle number and size distribution was quantified using Nano Sight. **(A)** Size distribution and quantification REVs and PLEVs. **(B)** NO consumption assay in isolated REVs. All values are mean values ±

SEM (n = 9-10 per group). *, $P \leq 0.05$. Student t-test was used to compare the particles concentration between anemic and non-anemic patients. CAD: Stable coronary artery disease.

Increased release of REVs promote ED by cellular crosstalk

EVs are known to be uptaken by recipient cells (*11)*. To assess the interaction of REVs and PLEVs with endothelial cells, we examined whether the isolated REVs and PLEVs were taken up by endothelial cells under *in vitro* conditions. After 18 h of co-incubation, PKH67-labeled REVs and PLEVs were widely distributed in HUVECs (Fig. 3A-B). Additionally, RBCs were stained with PKH67, and vesiculation was allowed for 48 h. After this period, the isolated REVs, which contained membrane labelling from the parental cells, were incubated with HUVECs. As expected, after 18 h of co-incubation, PKH67-labeled REVs were widely distributed in HUVECs (Fig. 3C). This further confirms that RBC-derived EVs are taken up by recipient cells, such as endothelial cells.

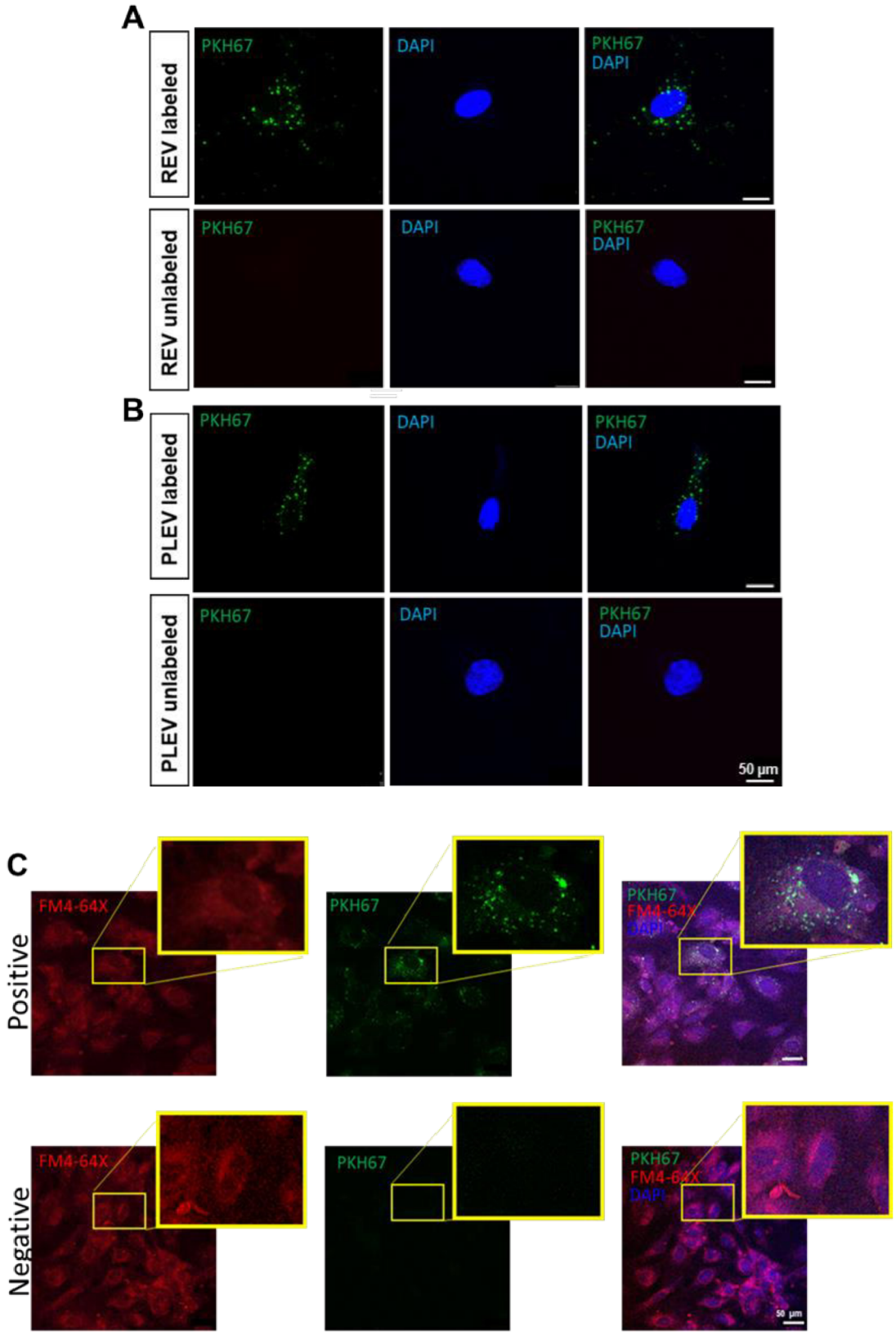


Figure 3: Both RBC-derived (REVs) and plasma-derived extracellular vesicles (PLEVs) are taken up by endothelial cells. Both REVs and PLEVs were isolated from healthy CAD patients. **(A-B)** Representative images of HUVECs incubated with either REVs or PLEVs labelled with PKH67 (membrane stain). Nuclei of HUVECs were stained with DAPI. Unlabelled EVs from same patients are used as negative controls. **(C)** Representative images of HUVECs incubated with PKH67-labeled REVs. Unlabelled EVs from same patients are used as negative controls.

The membrane was stained with FM4-64X, and the nuclei were stained with DAPI. CAD: stable coronary artery disease.

To evaluate the role of anemic REVs in mediating endothelial dysfunction (ED), we assessed both endothelium-dependent and -independent relaxation responses in murine aortic segments, which were co-incubated with REVs from anemic and non-anemic CAD patients. In the presence of indomethacin, aortic rings incubated with REVs from anemic patients showed significantly increased contractile responses to phenylephrine (E_{max}: 8.72 ± 0.88%) compared to non-anemic controls (E_{max}: 6.07 ± 0.61%) (Fig. 4A, Suppl. Table 1). Additionally, endothelium-dependent relaxation responses to acetylcholine, in the presence of indomethacin, were significantly reduced in aortic segments treated with anemic REVs (E_{max}: 48.90 ± 13.18%) compared to non-anemic REVs (E_{max}: 81.09 ± 5.14%), indicating impaired endothelial-dependent relaxation (Fig. 4C, Suppl. Table 1). The relaxation responses were equally and completely inhibited in the presence of the NOS inhibitor L-NAME (100 µM), confirming that these relaxation responses are entirely mediated by NO (Fig. 4D, Suppl. Table 1). Additionally, the relaxation responses to SNP were similar in both groups (Fig. 4E, Suppl. Table 1).

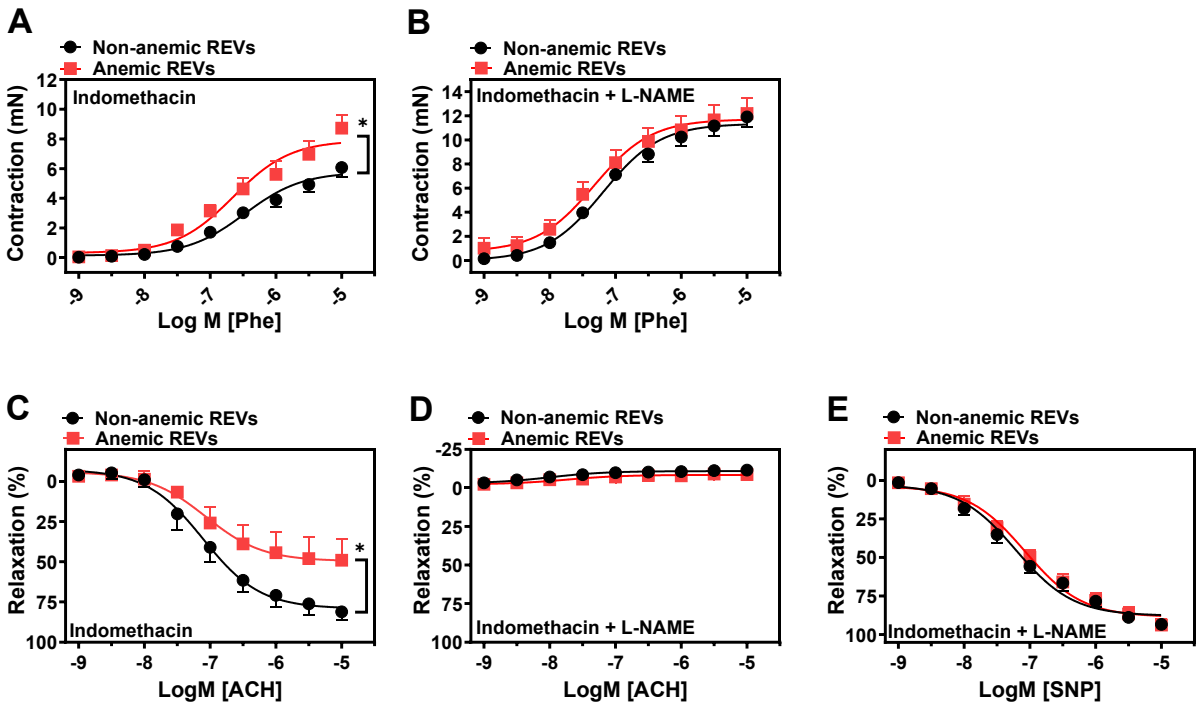


Figure 4: REVs from anemic CAD patients induce endothelial dysfunction in murine aortic segments. REVs were isolated from anemic (red squares) and non-anemic patients (black circles) and incubated with murine aortic rings. After 18 h, segments were mounted in a wire myograph system. **(A)** Contractile responses (mN) to phenylephrine in the presence of indomethacin (10 µM, COX inhibitor). **(B)** Contractile responses in the presence of indomethacin and L-NAME. **(C)** Relaxation responses (%) to acetylcholine in the presence of indomethacin. (**D)** Relaxation responses to acetylcholine in the presence of indomethacin and L-NAME. **(E)** Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 µM) in the presence of indomethacin and L-NAME. Values are shown as

means ± SEM (n = 6-7 per group). *, P ≤ 0.05. CRCs were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test. CAD: stable coronary artery disease. REVs: RBC-derived extracellular vesicles.

Furthermore, we investigated the effects of PLEVs from anemic and non-anemic patients on endothelial function. Similar to the REVs experiment, PLEVs from both anemic and non-anemic patients were co-incubated with WT mouse aortic segments for 18 h. The vascular function assessment revealed that the contractile responses of the aortic rings incubated with PLEVs were comparable between the anemic and non-anemic groups (Fig. 5A-B, Suppl. Table 1). Likewise, the relaxation responses in the presence of indomethacin were similar for both anemic and non-anemic CAD patients (Fig. 5C, Suppl. Table 1). In the presence of L-NAME, the relaxation responses were equally and completely inhibited in both groups (Fig. 5D, Suppl. Table 1). Additionally, the relaxation responses to SNP were similar in both groups (Fig. 5E, Suppl. Table 1). In summary, these results indicate that REVs, but not PLEVs, from anemic CAD patients contribute to endothelial dysfunction (ED).

