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An Oral Carbon Monoxide-Releasing Molecule Protects against Acute Hyper-hemolysis in Sickle Cell Disease

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Abstract:
Acute hyper-hemolysis is a severe life-threatening complication in patients with sickle cell disease (SCD) that may occur during delayed hemolytic transfusion reaction (DHTR), or vaso-occlusive crises associated with multi-organ failure. Here, we developed in vitro and in vivo animal models to mimic endothelial damage during the early phase of hyper-hemolysis in SCD. We then used the carbon monoxide (CO)-releasing molecule CORM-401 and examined its effects against endothelial activation, damage, and inflammation inflicted by hemolysates containing red blood cell membrane-derived particles. The in vitro results revealed that CORM-401: 1) prevented the up-regulation of relevant pro-inflammatory, and pro-adhesion controlled by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and 2) abolished the expression of the nuclear factor erythroid-2-related factor 2 (Nrf2) that regulates the inducible antioxidant cell machinery. We also show in SCD mice that CORM-401 protects against hemolysate-induced acute damage of target organs such as the lung, liver, and kidney through modulation of NF-kB pro-inflammatory and Nrf2 antioxidant pathways. Our data demonstrate the efficacy of CORM-401 as a novel therapeutic agent to counteract hemolysate-induced organ damage during hyper-hemolysis in SCD. This approach might be considered as possible preventive treatment in high-risk situations such as SCD patients with history of DHTR.

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available on request from the corresponding author, Pablo Bartolucci (pablo.bartolucci@aphp.fr)

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Keywords: Delay hemolysis transfusion reaction (DHTR), sickle cell disease (SCD), intravascular hemolysis, endothelial damage, transfusion, carbon monoxide (CO), CO-releasing molecules (CO-RMs)

Key Points

- Red blood cell membrane-derived particles cause endothelial dysfunction and organ damage at the onset phase of hyper-hemolysis in SCD.
- Carbon monoxide-releasing molecule CORM-401 counteracts organ damage at the onset phase of hyper-hemolysis in SCD.

ABSTRACT

Acute hyper-hemolysis is a severe life-threatening complication in patients with sickle cell disease (SCD) that may occur during delayed hemolytic transfusion reaction (DHTR), or vaso-occlusive crises associated with multi-organ failure. Here, we developed *in vitro* and *in vivo* animal models to mimic endothelial damage during the early phase of hyper-hemolysis in SCD. We then used the carbon monoxide (CO)-releasing molecule CORM-401 and examined its effects against endothelial activation, damage, and inflammation inflicted by hemolysates containing red blood cell membrane-derived particles. The *in vitro* results revealed that CORM-401: 1) prevented the up-regulation of relevant pro-inflammatory, and pro-adhesion controlled by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and 2) abolished the expression of the nuclear factor erythroid-2-related factor 2 (Nrf2) that regulates the inducible antioxidant cell machinery. We also show in SCD mice that CORM-401 protects against hemolysate-induced acute damage of target organs such as the lung, liver, and kidney through modulation of NF-kB pro-inflammatory and Nrf2 antioxidant pathways. Our data demonstrate the efficacy of CORM-401 as a novel therapeutic agent to counteract hemolysate-induced organ damage during hyper-hemolysis in SCD. This approach might be considered as possible preventive treatment in high-risk situations such as SCD patients with history of DHTR.

INTRODUCTION

Sickle cell disease (SCD) is a common inherited red blood cell (RBC) disorder characterized by chronic hemolysis and acute vaso-occlusive events.[1] RBC transfusion is still a key therapeutic intervention in the clinical management of both acute and chronic sickle cell-related complications.[2]

Acute hyper-hemolysis is an extremely severe situation encountered in SCD during different situations including severe vaso-occlusive crises (VOC) with multi-organ damage and delayed hemolytic transfusion reaction (DHTR), a life-threatening complication of RBC transfusion with high incidence among SCD patients,[3–5] which contributes to 6% of mortality.[6] DHTR is characterized by dramatic hemolysis with a drop in hemoglobin (Hb) levels due to the destruction of both transfused RBCs and patients' RBCs (bystander effect).[4,7] Evolution during the first 24-48 hours after DHTR onset is crucial in terms of organ damage and prognosis. During DHTR, hemolysis is generally caused by alloimmunization via antibodies against RBCs, which are not identified in almost 30% of DHTR cases.[7,8] The risk of DHTR following a blood transfusion can be evaluated by considering a patient's history of DHTR, the presence of alloimmunization, or a low number of prior transfusions.[4,9] DHTR results in severe intravascular hemolysis and activation of the complement system.[10–12] Free heme and free Hb during DHTR promote intravascular oxidative stress, leading to (i) endothelial damage with up-regulation of pro-adhesive and chemotaxis factors such as vascular cell adhesion molecule 1 (VCAM-1) or monocyte chemoattractant protein 1 (MCP-1);[11,13–16] (ii) reduction in nitric oxide (NO) bioavailability;[17–19] and (iii) abnormal activation of the alternative complement pathway.[11,12] Previous studies in cell- and animal-based DHTR models used purified Hb or hemin to mimic the effects

of severe intravascular hemolysis,[13,14,20,21] which do not take into account the contribution of RBC membrane-derived particles in amplifying and sustaining free heme/Hb-induced vascular endothelial damage.

To limit the deleterious effects of free heme/Hb in SCD, different therapeutic strategies have been proposed including infusion of hemopexin, which neutralizes heme, or haptoglobin, a scavenger of hemoglobin. Carbon monoxide (CO), a byproduct of heme metabolism catalyzed by heme oxygenase-1 enzyme, evokes anti-inflammatory effects on the vasculature, and in SCD mice has been demonstrated to elicit beneficial therapeutic effects.[22–24] Both CO inhalation and CO-releasing molecules (CO-RMs) are promising candidates for the treatment of inflammatory vasculopathy.[23,25–27] Among the pharmacologically active CO-RMs, CORM-401 is a recently characterized Mn-containing compound that is stable and delivers precise CO quantities with high efficiency both *in vitro* and when orally administered *in vivo*.[27,28] Previous studies showed that endothelial cells exposed to CORM-401 accumulate intracellular CO, leading to endothelial calcium signaling and increased NO bioavailability.[29]

Here, we studied the effects of CORM-401 on acute hyper-hemolysis onset *in vitro* and in a humanized SCD mouse model.[30] Using approaches that mimic the onset phase of hyper-hemolysis, we found a protective effect of CO delivered by CORM-401 in hemolysis-induced endothelial dysfunction and organ damage, with significant reduction of inflammation and acute response systems. Thus, CORM-401 may be considered an interesting therapeutic agent to alleviate hyper-hemolysis-mediated vascular and organ dysfunction occurring during DHTR in SCD patients.

MATERIALS AND METHODS

In vitro cell-based assays

Blood samples were collected from healthy blood donors (AA) from the "Etablissement-Français-du-Sang" and from SCD patients (SS) with consent and local-Institutional-Review-Board (CPP–Creteil) approval (collection protocol ERYTHROPEDIE), excluding patients in chronic transfusion program.

Fluidic model to mimic DHTR

Flow culture of human umbilical vein endothelial cells (HUVECs) (Step 1, Figure 1A). HUVECs isolated from neonate umbilical vein (see Supplementary Methods) were seeded in fibronectin-coated µ-Slides (Sigma-Aldrich). After 2H of static culture, cells were exposed to shear stress 1 dyne.cm−2 overnight using the Kima pump (Cellix). For hemolysate preparation, freshly washed AA-RBCs were resuspended in autologous serum at 2.5% (V/V) then lysed by sonication. This hemolysate reproduces the early phase of DHTR observed in SCD patients, when almost 10% of RBCs are destroyed within a few hours with a drop in Hb concentration[6]. In all experiments, lysis of 10% RBCs corresponds to free Hb levels of 7.022 g/L. MetHb, and heme were extremely low (<0.1%, and <0.17 µM respectively).