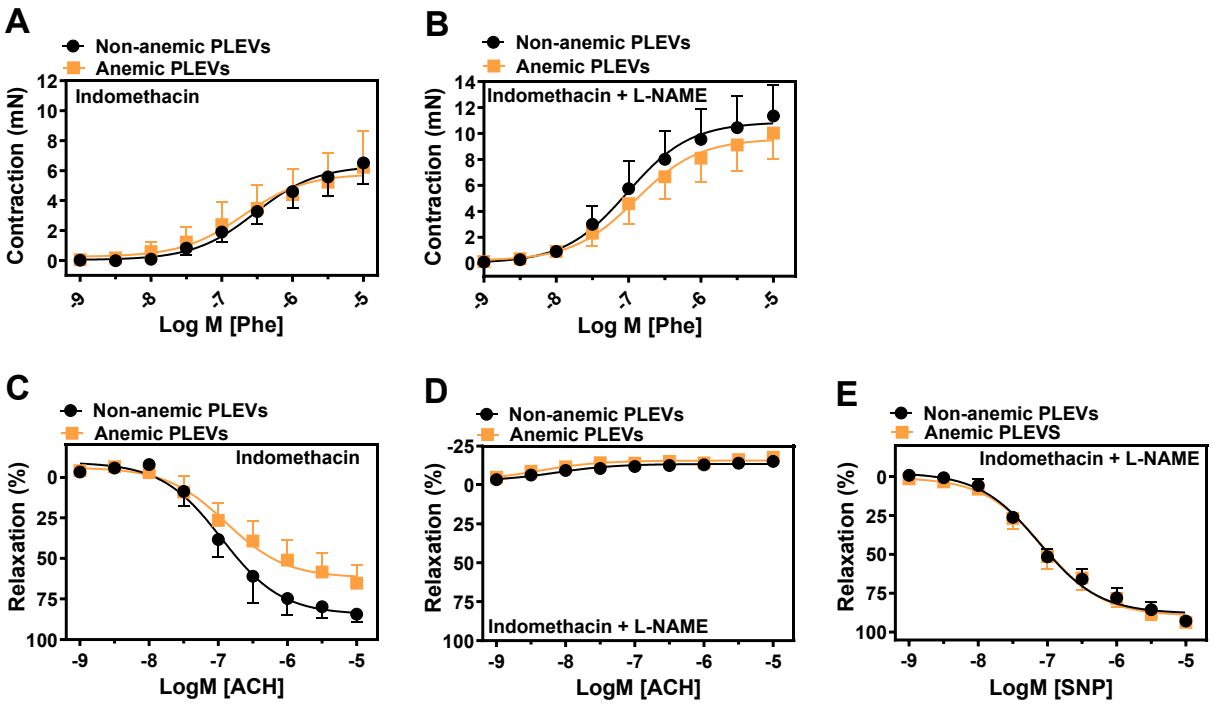
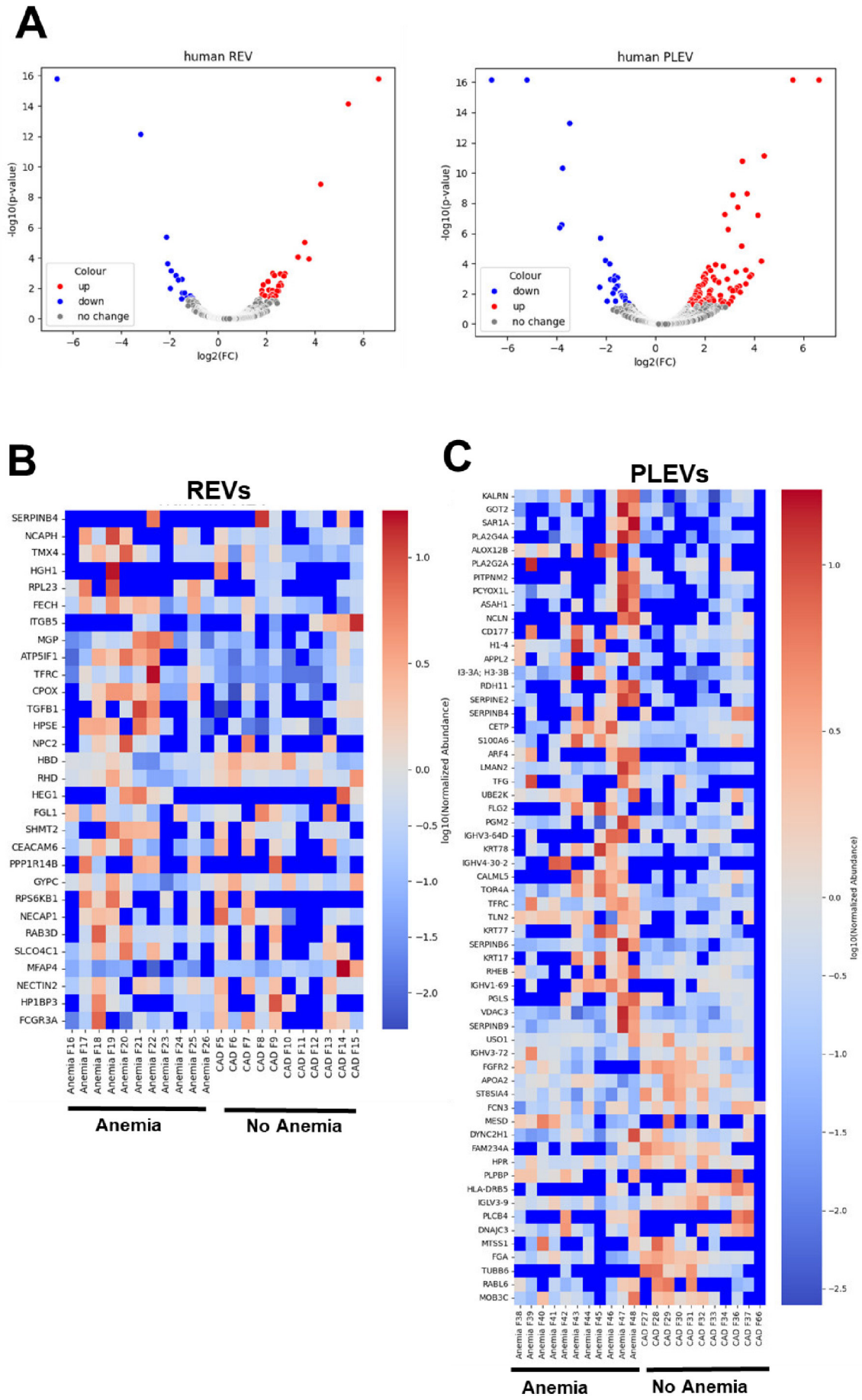


Figure 5: PLEVs from anemic CAD patients do not alter endothelial function. PLEVs were isolated from anemic (yellow squares) and non-anemic patients (black circles) and incubated with murine aortic rings. After 18 h of incubation, aortic segments were mounted in a wire myograph system. **(A)** Contractile responses (mN) to phenylephrine in the presence of indomethacin (10 µM, COX inhibitor). **(B)** Contractile responses in the presence of indomethacin and L-NAME. **(C)** Relaxation responses (%) to acetylcholine in the presence of indomethacin. **(D)** Relaxation responses to acetylcholine in the presence of indomethacin and L-NAME. **(E)** Relaxation responses to sodium nitroprusside (SNP, 10 nM-10 µM) in the presence of indomethacin and L-NAME. Values are shown as means ± SEM (n = 4 per group). CRCs were analyzed by Two-Way ANOVA and Bonferroni's post-hoc test. PLEVs: plasma-derived extracellular vesicles. CAD: stable coronary artery disease.

Increased release of REVs promote ED by cellular crosstalk

To gain deeper insights into the content of REVs and PLEVs, we performed a proteomic analysis. Due to the partly uneven distribution of proteins in our isolated EVs, we applied specific filter criteria for the analysis. Only proteins that were abundant in at least five out of eleven patients in one of the groups were considered for further analysis. After filtration, 2369 out of 2389 total proteins detected in REVs were included in the analysis. In the case of PLEVs, 2044 out of 2071 total proteins were considered after filtration. The volcano plots represent all proteins, including differentially regulated ones in REVs and PLEVs (Fig. 6A). Next, we selected all differentially expressed proteins in REVs and PLEVs. When comparing anemic to non-anemic patients, approximately 30 proteins were differentially expressed in REVs (Fig. 6B) and 63 proteins in PLEVs (Fig. 6C), revealing differences in the EV cargo content between the two groups.


A
human REV
human PLEV
-log10(p-value)
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Colour
up
down
no change
B
REVs
SERPINB4
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TMX4
HGH1
RPL23
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ITGB5
MGP
ATP5IF1
TFRC
CPOX
TGFB1
HPSE
NPC2
HBD
RHD
HEG1
FGL1
SHMT2
CEACAM6
PPP1R14B
GYPC
RPS6KB1
NECAP1
RAB3D
SLCO4C1
MFAP4
NECTIN2
HP1BP3
FCGR3A
Anemia
No Anemia
C
PLEVs
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Anemia
No Anemia

Figure 6: Proteomic analysis of RBC-derived (REVs) and plasma-derived extracellular vesicles (PLEVs) isolated from anemic and non-anemic CAD patients. (A) Volcano plots displaying the enrichment of proteins between anemic and non-anemic patients; proteins with occurrence ≥5 in anemic samples or ≥5 in non-anemic samples are considered and selected for further analyses. **(B, C)** Heatmap of the normalized abundances of selected proteins isolated from anemic and non-anemic patients**,** in REVs **(B)** and PLEVs **(C)**; proteins in the heatmap are ordered according to their enrichment between anemic and non-anemic samples. CAD: stable coronary artery disease.

REVs and PLEVs from anemic patient's cargo variety of proteins

Interestingly, in both PLEVs and REVs, we identified specific proteins that were enriched exclusively in anemic patients (Suppl. Table 2). In anemic REVs, we identified NADH-cytochrome b5 reductase 1, which plays a role in the reduction of methemoglobin, and immunoglobulin kappa variable 1-17, a protein involved in immune response (Suppl. Table 2). In PLEVs, we found AP-3 complex subunit beta-1 and tetraspanin-15 enriched in anemic patients, both involved in vesicle formation, as well as other proteins such as heat shock protein 75 kDa, ubiquitin carboxyl-terminal hydrolase, and 2-hydroxyacyl-CoA lyase 2 (Suppl. Table 2). Concluding that these proteins can be used as potential role as biomarkers in anemia.

REVs are known to carry redox proteins, so we specifically analyzed the redox proteins expressed in both REVs and PLEVs and compared them between anemic and non-anemic patients (Fig. 7A-B). The results suggest that REVs from anemic patients are associated with an increased average abundance of redox-regulating proteins compared to REVs from non-anemic patients. Due to high variability between samples, these redox proteins did not significantly differ between the anemic and non-anemic patients. Interestingly, several antioxidants such as superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPX1), catalase (CAT), were found to be reduced in anemic REVs compared to those from non-anemic patients (Fig. 7A). Noteworthy, we also observed an increased abundance of myeloperoxidase (MPO) in anemic REVs compared to non-anemic REVs. No specific pattern in the distribution of redox proteins was observed in PLEVs, except for glutathione S-transferase mu 2 (GSTM2), which was notably enriched in anemic PLEVs compared to non-anemic ones (Fig. 7B). These results suggest a potential role of redox proteins in promoting endothelial dysfunction in anemia.