Hemolysate preconditioning (Step 2, Figure 1A). Flow cultured HUVECs were exposed to hemolysate containing RBC membrane-derived particles for 4H at shear stress 1 dyne.cm−2. HUVECs were then washed in culture medium before functional assays or other studies.

Functional assays (Step 3, Figure 1A). AA/SS whole blood (WB) containing 10% (V/V) of the supernatant from the hemolysate preconditioning step was perfused onto

preconditioned HUVECs to study RBC adhesion and platelet thrombus formation (see Supplementary Methods).

Determination of Hb, MetHb, heme and carboxyhemoglobin (COHb) were performed as previously reported.[31]

Transcriptome analysis of HUVECs: See Supplementary Methods.

CORM-401 or inactive CORM (iCORM)

CORM-401 was synthetized as described previously.[27,29] iCORM, which does not contain CO, was used as a negative control and was prepared by mixing equimolar amounts of the dithiocarbamate ligand present in CORM-401 ($Na[S_2CN(CH_3)CH_2COONa]x2CH_3OH$) in combination with Mn_2SO_4. Treatment with CORM-401 (50 or 100 µM) or iCORM (100 µM) in the hyper-hemolysis syndrome-mimicking model was performed 1 hour before and during Step 2. CORM-401 or iCORM were then incubated with WB for 30 min before perfusion on HUVECs (Step 3).

Mouse model and experimental design

Experiments were performed on 4 months old sex matched healthy control (Hbatm1(HBA)Tow Hbbtm3(HBG1, HBB)Tow) and SCD (Hbatm1(HBA)Tow Hbbtm2(HBG1,HBB*)Tow) (SS) mice. The animal protocol was approved by the Animal Care and Use Committee at the University of Verona (CIRSAL). Hematological parameters and COHb levels were assessed using a Sysmex XN-1000 Hematology Analyzer and a GEM Premier 4,000 blood gas analyzer, respectively. Blood urea nitrogen (BUN) and creatinine were measured in plasma.

Hemolysate preparation involved collecting blood from AA or SCD mice. Washed RBCs were lysed in water (see Supplementary Methods). The resulting hemolysate

was infused intravenously in AA (560 mg/kg) and SCD mice (350 mg/kg). Vehicle (PBS) or CORM-401 (30 mg/kg) was orally administered three times weekly for 3 weeks. When indicated, mice underwent acute hemolysate infusion 5 hours before sacrifice.

Reticulocytes, Annexin V positive cell, organ histology, immunoblot analyses and quantification of hepatic CO accumulation

Detailed in Supplementary Methods.

Statistical analysis

Detailed in Supplementary Methods.

Blood samples were collected from healthy blood donors (AA) from the "Etablissement -Français-du-Sang" and from SCD patients (SS genotype) with consent and local-Institutional-Review-Board (CPP-Creteil) approval (collection protocol ERYTHROPEDIE) The Institutional Animal Experimental Committee of University of Verona (CIRSAL) and the Italian Ministry of Health approved the experimental protocols (56DC9.64), following European directive 2010/63/EU and the Federation for Laboratory Animal Science Associations guidelines and recommendations.