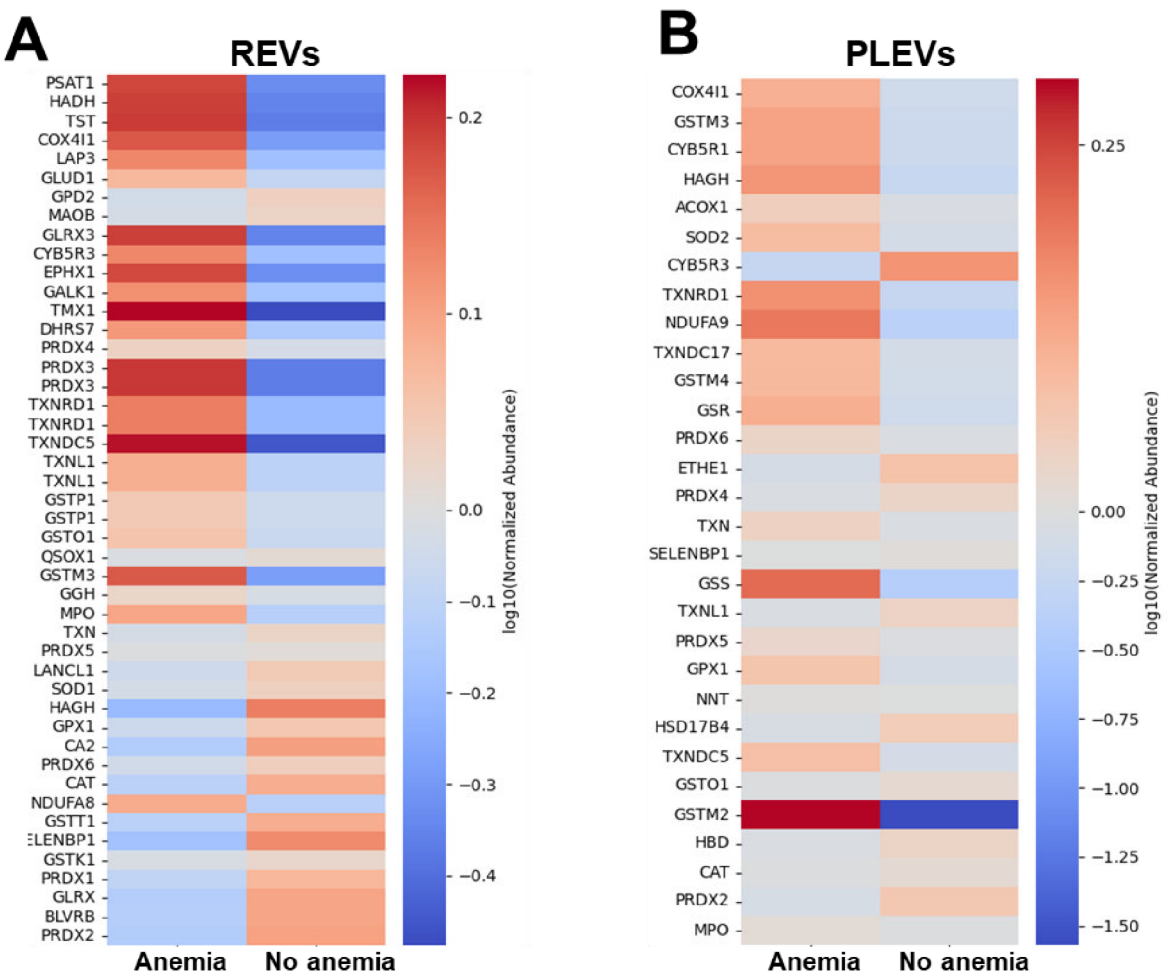

Figure 7: Abundance of redox proteins in RBC-derived (REVs) and plasma-derived extracellular vesicles (PLEVs) isolated from anemic and non-anemic patients. (A-B) Heatmap of normalized abundance of selected redox proteins isolated from anemic and non-anemic patients in REVs **(A)** and PLEVs **(B)**. Proteins in the heatmap are ordered according to their enrichment between anemic and non-anemic samples, and are normalized accordance with the average of a protein across different samples is 1. Dark red represents high abundance, grey indicates no difference, dark blue demonstrates low abundance, and white denotes no abundance.

Discussion

In this study, we examined the effect of REVs and PLEVs from anemic patients on endothelial function. The key findings of the study are: (1) We successfully characterized the isolated particles from RBCs and plasma as EVs; (2) Increased RBC vesiculation was observed in anemic CAD patients compared to non-anemic CAD patients; (3) Anemic REVs showed increased NO consumption compared to REVs from non-anemic CAD patients; (4) Both PLEVs and REVs were taken up by endothelial cells *in vitro*; (5) REVs, but not PLEVs, from anemic CAD patients induced ED in isolated murine aortic rings after co-incubation; (6) Proteomic analysis revealed several differentially expressed proteins in PLEVs and REVs, with an altered abundance of redox proteins in REVs from anemic CAD patients compared to non-anemic patients.

In this study, we used a modified method to isolate REVs from human samples. Our isolation protocol includes 48 h of storage of the RBC suspension at cold temperature (4°C). This process allows us to obtain higher concentrations of REVs from RBCs compared to freshly isolated RBCs. Previous studies have shown that exosomes are primarily released during the maturation of reticulocytes into erythrocytes, while microvesicles are mostly released from mature RBCs (*20*). The EV content is more uniform in mature RBCs than in reticulocytes. Based on this, we specifically focused on large EVs rather than exosomes, which are mainly released from mature RBCs. We performed FACS analysis to check the purity of our RBCs before starting the experiments. The results showed that reticulocytes constituted less than 1% of freshly isolated blood and decreased further after 48 h of incubation (Suppl. Fig. 3A). The incubation step in our protocol not only facilitate RBC maturation but also enrich the EV numbers for the experiments. Our NanoSight analysis revealed an increased vesiculation (REVs) in the RBCs from anemic patients compared to RBCs from non-anemic patients. These findings are consistent with previous studies, which have shown that various pathological conditions, such as cardiovascular disease (CVD), metabolic diseases, sickle cell anemia, and thalassemia intermedia, are also associated with increased RBC vesiculation (*20,22*). In contrast to the increase in REVs, we did not observe an increase in PLEVs in anemic patients compared to non-anemic patients. This may be due to the fact that plasma contains EVs from multiple cell types, including the endothelium, platelets, and red blood cells (*23*). The total number of plasma EVs also depends on associated comorbidities and their effects on different cell types.

Recent studies demonstrated that REVs induce ED in pathological conditions such as diabetes and intermittent hypoxia (*24*). To further explore the potential role of anemic REVs in anemia, we first investigated whether REVs isolated from humans interact with target recipient cells, specifically endothelial cells. In our *in vitro* uptake assays, we demonstrated that both REVs and PLEVs are taken up by HUVEC cells, suggesting that REVs may also be taken up by endothelial cells *in vivo*. However, an *in vivo* biodistribution assessment is crucial to confirm our *in vitro* findings, warranting further investigation. To explore their potential role in endothelial function, we co-incubated REVs and PLEVs with murine aortic rings to assess their effects on endothelial function in isolated aortic tissue. Aortic rings incubated with anemic REVs, but not PLEVs, showed significantly reduced NO-dependent relaxation responses. These findings are consistent with previous studies suggesting that EVs mediate ED and vascular inflammation by altering NO production, oxidative stress, inflammation, and coagulation (*24*). These new findings suggest that EVs could be a potential mechanism or a part of a mechanism by which dysfunctional RBCs from anemic patients induce ED.

It has been shown that large EVs (microparticles, microvesicles) derived from RBCs act as potent scavengers of NO, similar to free Hb (*25*). The scavenging ability of microvesicles is 1000-times faster than that of intact RBCs. RBC hemolysis and microparticles formation have

been proposed to contribute to poor outcomes associated with transfusion of older stored blood due to NO scavenging (*26*). In line with these findings, REVs from anemic patients demonstrated a trend toward increased NO consumption. We strongly believe that the increased vesiculation and enhanced NO consumption may contribute to altered NO production and bioavailability, especially *in vivo* in anemia.

As demonstrated in previous studies, promoting oxidative stress may be one of the potential mechanism by which RBCs contribute to ED in anemia (*14*). Consistent with these findings and previous studies on REVs, we identified increased abundance of several proteins involved in redox homeostasis, including glutathione S-transferase, thioredoxin, and peroxiredoxin in anemic REVs (*16*). Furthermore, oxidative stress-promoting enzymes, such as MPO was also found to be abundant in REVs isolated from anemic patients. MPO is associated with vascular endothelial damage and contributes to ED by promoting the formation of ROS, which in turn reduces nitric oxide (NO) bioavailability (*23, 24*). The release of MPO from REVs into recipient cells possibly promotes oxidative stress, contributing to the development of ED in anemia. The observed decreased abundance of important antioxidant enzymes such as SOD, GPX1, and CAT in anemic REVs compared to non-anemic may suggest a potential redox imbalance within the cargos of anemic REVs. Additionally, these findings suggest changes in redox regulation in RBCs from anemic patients, as the cargo in REVs originates from the parental cells. While these findings provide a valuable overview and identify potential targets for future research, further investigation is required to confirm the specific involvement and mechanism.

Summary

Our data suggest that anemia is associated with increased vesiculation of red blood cell-derived extracellular vesicles (REVs) but not plasma-derived extracellular vesicles (PLEVs) in patients with stable coronary artery disease. The anemic REVs exhibit increased nitric oxide (NO) consumption, which may regulate systemic NO bioavailability. In co-incubation experiments, we demonstrated that anemic REVs, but not PLEVs, induce endothelial dysfunction (ED) in isolated murine aortic rings. Furthermore, REVs from anemic patients show an increased abundance of several redox proteins, including oxidative stress-promoting enzymes such as myeloperoxidase (MPO). Taken together, these findings indicate that anemic REVs contribute to ED by consuming NO and altering the redox state of the endothelium through their content

Data availability statement

All the raw data supporting results and conclusions of this manuscript will be made available by the authors, without undue reservation.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RC designed the study. IS, VY, LH, ST, AB, and MRH contributed to EV isolation and characterization. IS and RC contributed to the myograph experiments. PW arranged the CAD patient samples. RC and IS contributed to immunohistochemistry. AS conducted the mass spectrometry experiments. TP, RC, AL, and MRH contributed to proteomics analysis. SB contributed to anemia induction. IS, RC, and MRH interpreted the data and drafted the manuscript. MK and RC secured funding. MRH, CJ, NG, MK, and RC critically revised the manuscript. All authors contributed to the article and approved the final version for submission.

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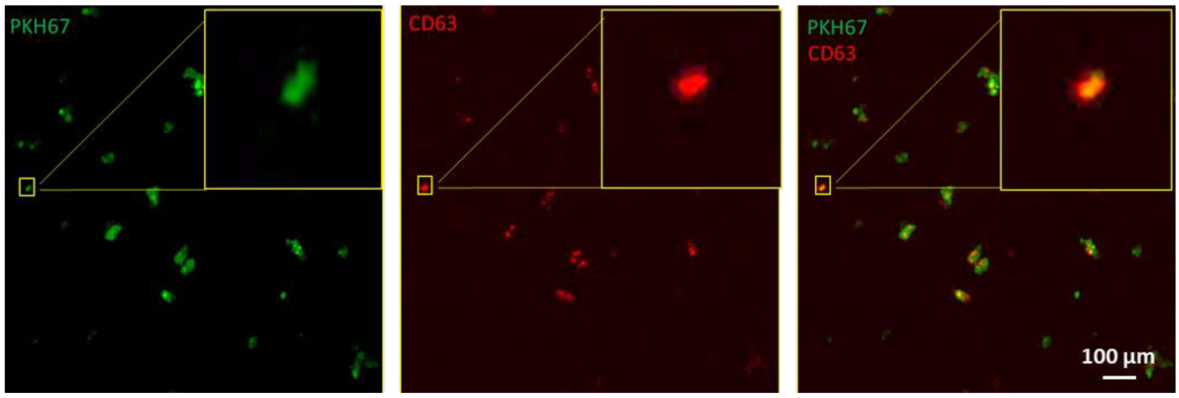
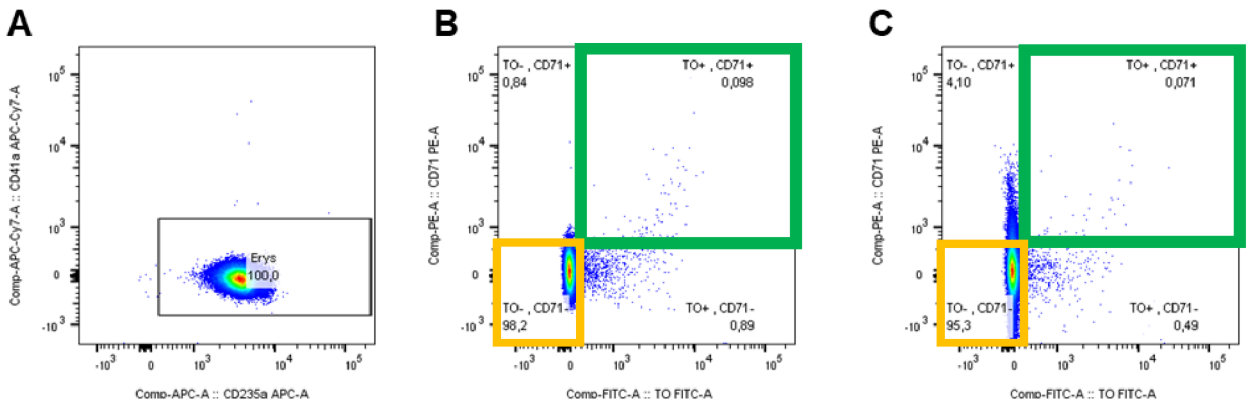
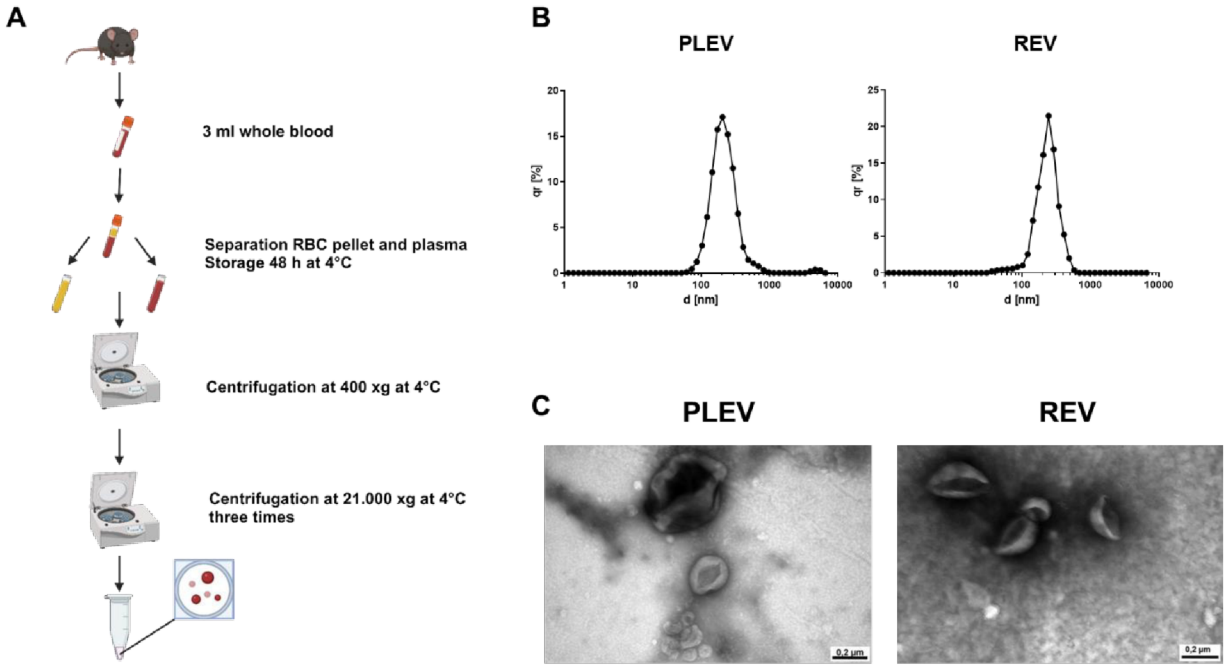
Supplementary data



Supplementary Figure 1: Characterization of REVs with specific EV marker CD63. Representative microscope images of isolated human REVs labelled with PKH67 (green) and CD63 (red).



Supplementary Figure 2: Analysis of mature RBCs and reticulocytes in the whole blood. (A) Gating for RBCs by using CD235a (specific RBC maker) and CD41a (specific platelet marker). **(B)** Gating for CD71 positive cells (green square), a marker for reticulocytes (immature RBCs) and negative (mature RBCs, yellow square)**. (C)** Fresh isolated RBCs from patients**.**



Supplementary Figure 3: Characterization of RBC- and plasma-derived EVs form mice. **(A)** Isolation protocol for REV and PLEV from whole mouse blood. **(B)** Size distribution of REVs and PLEVs isolated from mice. **(C)** Representative TEM pictures of REVs and PLEVs isolated from mice.

Supplementary Table 1. The effect of anemic REVs and PLEVs on contraction and endothelial-dependent and -independent relaxation responses. The contraction responses to PHE are presented as maximal contraction (E_{max}) and pEC50 values. The maximal relaxation responses to ACH or SNP are expressed as a percentage reduction of the maximal contractile response to 10 µM PHE. As aortic rings did not show any relaxation in the presence of L-NAME in all experiments, the data is excluded from the table. All values are shown as mean ± SEM. Emax and pEC_{50} between the two groups are com-pared using Student's t-test, and the significant values are represented in the table. ACH, acetylcholine; INDO, indo-methacin; SNP, sodium nitroprusside; NAC, N-acetyl cysteine

REVs	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n	p-value pEC_{50}	p-value E_{max}
PHE (Indomethacin)								
Non-anemic vs. anemia	6.47 ± 0.11	6.07±0.61	7	6.65 ± 0.14	8.72±0.88	6	0.33	**0.03**
PHE (Indomethacin +L-NAME)								
Non-anemic vs. anemia	7.19 ± 0.08	11.91±0.85	7	7.33 ± 0.16	12.19±1.25	6	0.43	0.85
ACH (Indomethacin)								
Non-anemic vs. anemia	7.11 ± 0.13	81.09±5.14	7	7.07 ± 0.28	48.90±13.18	6	0.89	**0.03**
SNP								
Non-anemic vs. anemia	7.21 ± 0.08	93.35±1.74	7	7.07 ± 0.09	93.74±4.07	6	0.27	0.93
PLEVs	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n	p-value pEC_{50}	p-value E_{max}
PHE (Indomethacin)								
Non-anemic vs. anemia	6.53 ± 0.22	6.51±1.43	4	6.70 ± 0.41	6.23±2.39	4	0.73	0.92
PHE (Indomethacin +L-NAME)								
Non-anemic vs. anemia	7.02 ± 0.26	11.35±2.40	4	6.90 ± 0.24	10.03±2.01	4	0.75	0.69
ACH (Indomethacin)								
Non-anemic vs. anemia	6.95 ± 0.15	84.34±5.06	4	6.89 ± 0.22	65.18±11.19	4	0.83	0.17
SNP								
Non-anemic vs. anemia	7.12 ± 0.08	92.75±3.51	4	7.06 ± 0.10	93.62±3.68	4	0.66	0.87

Supplementary Table 2: List of proteins that are specifically abundant in the REVs and PLEVs of anemic patients.

RBC-derived extracellular vesicles	Plasma-derived extracellular vesicles
Tubulin beta-2A chain	Exportin-7
NudC domain-containing protein 3	Tetraspanin-15
Cytochrome b-c1 complex subunit 6	Heat shock protein 75 kDa
Ornithine aminotransferase	26S proteasome non-ATPase regulatory subunit 3
Immunoglobulin kappa variable 1-17	Protein SGT1 homolog
NADH-cytochrome b5 reductase 1	ATP synthase F(0) complex subunit B1
	AP-3 complex subunit beta-1
	Histone H1.5
	GTP-binding protein Rit1
	Ubiquitin carboxyl-terminal hydrolase 47
	Proteasome adapter and scaffold protein ECM29
	Carnitine O-palmitoyltransferase 2
	2-hydroxyacyl-CoA lyase 2

Supplementary Table 3. Patient characteristics of coronary artery disease (CAD) with anemia and without anemia used in the study.

Parameter	Non-anemic CAD patients	Anemic CAD patients
Age, year (mean ± SD)	73 ± 9	77 ± 9
Sex, male (%)	60%	54%
Arterial Hypertension, n (%)	71%	63%
Smoker, n (%)	15%	17%
Hemoglobin, g/dl (mean ± SD)	14.4 ± 0.3	10 ± 0.8
Glomerular filtration rate, ml/min/1.73m^2 (GFR;mean ± SD)	69.8 ± 16.8	64.2 ± 15.4

Chapter 5

General discussion

Abbreviations

Discussion

Anemia is known to be associated with poor prognosis in cardiovascular diseases (CVD) such as acute myocardial infarction (AMI), leading to higher rates of morbidity and mortality. It is largely unknown how exactly anemia results in worsening of outcome after AMI. Endothelial dysfunction (ED) is a hallmark of CVD.

The studies in this thesis aimed to find the effects of blood-loss anemia on endothelial function before and after AMI, and have led to following findings:

1. Chronic anemia is associated with systemic endothelial dysfunction **(Chapter 2)**

2. Anemia aggravates endothelial dysfunction after acute myocardial infarction **(Chapter 3)**

3. Red blood cell-derived extracellular vesicles promote endothelial dysfunction in anemia **(Chapter 4)**

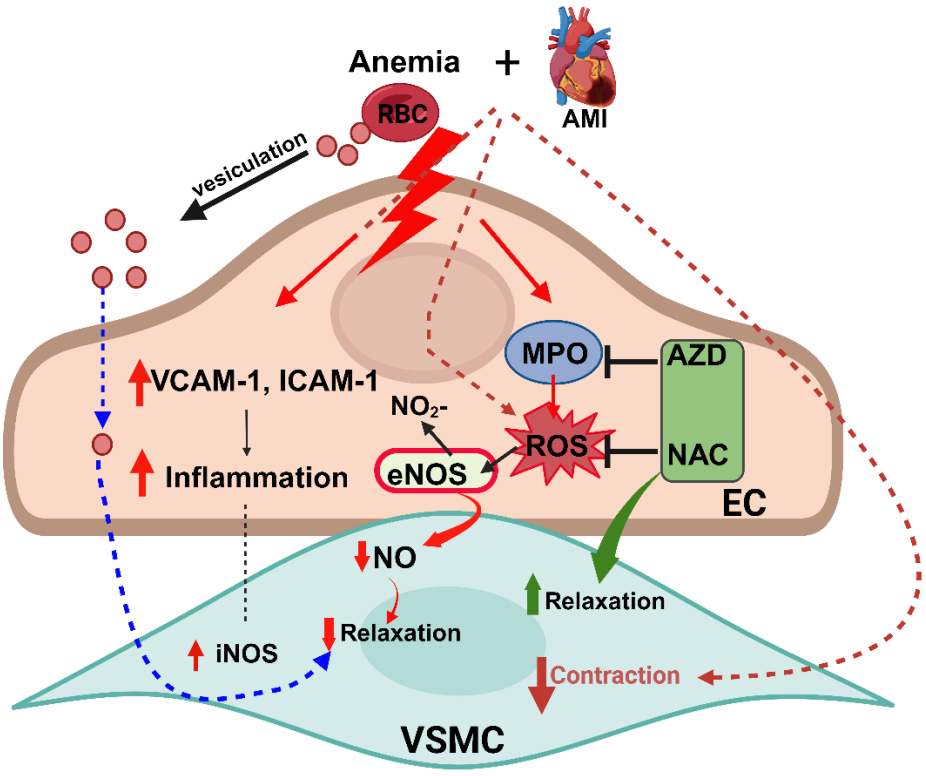


Figure 1: General conclusion of all chapters: Anemia either alone or in combination with AMI is leading to endothelial dysfunction through different mechanisms. Chronic anemia is associated with elevated inflammation (e.g., intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and inducible nitric oxide synthase (iNOS)) and increased reactive oxygen species (ROS) production. The increased ROS production leads to endothelial nitric oxide synthase (eNOS) uncoupling and reduced vascular smooth muscle cell (VSMC) relaxation, resulting in endothelial dysfunction (ED). Inhibition of ROS production or myeloperoxidase (MPO) (e.g., with AZD or N-Acetyl-L-Cystein (NAC) improves endothelial function. Anemia in combination with acute myocardial infarction (AMI), is associated with inflammation, ROS formation, aggravated ED and altered VSMC contraction. NAC treatment improves vascular function. Red blood cells (RBCs) release extracellular vesicles, which contribute to ED either by NO consumption or promoting oxidative stress. Created with BioRender.