RESULTS

HUVECs display a pro-adhesion and pro-inflammatory phenotype when exposed to hemolysis in a fluidic model

To mimic DHTR, we exposed HUVECs to hemolysate in a fluidic model for 4 hours (Figure 1A). MetHb was negligible (<0.1 %) and heme undetectable (<0.17 µM), indicating that oxidation of Hb does not occur under these conditions. Exposure of HUVECs to hemolysate upregulated ICAM-1 (CD54) and VCAM-1 (CD106), although less markedly than tumor necrosis factor alpha (TNFα), a positive control for HUVECs activation, compared to serum-treated cells (Figure 1B). Moreover, we observed a reorganization of HUVECs exposed to hemolysate compared to serum or TNFα, which might favor cell detachment under flow conditions, as shown by the increased percentage of non HUVEC-covered surfaces (Supplementary Figure 1S). In these cell-detached areas, we detected Annexin Vpos without nuclear and PECAM-1 staining due to either residual HUVEC membrane or deposition of phosphatidylserine (PS) from RBC membranes in hemolysate (Figure 1B, Supplementary Figure 2S). The merged image also showed a colocalization of vascular activation (ICAM-1 and or VCAM-1) indicating that hemolysate induces inflammatory response. Some of these cells also positive for Annexin V indicating an apoptotic response in the same cell (Figure 1B, Supplementary Figure 2S).

We investigated the effect of hemolysate on gene expression profile in HUVECs by mRNA sequencing. We found a significant up-regulation of inflammatory genes such as *VCAM-1, ICAM-1, NFKβ1, NFKβ2, E-Selectin-, IL-6, IL-8* and *Hmox1* in hemolysate-treated HUVECs compared to serum-treated cells (fold change > 1.5 and False Discovery Rate (FDR) < 0.05) (Figure 1C).

We then evaluated adhesion of blood cells from AA WB to HUVECs pre-exposed to either serum, hemolysate, or TNFα (Step 3, Figure 1A). RBC adhesion increased significantly in hemolysate or TNFα–treated HUVECs compared to serum only (Figure 2A). This adhesion occurred mainly on HUVECs positive for ICAM-1 and/or VCAM-1 and/or Annexin V rather than on non-HUVEC-covered surfaces resulting from cell detachment (without PECAM-1 and nuclear marker) (Supplementary Figure 2S). Interestingly, pre-treatment with AA hemolysate tended to increase the adhesion of RBCs from SS patients compared to AA controls (Supplementary Figure 3S). We noted a negligible leukocyte adhesion on hemolysate-treated HUVECs (data not shown).

The formation of platelet thrombus on damaged endothelium was evaluated by staining platelet and endothelial cells after AA WB perfusion on preconditioned HUVECs for 10 minutes. We observed that hemolysate induced aggregation (Figure 2B) and activation (Figure 2C) of platelets at injury sites. This platelet recruitment was significantly reduced by pre-treating WB with integrilin, a GPIIbIIIa antagonist (Figure 2D). Interestingly, when SS WB was perfused, we noted a massive platelet recruitment in non-HUVEC-covered areas, which was completely abolished by intergrilin treatment (Supplementary Figure 4S). These results suggest a role for platelet-platelet interaction in hemolysate-induced thrombus formation on damaged endothelium.

CORM-401 prevents hemolysate-induced activation of HUVECs and inflammatory response

Previous studies have highlighted the importance of CO against inflammatory vasculopathy related to SCD.[22,32] We therefore evaluated the effects of CORM-401

by exposing HUVECs to this CO-releasing agent[27–29,33] either: (i) before hemolysate infusion, (ii) during hemolysate infusion or (iii) before and during hemolysate infusion Protocol (iii) was the most effective in preventing HUVECs activation (Supplementary Figure 5S) and was chosen for the following experiments.

Thus, we pre-treated HUVECs with CORM-401 or its inactive counterpart (iCORM) for 1 hour before exposure to hemolysate incubated with CORM-401 or iCORM, which contained similar Hb levels (Supplementary Figure 6SA). CORM-401 (50 and 100 µM) increased COHb from 0% to 8.88% and 14.74%, respectively, while 100 µM iCORM did not (Supplementary Figure 6SB). A two-step treatment with 100 µM CORM-401 significantly reduced ICAM-1, VCAM-1 expression (Figure 3A) and tended to reduce reactive oxygen species (ROS) generation (Supplementary Figure 7S), suggesting a reduction of hemolysate-induced HUVEC activation and oxidative stress. CORM-401 had no significant effect on annexin V or PECAM-1 staining (Figure 3A), maintaining a similar proportion of non-HUVEC-covered surfaces (Supplementary Figure 8S). Noteworthy, hemolysate-induced RBC and platelet adhesion on HUVECs decreased with the two-step treatment compared to untreated hemolysate (Figure 3B).