Blood-loss anemia is associated with endothelial dysfunction

Endothelial dysfunction (ED) is a hallmark of cardiovascular diseases *(1)*. Clinical studies, as well as our recent research work, have shown that acute blood-loss anemia is associated with a compensatory transient increase in endothelial function evidenced by enhanced flow-mediated dilation (FMD) responses *(2)*. However, the prolonged effects of blood-loss anemia on vascular function and its implications after AMI have never been investigated. Therefore, in **Chapter 2** of this PhD thesis, we investigated the effect of chronic blood-loss anemia on vascular function. In this chapter, we showed that the compensatory increase in vascular function (FMD) observed in acute anemia (AA) was compromised in chronic blood-loss anemia. In chronic anemia (CA), we observed a progressive loss of endothelial function with increasing duration of anemia. We further explored the mechanisms behind the deterioration of endothelial function in CA. We observed that chronic inflammation and increased oxidative stress contributed to the altered ED. We also ruled out the potential role of arginases in mediating ED in CA. Additionally, we found that myeloperoxidase (MPO), which promotes oxidative stress, is upregulated in the vessels of CAmice. Pharmacological inhibition of MPO *ex vivo* or supplementation of CA mice with N-Acetyl-L-Cystein (NAC) improved endothelial function. These results conclude that chronic blood-loss anemia is associated with ED due to increased reactive oxygen species (ROS), which might be partly mediated by MPO. Furthermore, using a newly established co-incubation protocol, we demonstrated that red blood cells (RBCs) from anemic mice and anemic patients induce ED in murine aortic rings. This highlights the novel role of anemic RBCs in mediating ED in anemia.

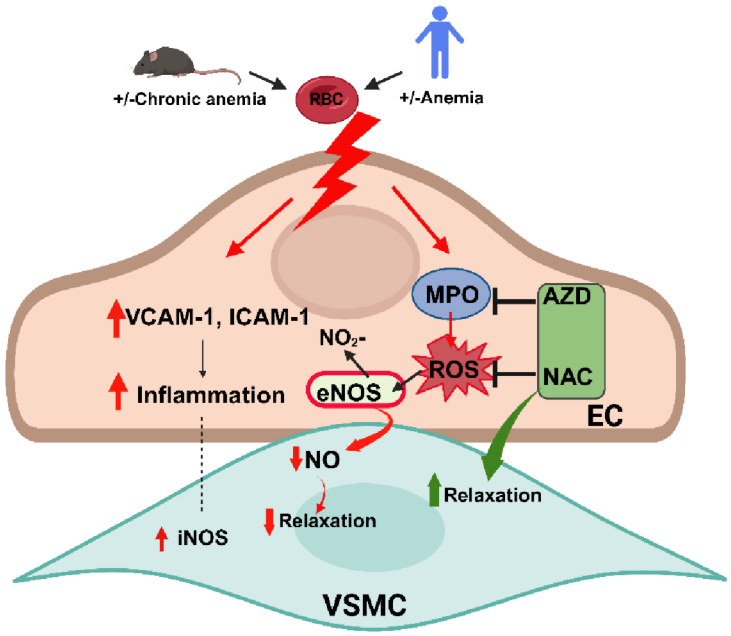


Figure 2: Conclusion of chapter 2: Chronic anemia is associated with increased ROS formation and inflammation, resulting in ED. Chronic anemia is associated with increased inflammation (e.g., intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and inducible nitric oxide synthase (iNOS)) and increased reactive oxygen species (ROS) production, leading to endothelial nitric oxide synthase (eNOS) uncoupling and reduced vascular smooth muscle cell (VSMC) relaxation, resulting in endothelial dysfunction (ED). Inhibition of ROS production or myeloperoxidase (MPO) (e.g., with AZD or N-Acetyl-L-Cystein (NAC)) improves endothelial function. Created with BioRender.

Clinical studies have demonstrated that anemia worsens the prognosis of CVD and after AMI *(3,4)*. However, the underlying mechanisms in relation to ED has not been studied. Based on the findings from **Chapter 2**, we next investigated the effects of short-term acute blood-loss anemia and chronic blood-loss anemia on endothelial function after AMI. In **Chapter 3**, we showed that, after AMI, endothelial function deteriorates in AA mice across different types of blood vessels, accompanied by decreased FMD responses and nitric oxide (NOx) metabolites. We further observed that increased oxidative stress in AA mice after AMI contributes to ED. Additionally, CA mice after AMI exhibited both endothelial and smooth muscle dysfunction, evidenced by decreased FMD responses and contractile responses in large and small resistance arteries. We also observed decreased NOx metabolites in CA mice indicating decreased NO bioavailability. We also showed that vessels from both AA and CA mice show increased oxidative stress. Supplementation of NAC improved vascular function in both AA and CA mice. Furthermore, we demonstrated the potential role of anemic RBCs after AMI in mediating both ED and vascular smooth muscle dysfunction.

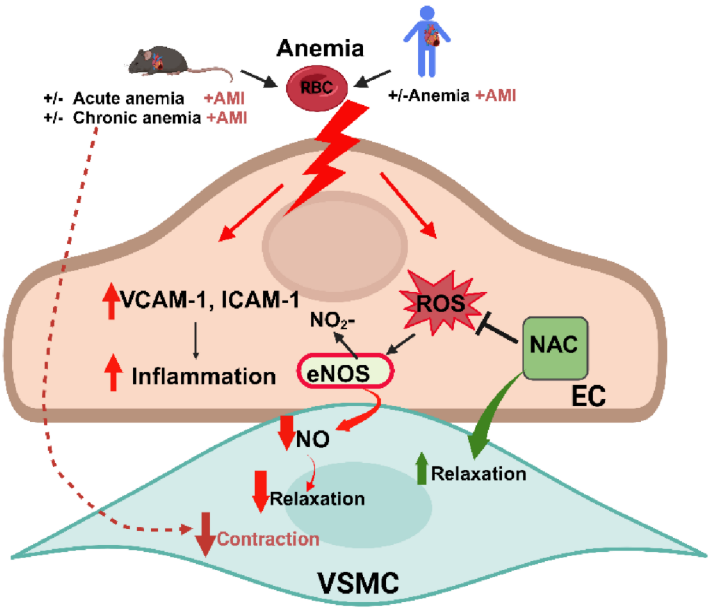


Figure 3: Conclusion of chapter 2: Anemia 24 h post AMI is associated vascular dysfunction. Both acute and chronic anemia is associated with increased inflammation, marked by elevated levels of intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), as well as increased production of reactive oxygen species (ROS). This leads to endothelial nitric oxide synthase (eNOS) uncoupling and reduced vascular smooth muscle cell (VSMC) relaxation, ultimately resulting in endothelial dysfunction (ED). Chronic anemia in mice post-AMI is associated with impaired VSMC contraction. N-Acetyl-L-Cystein (NAC) treatment improves vascular function in both mouse models of anemia after AMI. Created with BioRender.

Based on the findings from **Chapters 2 and 3**, we have evidence that anemic RBCs play a crucial role in mediating ED. However, the underlying mechanisms are not clearly understood. In **Chapter 4**, we investigated the potential role of RBC-derived extracellular vesicles (REVs) in mediating ED in anemia. In this chapter, we established a new protocol to isolate REVs, which we extensively characterized using different methods such as dynamic light scattering (DLS), Nanoparticle Tracking Analysis (NTA), Transmission electron microscopy (TEM), and western blotting. We then explored the role of anemic REVs in mediating ED using a newly established co-incubation protocol. Interestingly, REVs, but not plasma-derived extracellular vesicles (PLEVs), induced ED. Furthermore, REVs from anemic patients show a trend towards increased NO consumption, suggesting increased vesiculation and associated NO consumption might contribute to ED in anemia. Proteomic analysis of REVs demonstrated that REVs from anemic patients are associated with increased abundance of proteins involved in redox regulation, including oxidative stress-promoting proteins such as MPO, compared to REVs from non-anemic patients. Notably, several antioxidants, such as superoxide dismutase 1 (SOD1), lanthionine glutathione peroxidase 1 (GPX1), and catalase (CAT), were reduced in anemic REVs compared to those from non-anemic patients. These findings suggest that REVs

induce ED by NO consumption and redox alteration in recipient cells. However, the precise underlying mechanisms remain to be clarified.

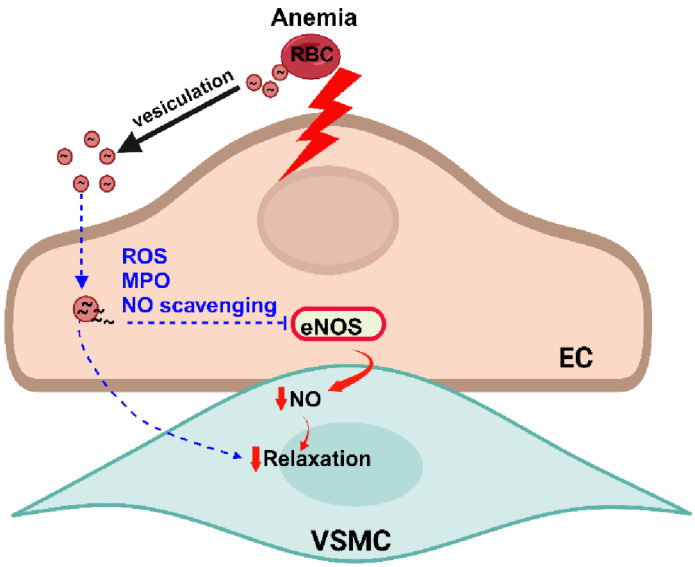


Figure 4: RBC-derived extracellular vesicles promote ED in anemia. Red blood cells (RBCs) release RBC-derived extracellular vesicles (REVs), which are taken up by endothelial cells. These REVs carry various redox enzymes, including oxidative stress promoting myeloperoxidase (MPO), leading to endothelial nitric oxide synthase (eNOS) uncoupling and a reduced vascular smooth muscle cell (VSMC) response. In addition, increased vesiculation and associated enhanced NO consumption contribute to NO dysregulation. Created with BioRender.

Blood-loss anemia mouse models

Anemia is frequently observed in patients with CVD and occurs in different types including hemoglobinopathies such as sickle cell anemia and beta-thalassemia, as well as blood-loss anemia, and iron-deficiency anemia *(5,6)*. To study different types of anemia, multiple mouse models are widely used, with each model contributing to the understanding of specific types of anemia. Given the clinical relevance of hospital-acquired anemia (HAA), we used a blood-loss anemic mouse model in this study to mimic HAA in a clinical setting. HAA is a frequently observed condition in patients linked to poorer clinical outcomes, including extended hospital stays and increased morbidity and mortality *(7)*. It is defined as the development of anemia during hospitalization or the worsening of existing anemia at the time of admission, as a result of clinical interventions *(8,9)*. In our mouse models, anemia was induced by drawing blood equivalent to 10% of the total body weight, which may also result in the loss of plasma proteins, including vasoactive compounds. However, our previous studies have shown that protein loss during blood withdrawal in this model is mild and does not cause vascular edema or leakage *(2)*. The effect of HAA on vascular function is largely unknown. In this thesis, we explored the

effects of this mouse model. Our studies have demonstrated that blood-loss anemia induces ED in anemic mice and provides clear evidence of RBC dysfunction. In clinical settings, patients often suffer from additional comorbidities and pathologies (e.g. hypertension, diabetes, CKD), which also influence vascular function. Therefore, our blood-loss mouse models are of significant value as they enable precise investigation of the effects of anemia alone on vascular function. In our anemic mouse models, we demonstrated that anemia aggravates ED in the context of AMI, further underscoring anemia mediated ED as a potential contributor to adverse outcomes.

Several mouse models of anemia are available to investigate the effects on endothelial function *(10–12)*. One such model is anemia of inflammation, commonly associated with infections, systemic inflammatory disorders, and also in patients admitted in intensive care units *(13)*. The frequently used mouse model for anemia of inflammation is an infection model in which mice are injected with heat-inactivated *Brucella abortus*. This leads to severe acute and chronic inflammation, which drastically affects the entire vascular system, including endothelial cells. *(13)*. Furthermore, another frequent type of anemia frequently observed in elderly patients is iron deficiency anemia *(14)*. Intestinal-specific ferroportin knockout mouse model is frequently used model to study iron deficiency anemia (*15*). This mouse model shows severe iron deficiency which resulted in unspecific effects on the whole vascular system (*12*). Compared to these two anemic mouse models, our blood-loss anemic mouse model represents low-grade inflammation and mild iron deficiency, characteristics that are commonly observed in many anemic patients. To ensure consistency with patient samples, we used Hb levels as an exclusion criterion. Since Hb levels are consistently maintained in our mouse, it is well-suited for uniformly studying anemia associated changes in vascular function. However, it is important to note that this approach does not provide a fully comprehensive representation of all endotypes of anemia.

Altered FMD responses in anemic mice and humans

The assessment of FMD responses is the gold standard for evaluating endothelial function in clinical settings. The FMD responses are mainly mediated by endothelium-dependent NO release. Impaired FMD responses are correlated with cardiovascular diseases (CVD) and their progression *(16–18)*. In this thesis, we evaluated the FMD responses to assess *in vivo* endothelial function in our mouse model. In the context of anemia, the available literature has been summarized in the table.

Table 1. Literature on flow-mediated dilation (FMD) measurements in different types of anemic mouse models and humans.

Disease/title	Species/Region	FMD	Author	Citation
Sickle cell disease	Humans /Brachial artery	Impaired endothelial function	Montalembert, Agguon et al. 2007	Haematologica. 2007 Dec;92(12):1709-10
Impaired nitric oxide-mediated vasodilation in **transgenic sickle** mouse	Mouse	Impaired Ex vivo NO relaxation Responses	Kaul, Liu et al. 2000	Am J Physiol Heart Circ Physiol. 2000 Jun;278(6):H1799-806.
Hemoglobin is inversely related to flow-mediated dilatation in **chronic kidney disease**	Humans /Brachial artery	Impaired	Yilmaz, Sonmez et al. 2009	Kidney Int. 2009 Jun;75(12):1316-1321
Up-regulation of renal and vascular nitric oxide synthase in **iron-deficiency anemia**	Rats	eNOS, iNOS, NOx increased	Ni, Morcos et al. 1997	Kidney Int. 1997 Jul;52(1):195-201
Erythropoietin therapy improves endothelial function in patients with non-dialysis **chronic kidney disease** and anemia (EARNEST-CKD): A clinical study	Humans /Brachial artery	Improved FMD after erythropoietin therapy	Lim, Yu et al. 2021	Medicine (Baltimore). 2021 Oct 22;100(42):e27601
Beta-thalassemia	Humans /Brachial artery	Impaired FMD, endohelial function	Solmaz, Cabuk et al. 2021	Echocardiography. 2021 Jun;38(6):825-833
The relationship between hemoglobin levels and endothelial functions in **diabetes mellitus**	Humans /Brachial artery	Endothelial function is inversely associated with Hb levels	Sonmez, Yilmaz et al. 2010	Clin J Am Soc Nephrol. 2010 Jan;5(1):45-50
Endothelium-derived relaxing factor is important in mediating the high output state in **chronic severe anemia**	Humans /Brachial artery	Increased	Anand, Chandrashekhar et al. 1995	J Am Coll Cardiol. 1995 May;25(6):1402-7
Effect of transfusion on the endothelium dependent dilatation of brachial artery in patients with **chronic anemia**	Humans /Brachial artery	FMD not improved after transfusion in anemic patients	Nagy, Tóth et al. 1999	Clin Hemorheol Microcirc. 1999;20(3):145-50.

Some of these studies indicate that chronic anemia alone (without co-morbidities) leads to an sustained or transient increase in FMD responses *(19)*. However, when combined with co-morbidities such as diabetes and CKD, anemia is linked to a deterioration in FMD responses *(20,21)*. Furthermore, our findings indicate that prolonged blood-loss anemia is associated with a further decline in FMD responses *(22)*. Similarly, blood-loss anemia in the context of pathologies, such as AMI, also contributes to worsening FMD responses *(23)*. Based on our study and existing literature, we can conclude that sustained or chronic anemia worsens FMD responses in various pathological conditions. These results highlight the importance of co-morbidities, including anemia, when evaluating FMD responses in clinical settings. Based on the findings of this study, we believe that anemia (on top of other pre-existing CV risk factors) aggravate ED and thus may further contribute to worsening of prognosis in CVD.

The role of red blood cells in endothelial dysfunction

Under physiological conditions, RBCs are essential not only for gas transport but also for various other functions, including their roles in vascular homeostasis, maintaining integrity, and

secreting vasoactive and cardioprotective molecules *(24,25)*. RBCs are known to release vasoactive factors such as adenosine triphosphate (ATP), sphingosine-1-phosphate (S1P), and cyclic guanosine monophosphate (cGMP) which play a crucial role in regulating endothelial function *(26–29)*. Further studies have revealed their involvement in pathologies, particularly in cardiovascular and metabolic diseases, including hypertension, heart failure and coronary heart disease *(24,30,31)*.

Since our anemic mouse models demonstrated a clear induction of ED in anemia in **Chapters 2** and **3**, we further explored the specific role of RBCs in mediating ED. Previous studies from our working group already have shown that RBCs from our acute anemic blood-loss model are associated with RBC dysfunction by decreasing NO bioavailability, increasing ROS formation, compromising membrane integrity, and enhancing NO scavenging by free plasma Hb *(2)*. Further, studies have demonstrated the influence of RBCs in mediating ED in different diseases such as diabetes, hypercholesterolemia and dyslipidemia *(32,33)*. Similarly, our co-incubation studies using isolated RBCs from anemic mice and patients demonstrated impaired endothelial function in murine aortic segments, suggesting that RBCs serve as mediators of ED.

However, the underlying mechanism how RBCs mediate ED is poorly understood. One of the proposed mechanisms is that ROS derived from RBCs promote ED by enhancing vascular arginase activity in endothelial cells in disease states such as diabetes *(31)*. Other studies also support the view that RBCs mediate endothelial function in other disease states such as hypercholesterolemia and dyslipidemia *(33,34)*. In line with these studies, we also showed that anemic RBCs promote oxidative stress in the endothelium. However, considering the short life span of oxygen radicals, the downstream signalling in endothelium in relation to ED yet need to be established. In **Chapter 2**, we showed that MPO abundance is increased in CA mice vessels. MPO is known to promote oxidative stress *(35)*. Studies have suggested that apart from neutrophils, RBCs also carry MPO *(36)*. This finding is further supported by our proteomics data where REVs isolated from anemic patients show an abundance of MPO. The specific role of RBC- derived MPO in mediating ED in anemia need to be further investigated.

The NO scavenging ability of free Hb is known to alter the NO bioavailability *(37)*. During RBC hemolysis, free Hb is released into the bloodstream, primarily in the reduced Fe(II)O2 state, which reacts with NO, reducing its bioavailability. This process also contributes to oxidative damage of cells, both leading to ED *(38,39)*. Previously, we demonstrated that our anemic mouse models are associated with mildly elevated levels of cell-free Hb *(21,2)*. This may contribute not only to NO scavenging but also to endothelial nitric oxide synthase (eNOS) inhibition by cell-free Hb (*40*), might lead to the observed reduction in FMD responses *in vivo* in anemic mice. However, the exact mechanism and contribution of cell-free Hb in blood-loss anemia-mediated ED need to be further investigated.

Potential role of red blood cell-derived extracellular vesicles in anemia-associated ED and therapeutic approaches

EVs are known to play a role in various physiological processes, including cellular communication, tissue regeneration, inflammatory regulation, and immune response *(41)*. Furthermore, EVs also contribute to various disease states including CVD such as coronary artery disease, acute coronary syndrome, and atherosclerosis *(42,43)*.

In our studies, we demonstrated that RBCs play a critical role in mediating ED in anemia. Additionally, we identified that REVs also play a potential role in this process. We demonstrated that REVs isolated from anemic patients impaired endothelial function in co-incubated murine aortic segments. Furthermore, consistent with previous studies, we also identified increased abundance of several proteins involved in redox homeostasis in our isolated REVs, including glutathione S-transferase, thioredoxin, and peroxiredoxin, with even higher abundance in anemic REVs *(44)*. The oxidative stress-promoting enzyme MPO was also identified among various redox-regulating enzymes in anemic REVs. Given that MPO is linked to vascular endothelial damage and progression of ED by promoting ROS formation and reducing NO bioavailability, the release of MPO from REVs into recipient cells may contribute to the development of ED in anemia *(35)*. Interestingly, decreased abundance of critical antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase 1 (GPX1), and catalase (CAT), was observed in anemic REVs compared to those from non-anemic patients. These enzymes are recognized as key components of the first-line antioxidant defense system, playing a fundamental role in overall defense mechanisms within biological systems *(45)*. This decreased abundance may indicate a potential redox imbalance within the cargo of anemic REVs, which could also affect the redox state of recipient cells. Redox proteins could potentially become key targets for further analysis, with enzymes like MPO offering valuable insights into the possible mechanisms by which RBCs and REVs contribute to ED. Another notable finding in our studies was the demonstration that, under *in vitro* conditions, REVs are actively taken up by endothelial cells, suggesting that similar uptake may occur *in vivo*. These findings highlight the potential relevance of REVs in the context of CVD, as they may represent a promising therapeutic strategy for promoting tissue repair and healing.

Interestingly, we identified specific proteins that were enriched exclusively in anemic patients, suggesting their potential role as biomarkers for anemia associated ED. In anemic REVs, we identified NADH-cytochrome b5 reductase 1, which plays a role in the reduction of methemoglobin, and immunoglobulin kappa variable 1-17, a protein involved in immune response *(46,47)*. In PLEV we found AP-3 complex subunit beta-1 and tetraspanin-15 enriched in anemic patients, both involved in vesicle formation but also other proteins such as heat shock protein 75 kDa, ubiquitin carboxyl-terminal hydrolase, and 2-hydroxyacyl-CoA lyase 2. The

exact effects of these proteins in the recipient cells, as well as the reason for their specific enrichment in anemic patients, remains unknown yet and requires further investigation.

EVs are considered suitable for therapeutic applications due to their properties, such as low immunogenicity and toxicity. Additionally, their ability to deliver genetic material while protecting it from degradation and crossing biological barriers, like the blood-brain barrier, makes them ideal carriers for therapeutic strategies *(41)*. Their versatile properties, heterogeneity, and the potential for modifying their cargo and targeting mechanisms position EVs as powerful tools for advancing both diagnosis and treatment *(48)*. This includes applications such as targeted drug delivery, gene therapy, and regenerative medicine *(48)*.

Especially REVs are proposed to operate as potential delivery vectors for therapeutic strategies, offering several advantages over vesicles derived from other sources *(49,50)*. A key consideration in therapeutic approaches is safety. Since mature RBCs lack a nucleus, they are considered safer, as their delivered content in REVs is more homogenous. This property is reducing the risk of genetic mutations and, consequently, the potential for unpredictable effects *(49,51)*. This makes REVs potentially safer compared to EVs isolated from other cell types. Another advantage of REVs lies in their readily available source, as they can be easily isolated from standard blood donations. Through the use of calcium ionophore treatment, a large quantity of REVs can be produced efficiently for therapeutic applications *(49)*. By connecting these advantages to our findings, which demonstrate the influence and uptake of REVs by endothelial cells, the use of REVs could represent a novel therapeutic approach in the field of CVD.