Transcriptomic analysis showed that CORM-401 prevented hemolysate-induced up-regulation of pro-inflammatory markers (Figure 4A). As shown in Figure 4B, hemolysate significantly upregulated the TREM1, IL-1, HMGB1, and IL-8 signaling pathways (z-score>2, large red dots) and tended to increase NFkB, IL-6, acute inflammatory phase signaling (z-score between 1 and 2, small red dots). Noteworthy, CORM-401 inhibited all these pathways (z-score<-2, Figure 4B, large green dots) except apoptosis which was non-significantly increased (Figure 4B, z-score=1, small green dots). In contrast, iCORM had no significant effect on 28/33 genes compared

to serum conditions (fold change < 1.5 Supplementary Figure 9S, green graph). Therefore, CO released from CORM-401 appears to counteract the negative effects of hemolysate on pro-inflammatory, pro-adhesion, and pro-oxidant signaling pathways.

These data suggested that CORM-401 could be an interesting candidate for pre-clinical studies. Thus, we used a humanized mouse model of SCD challenged with acute hyper-hemolysis to evaluate the effects of CORM-401 on lung, liver, and kidney, which are commonly affected during DHTR.[6,34,35]

CORM-401 reduces lung damage and pulmonary inflammatory vasculopathy induced by acute hemolysis in humanized SCD mice

To evaluate CORM-401's potential therapeutic role in hyper-hemolysis induced inflammatory vasculopathy in SCD, we orally administered 30 mg/kg CORM-401 three times a week to humanized SCD (SS) mice for 21 days (dose based on previous studies).[28,36] CORM-401 did not significantly affect Hb or reticulocyte counts in both healthy AA and SS mice (Supplementary Figure 10SA). As expected, COHb levels notably increased on days 7 and 14 after CORM-401 treatment in both mouse strains (Supplementary Figure 10SB). Noteworthy, we did not find changes in liver hemopexin expression in SCD mice treated with either vehicle or CORM-401 (Supplementary Figure 10SC), suggesting no major effect of CORM-401 on chronic hemolysis. Indeed, there were no changes in expression of VCAM-1, a marker of inflammatory vasculopathy, in target organs for SCD such as lung, liver, or kidney of CORM-401-treated SCD mice when compared to vehicle-treated animals (Supplementary Figure 10SD). We also found lower ICAM-1 expression in lung and kidney from CORM-401-treated SCD than in vehicle-treated SCD mice

(Supplementary Figure 10SD). Conversely, no effect on liver ICAM-1 expression was detected in CORM-401-treated SCD mice *vs* vehicle-treated animals (Supplementary Figure 10SD).

To simulate our *in vitro* experiments, at day 21 after administration with CORM-401 or vehicle mice were exposed to hemolysate to mimic acute hyper-hemolysis as in DHTR. No major changes in Hb or in the amount of Annexin-V+ red cells were observed after hemolysate infusion in either vehicle or CORM-401 treated mice (Supplementary Figure 11SA). However, we found lower total leukocyte count in CORM-401-treated than in vehicle-treated SS mice exposed to hemolysate (Supplementary Figure 11SB). Noteworthy, CORM-401-treated SS mice exposed to hemolysate exhibited a significant increase in plasma hemopexin and a reduction in liver hemopexin expression when compared to vehicle-treated SS mice (Supplementary Figure 12SA, B).