Clinical relevance of the current research

Acute myocardial infarction is one of the leading diseases and causes of sudden death worldwide *(52)*. It occurs when a blockage in the coronary arteries restricts blood and oxygen supply to the heart muscle, leading to the death or injury of cardiomyocytes and ultimately resulting in necrosis *(53)*. Previous studies have demonstrated that anemia, and HAA, are associated with worse outcomes in affected patients *(54)*. To gain insights into the potential mechanisms by which anemia contributes to these outcomes, we focused on alterations in endothelial function using mouse models. Our studies demonstrated that ED, which is mediated by anemia, becomes even more pronounced following AMI. These results also hint that deterioration of endothelial function may play a significant role in AMI and might contribute to a worse prognosis in anemic patients. These findings propose anemia and anemia-related ED as critical therapeutic targets in anemic patients following AMI.

Furthermore, our studies revealed that NAC treatment has a beneficial effect in our anemic mouse models on ED, which underscores the important role of ROS formation in anemia. NAC

is known for its antioxidant properties and has been identified as potential treatment in clinical settings. It has been proposed for the usage in pathologies such as diabetic cardiomyopathy, CAD, heart failure and AMI due to its cardioprotective function *(55)*. In our studies, we could show the potential benefits of NAC on endothelial function in anemia. Based on our findings, we strongly believe that NAC supplementation may improve systemic vascular function in HAA patients. Given that NAC is safe and widely used, it is worth considering clinical trials in HAA patients to check improvements vascular and cardiac function. In conclusion, we propose that antioxidants could serve as effective therapeutic options for preventing vascular complications associated with anemia, while preserving the cardioprotective properties of RBCs.

In the long term, we aim to develop a novel, easily quantifiable diagnostic panel based on the data collected, which will help to detect the extent of ED mediated by dysfunctional RBCs and REVs in these patients. Additionally, we aim to further investigate REVs as potential tools and biomarkers for future diagnostic and therapeutic applications in anemia. The findings of this thesis pave the way for further research, aiming to develop potential biomarkers and therapeutics.

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Abbreviations

4-HNE	4-Hydroxynonenal
AA	Acute anemia
ACH	Acetylcholine
AMI	Acute myocardial infarction
BH_4	Tetrahydrobiopterin
CA	Chronic anemia
CAD	Coronary artery disease
CaM	Calmodulin
CAT	Catalase
Ca^{2+}	Calcium
CCS	Chronic coronary syndrome
cGMP	Cyclic guanosine monophosphate
CLD	Chemiluminescence detector
CRC	Concentration-response curve
CVD	Cardiovascular disease
DLS	Dynamic light scattering
ECG	Electrocardiography
ED	Endothelial dysfunction
eNOS	Endothelial nitric oxide synthase
EV	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FMD	Flow-mediated dilation
FMN	Flavin mononucleotide
GTP	Guanosine triphosphate
HAA	Hospital-acquired anemia
Hb	Hemoglobin
HCT	Hematocrit
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
INDO	Indomethacin
iNOS	Inducible nitric oxide synthase

IR	Ischemia-Reperfusion
KRB	Krebs-Ringer bicarbonate-buffered salt solution
L-NAME	N(gamma)-nitro-arginine methyl ester
MPO	Myeloperoxidase
NAC	N-acetylcysteine
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NTA	Nanoparticle tracking analysis
$ONOO^-$	Peroxynitrite
O_2^-	Superoxide
PHE	Phenylephrine
PLEV	Plasma-derived EVs
RBCs	Red blood cells
REV	Red blood cell-derived extracellular vesicles
ROS	Reactive oxygen species
Ser	Serine
sGC	Soluble guanylate cyclase
SMCs	Smooth muscle cells
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STEMI	ST-elevation myocardial infarction
TEM	Transmission electron microscopy
Thr	Threonine
Tyr	Tyrosine
VCAM-1	Vascular cell adhesion molecule 1
VSMCs	Vascular smooth muscle cells
WHO	World Health Organization
WT	Wildtype

Chapter 6

Summary | Zusammenfassung

Acknowledgements

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Appendix

Summary

Cardiovascular diseases (CVD), including acute myocardial infarction (AMI), are the leading cause of death worldwide. Various pathologies and cardiovascular risk factors contribute significantly to the development of AMI. Endothelial dysfunction (ED) is very well known to be involved in the progression and development of atherosclerosis leading to AMI. Clinically, anemia, specifically hospital-acquired anemia (HAA), is frequently diagnosed in patients following AMI, often resulting in poor outcomes. However, the precise underlying mechanisms of this this association remain poorly understood.

In this thesis, we primarily focused on the role of anemia in relation to endothelial function, both independently and in combination with AMI. A key aspect of our research was to investigate the potential role of red blood cells (RBCs) in mediating ED associated with anemia. To address the research questions, we utilized established blood-loss anemic mouse models to mimic HAA observed in patients, in addition also analysed samples from anemic patients and their respective control groups.

In **Chapter 2**, we investigated the effect of prolonged chronic blood-loss anemia on endothelial function. Our findings revealed that chronic blood-loss anemia is associated with impaired endothelium-dependent relaxation responses both *in vivo* and *ex vivo*, which was accompanied by increased systemic inflammation and reactive oxygen species (ROS) production, leading to ED. We also observed elevated abundance of myeloperoxidase (MPO), an enzyme that promotes oxidative stress, in the vessels of chronic anemic mice. The inhibition of MPO *ex vivo*, or supplementation of mice with N-acetylcysteine (NAC), a ROS scavenger, improved endothelial function, highlighting the potential role of ROS in anemia-mediated ED. Moreover, RBCs isolated from chronic anemic mice and anemic patients induced ED in isolated murine aortic segments, suggesting that dysfunctional RBCs contribute to ED in anemia.

In **Chapter 3**, we further investigated the effect of anemia on ED following AMI. We observed that post-AMI, endothelial function deteriorates, leading to aggravated ED in acute anemic mice across different types of aterial blood vessels, accompanied by decreased FMD responses. In addition, endothelial and smooth muscle function were altered in chronic anemic mice post-AMI, evidenced by decreased FMD responses and contractile responses in large and small resistance arteries. We also observed decreased nitric oxide (NOx) metabolites in both chronic and acute anemic mice indicating decreased NO bioavailability. Our findings further confirmed the potential role of RBCs in mediating ED, as co-incubation studies of RBCs from anemic mice 24 h post-AMI, as well as from ST-elevation myocardial infarction (STEMI) patients, induced ED in murine aortic rings. Together, the results from **Chapters 2** and **3** confirmed the role of dysfunctional RBCs in meditating ED in blood-loss anemia. Additionally, we observed that inflammation, ROS production, and reduced nitric oxide (NO) bioavailability are

key factors in this mechanism. In both chapters, we also demonstrated a beneficial effect of NAC on improving endothelial function in anemia. However, the precise mechanism by which dysfunctional RBCs mediate ED remains unclear.

To further investigate a potential mechanism, in **Chapter 4** we focused on the role of red blood cell-derived extracellular vesicles (REVs) in mediating ED. To address this research question, we developed a new isolation protocol for REVs and characterized them using various methods. We demonstrated that anemic REVs are taken up by endothelial cells *in vivo* and induce ED in isolated murine aortic segments. In addition, we demonstrated that vesiculation of REVs was increased in anemic patients and was associated with increased NO consumption, suggesting a potential role in regulating NO bioavailability. Proteomic analysis revealed an enriched abundance of redox-related proteins in REVs from anemic patients. These results support the hypothesis that REVs derived from circulating dysfunctional RBCs in anemia contribute to the development of ED; however, further detailed investigations are needed to fully understand the mechanisms.

In conclusion, the findings of this thesis contribute to a better understanding of how blood-loss anemia may worsen CVD and AMI prognosis in patients by influencing endothelial function. Furthermore, we suggest NAC treatment as a potential therapeutic strategy for preventing vascular complications associated with anemia, as we observed its beneficial effect on improving endothelial function in our anemic mouse models. Early insights into the role of REVs suggest that they may be involved in anemia-associated ED, warranting further investigation, and could serve as potential therapeutic targets in anemia and cardiovascular-related pathologies.

Zusammenfassung

Herz-Kreislauf-Erkrankungen (CVD, englisch: cardiovascular diseases), einschließlich des akuten Myokardinfarkts (AMI), sind die weltweit häufigste Todesursache. Verschiedene Pathologien und kardiovaskuläre Risikofaktoren tragen wesentlich zur Entwicklung des AMI bei. Es ist bekannt, dass Endotheliale Dysfunktion (ED) eine wichtige Rolle bei der Entstehung und dem Fortschreiten der zum AMI führenden Atherosklerose spielt. Klinisch wird Anämie, insbesondere die im Krankenhaus entwickelte Anämie (HAA, englisch: hospital-acquired anemia), häufig bei Patienten nach einem AMI diagnostiziert, was oft zu schlechteren Prognosen führt. Die genauen Mechanismen, die dieser Assoziation zugrunde liegen, sind jedoch noch unzureichend verstanden.

In dieser Dissertation konzentrierten wir uns in erster Linie auf die Rolle der Anämie in Bezug auf die Endothelfunktion, sowohl unabhängig als auch in Kombination mit AMI. Ein zentraler Aspekt unserer Forschung war die Untersuchung der potenziellen Rolle von roten Blutkörperchen (RBCs, englisch: red blood cells) bei der Induktion von ED, in der Anämie. Um die Forschungsfragen zu beantworten, nutzten wir etablierte Mausmodelle für blutverlustbedingte Anämie, um die bei Patienten beobachtete HAA nachzuahmen, und analysierten zusätzlich Proben von anämischen Patienten und ihren jeweiligen Kontrollgruppen.

In **Kapitel 2** untersuchten wir den Einfluss von chronischer Blutverlust-Anämie auf die Endothelfunktion. Unsere Ergebnisse zeigten, dass chronische Blutverlust-Anämie mit einer Beeinträchtigung der endothelabhängigen Relaxationsreaktionen sowohl *in vivo* als *auch ex vivo* verbunden ist. Dies ging einher mit einer erhöhten systemischen Entzündung und einer gesteigerten Produktion von reaktiven Sauerstoffspezies (ROS, englisch: reactive oxygen species), was zur ED führte. Zudem beobachteten wir eine erhöhte Menge an Myeloperoxidase (MPO), einem Enzym, das oxidativen Stress fördert, in den Gefäßen von chronisch anämischen Mäusen. Die Hemmung von MPO *ex vivo* oder die Supplementierung der Mäuse mit N-Acetylcystein (NAC), einem ROS-Scavenger, verbesserte die Endothelfunktion und unterstreicht die potenzielle Rolle von ROS bei der Anämie assoziierten ED. Darüber hinaus führten RBCs, die von chronisch anämischen Mäusen und anämischen Patienten isoliert wurden, zu ED in isolierten murinen Aorten-Segmenten, was darauf hindeutet, dass dysfunktionale RBCs zusätzlich zur ED bei der Anämie beitragen.

In **Kapitel 3** untersuchten wir weiter den Einfluss von Anämie auf die ED nach AMI. Wir stellten fest, dass die Endothelfunktion nach einem AMI weiter verschlechtert wird, was zu einer verstärkten ED bei akut anämischen Mäusen in unterschiedlichen arteriellen Blutgefäßen führt, begleitet von einer verringerten flussvermittelnden Dilatation (FMD, englisch: flow-mediated dilation). Darüber hinaus waren die Endothel- und glatte Muskelzellfunktionen bei chronisch

anämischen Mäusen nach einem AMI beeinträchtigt, was sich durch verringerte FMD-Antworten und Kontraktionsreaktionen in großen und kleinen Arterien zeigte. Wir beobachteten auch verminderte Nitrat- und Nitrit-Metaboliten (NOx) bei sowohl chronisch als auch akut anämischen Mäusen, was auf eine verringerte Stickstoffmonoxid (NO; Englisch: nitric oxide) -Bioverfügbarkeit hindeutet. Unsere Ergebnisse bestätigten erneut die potenzielle Rolle von RBCs bei der Vermittlung von ED, da Co-inkubationsstudien mit RBCs von anämischen Mäusen 24 Stunden nach AMI sowie von ST-Hebungs-Myokardinfarkt (STEMI, englisch: ST-elevation myocardial infarction) -Patienten eine ED in murinen Aortenringen auslösten. Insgesamt bestätigten die Ergebnisse aus den **Kapiteln 2** und **3** die Rolle dysfunktionaler RBCs bei der Vermittlung von ED bei Blutverlustanämie. Darüber hinaus zeigten wir, dass Entzündungen, ROS-Produktion und eine verringerte NO-Bioverfügbarkeit die Schlüsselfaktoren dieses Mechanismus sind. In beiden Kapiteln demonstrierten wir auch die positive Wirkung von NAC auf die Endothelfunktion bei Anämie. Der genaue Mechanismus, durch den dysfunktionale RBCs eine ED vermitteln, bleibt jedoch unklar.

Um einen möglichen Mechanismus weiter zu untersuchen, konzentrierten wir uns in **Kapitel 4** auf die Rolle von erythrozytenabgeleiteten extrazellulären Vesikeln (REVs, englisch: red blood cell-derived extracellular vesicles) bei der Vermittlung einer ED. Um diese Forschungsfrage zu beantworten, entwickelten wir ein neues Isolationsprotokoll für REVs und charakterisierten sie mit verschiedenen Methoden. Wir zeigten, dass anämische REVs von Endothelzellen *in vivo* aufgenommen werden und eine ED in isolierten murinen Aorten-Segmenten induzieren. Zudem stellten wir fest, dass die Vesikelbildung von REVs bei anämischen Patienten erhöht war und mit einem erhöhten NO-Verbrauch einherging, was auf eine potenzielle Rolle bei der Regulierung der NO-Bioverfügbarkeit hindeutet. Eine durchgeführte Proteomics-Analyse ergab eine Anreicherung von redoxbezogenen Proteinen in REVs aus anämischen Individuen. Diese Ergebnisse unterstützen die Hypothese, dass REVs aus zirkuierenden dysfunktionalen RBC in der Anämie zusätzlich zur Entwicklung von ED beitragen; dennoch sind weitere detaillierte Untersuchungen erforderlich, um ihre Beteiligungsmechanismen vollständig zu verstehen.

Zusammenfassend tragen die Ergebnisse dieser Dissertation zu einem besseren Verständnis darüber bei, wie Blutverlustanämie die Prognose von CVD und AMI bei Patienten die Endothelfunktion verschlechtern kann. Darüber hinaus könnte eine NAC-Behandlung eine potenzielle therapeutische Strategie zur Verhinderung von vaskulären Komplikationen im Zusammenhang mit Anämie darstellen, da wir in unseren anämischen Mausmodellen eine positive Wirkung auf die Verbesserung der Endothelfunktion beobachtet haben. Erste Erkenntnisse zur Rolle von REVs legen nahe, dass sie bei der anämiebedingten ED eine Rolle spielen könnten, was weitere Untersuchungen rechtfertigt und sie zu potenziellen therapeutischen Zielen bei Anämie und kardiovaskulären Erkrankungen macht.

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Curriculum vitae (CV)

Persönliche Daten

Name	Isabella Maria Solga-Kamp (geb. Solga)
Geburtsdatum	05. April 1995
Geburtsort	Mönchengladbach-Neuwerk
Staatsangehörigkeit	deutsch-polnisch

Hochschulbildung

2020 bis Aktuell	Promotion Heinrich-Heine-Universität Düsseldorf Im Forschungslabor der Klinik für Kardiologie, Pneumologie und Angiologie der Heinrich-Heine-Universität Düsseldorf Titel: "The role of red blood cells and red blood cell-derived extracellular vesicles in anemia-associated endothelial dysfunction"
2018 bis 2020	Masterstudium der Biologie (M.Sc.) Heinrich-Heine-Universität Düsseldorf Masterarbeit im Forschungslabor der Klinik für Kardiologie, Pneumologie und Angiologie der Heinrich-Heine-Universität Düsseldorf Titel: "Characterization of vascular and cardiac eNOS expression in acute and chronic anemia"
2014 bis 2018	Bachelorstudium der Biologie (B.Sc.) Heinrich-Heine-Universität Düsseldorf Bachelorarbeit am Institut für Molekulare Evolution Titel: „Kleptoplast-barcoding in Elysia timida, gesammelt im Mittelmeer"

Publikationen

Wischmann, P.; Chennupati, R.; **Solga, I**.; Funk, F.; Becher, S.; Gerdes, N.; Anker, S.; Kelm, M.; Jung, C. Safety and efficacy of iron supplementation after myocardial infarction in mice with moderate blood loss anaemia. ESC heart failure 2021, 8 (6), 5445–5455. DOI: 10.1002/ehf2.13639.

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with systemic endothelial dysfunction. Frontiers in cardiovascular medicine 2023, 10, 1099069. DOI: 10.3389/fcvm.2023.1099069.

Wischmann, P.; Chennupati, R.; **Solga, I**.; Yogathasan, V.; Langerbein, C.; Jäger, L.; Gerdes, N.; Kelm, M.; Jung, C. Red Blood Cell-Mediated Cardioprotection Is Impaired in ST-Segment Elevation Myocardial Infarction Patients With Anemia. JACC. Basic to translational science 2023, 8 (10), 1392–1394. DOI: 10.1016/j.jacbts.2023.06.010.

Solga, I.; Sahin, A.; Yogathasan, V.; Hofer, L.; Celik, F. G.; Rai, A. E.; Hosen, M. R.; Wischmann, P.; Becher, S.; Polzin, A.; Gerdes, N.; Jung, C.; Kelm, M.; Chennupati, R. Distinct effects of acute and chronic blood loss anemia on vascular function after acute myocardial infarction, 2024. DOI: 10.1101/2024.09.24.614629.

Poster

90. Deutsche Gesellschaft für Kardiologie (DGK)-Jahrestagung 3. April - 6. April 2024 | Mannheim

Isabella Solga, Aslihan Sahin, Vithya Yogathasan, Christian Jung, Malte Kelm, Ramesh Chennupati. Anemia aggravates the systemic endothelial dysfunction after acute myocardial infarction.

Eidesstattliche Versicherung

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Folgenden Versuche und Ergebnisse, wurden von Dritten bzw. in Kooperation mit Dritten erstellt :

- Die IR-Operationen und Anämie-Induktionen wurden von Stefanie Becher durchgeführt.

- Die Elektronmikrskopischen Aufnahmen wurden in Kooperation mit der Core Facility Elektronenmikroskopie (CFEM) der Heinrich-Heine-Uiniversität Düsseldorf von Dr. Ann Kathrin Bergmann durchgeführt

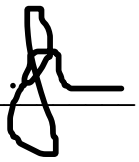
- Die experimentelle Durchführung und Analysen der Proteomics Proben wurden in Kooperation mit dem Biologisch-Medizinischem Forschungszentrum (BMFZ) an der Heinrich-Heine-Universität von Dr. Anja Stefanski durchgeführt. Zustätzlich haben Dr. Tin Yau Pang, Dr. Alexander Lang und Dr. Mohammed Rabiul Hosen zur weiteren Analyse der Proteomics-Daten beigetragen.

- Außerdem werden die von Dritten oder Ko-Autoren durchgeführten Experimente in jedem Manuskript unter dem Abschnitt „Author Contributions“ angegeben. Die von mir eigenständig durchgeführten Experimente sind entsprechend gekennzeichnet und im Anhang aufgeführt

Diese Dissertation wurde weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt. Außerdem erkläre ich, dass ich bisher noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Teile dieser Dissertation wurden bereits in einer wissenschaftlichen Zeitschrift veröffentlicht, als Manuskript hochgeladen oder auf wissenschaftlichen Konferenzen präsentiert (Aufgeführt im Anhang und CV).

Düsseldorf, den 25.11.2024



Unterschrift

Appendix

Contribution of Isabella Maria Solga-Kamp as an author in the manuscripts used in this dissertation

Manuscript 1: Chapter 2

(Published manuscript in Frontiers in Cardiovascular Medicine, 2023 (pp. 25–52 of Dissertation))

Chronic anemia is associated with systemic endothelial dysfunction

Chennupati R, **Solga I,** Wischmann P, Dahlmann P, Celik FG, Pacht D, Şahin A, Yogathasan V, Hosen MR, Gerdes N, Kelm M, Jung C. Chronic anemia is associated with systemic endothelial dysfunction. *Frontiers in Cardiovascular Medicine,* 2023, 10, 1099069. DOI: 10.3389/fcvm.2023.1099069.

(Shared first author)

Contribution:

The author of this dissertation contributed approximately 60% of the data in this publication. This includes the *ex vivo* assessment of endothelial function and analysis, as well as the evaluation of eNOS, iNOS, and ROS abundance in aortic tissue using Western blots and qPCR. Additionally, the author performed histological evaluation of eNOS, iNOS, and ROS staining. The author also contributed to the analysis of inflammatory markers in plasma from both mice and human samples using ELISA-based assays. The experiments were carried out by the author or with the help of co-authors under the author's supervision.

Manuscript 2: Chapter 3

(Preprint version of manuscript prepared for submission (pp. 53–90 of Dissertation))

Distinct effects of acute and chronic blood loss anemia on vascular function after acute myocardial infarction

Solga I, Şahin A, Yogathasan V, Hofer L, Celik FG, Rai AE, Hosen MR, Wischmann P, Becher S, Polzin A, Gerdes N, Jung C, Kelm M, Chennupati R. *Distinct effects of acute and chronic blood loss anemia on vascular function after acute myocardial infarction,* 2024. DOI: 10.1101/2024.09.24.614629.

(First author)

Contribution:

The author of this dissertation contributed 85% to the research work and manuscript completion. The author designed and conducted all animal experiments presented in

the manuscript. Additionally, the author performed immunohistochemistry and biochemical assays, contributed significantly to the analysis of the generated data, and co-drafted the manuscript with the corresponding author. The experiments were carried out by the author or with the help of co-authors under the author's supervision.

Manuscript 3: Chapter 4

(Manuscript in preparation for submission (pp. 91–119 of Dissertation))

Large extracellular vesicles derived from red blood cells promote endothelial dysfunction in patients with anemia

Solga I, Yogathasan V, Pang TY, Wischmann P, Temme S, Hofer L, Stefanski A, Bergmann AK, Becher S, Gerdes N, Hosen MR, Nickenig G, Jung C, Kelm M, Chennupati R.

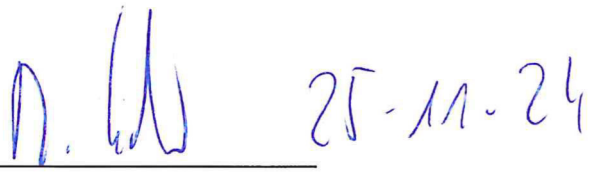
(First author)

Contribution:

The author of this dissertation contributed 85% to the manuscript completion. The author established the protocol for isolating vesicles from blood samples. The experiments for vesicle characterization were carried out by the author or with the help of co-authors under the author's supervision. Furthermore, the author prepared samples for proteomics analysis, which were processed and analyzed in collaboration with cooperation partners. The author also made significant contributions to drafting the manuscript together with corresponding author.

Confirmation of the supervisor regarding the above-listed contributions of Isabella Maria Solga-Kamp in the manuscripts used in this thesis:

I hereby confirm the above-mentioned contributions of Isabella Maria Solga-Kamp to the completion of the manuscripts used in this dissertation.



25.11.24

Prof. Dr. med. Malte Kelm