It has been reported that lungs are involved early during acute hyper-hemolysis such as in severe DHTR.[37] In SS mice treated with CORM-401 and exposed to hemolysate, lung inflammatory cell infiltrates, thrombi formation and iron accumulation in lung macrophages were significantly decreased (Table 1, Figure 5A, Supplementary Figure 13SA). This was associated with a decrease in the active form of Nrf2, an acute phase redox-sensitive transcription factor (Figure 5B) and down-regulation of Nrf2-dependent HO-1 and thromboxane synthase (TBXS) expression, which are linked to endothelial cell activation (Figure 5C). Furthermore, CORM-401 reduced the active form of NF-kB and decreased protein expression of VCAM-1, ICAM-1, and ET-1, in SS mice compared to vehicle-treated animals (Supplementary Figure 13SB, C).

In healthy AA mice treated with CORM-401, VCAM-1 expression was significantly lower compared to vehicle-treated animals. However, no change was observed in ICAM-1 and ET-1 expression in AA mice treated with vehicle or CORM-401 and exposed to hemolysate (Supplementary Figure 13SC).

Overall, these results provide *in vivo* evidence that CORM-401 protects SS mice against lung injury induced by acute hemolysis.

CORM-401 attenuates liver injury and the kidney inflammatory response in SS mice exposed to hemolysate

In CORM-401-treated SS mice exposed to hemolysate liver histopathology showed reduced inflammation, thrombi, and iron accumulation in Kupffer cells (Figure 6A, Supplementary Figure 14SA). Notably, hepatic CO content significantly increased in both AA and SS mice treated with CORM-401 compared to vehicle-treated animals (Figure 6B), indicating the effective delivery of CO by CORM-401 to organs *in vivo* (Figure 6B). Similarly, to the lung, CORM-401-treated SS mice exposed to hemolysate displayed significant reduction in the active forms of hepatic Nrf2 and NF-kB compared to vehicle-treated animals (Figure 6C). Hemolysate did not change liver VCAM-1 and ICAM-1 expression compared to mice on steady state (Supplementary Figure 14SB).

This might be related to the chosen experimental timeframe when compared to previous reports, exploring liver expression of pro-adhesion molecule after heme/hemin infusion (1 hour in Belcher et al.[13] and 6 hours in Vinchi et al.[38]). At this stage we cannot exclude a possible modulation of VCAM-1 or ICAM-1 liver expression in either shorter or longer time interval after the infusion of hemolysate.

In healthy AA mice, Nrf2 was activated by hemolysate and prevented by CORM-401, with no change in NF-kB activity or VCAM-1 expression (Figure 6C, Supplementary Figure 14SB). Increased liver ICAM-1 expression induced by hemolysate in AA mice was not impacted by CORM-401 (Supplementary Figure 14SB). In SS mice treated with CORM-401, HO-1, glutathione peroxidase-1 (Gpx1), and TBXS were down-regulated (Figure 6D). In healthy AA mice treated with CORM-401, HO-1 and of Gpx1 were significantly lower than in vehicle-treated animals. No change was observed in TBXS expression in AA mice treated with vehicle or CORM-401 and exposed to hemolysate (Figure 6D). Collectively, these data indicate that CORM-401 mitigates liver injury and modulates the amplified inflammatory and oxidative stress response induced by acute hemolysis.

Since the kidney is another major organ susceptible to damage during acute hyper-hemolysis in SCD patients,[39] we evaluated whether CORM-401 protected against hemolysate-induced kidney injury. Kidney sections from SS mice exposed to hemolysate and CORM-401 showed a marked reduction in glomerular inflammatory cell infiltration, with a slight reduction in tubular iron accumulation compared to vehicle-treated animals (Supplementary Figure 15SA, B). This led to a smaller hemolysate-induced increase in BUN plasma values compared to vehicle-treated SS animals (Supplementary Figure 15SC) with no major change in creatinine in both mouse strains (Supplementary Figure 15SC). Furthermore, Nrf2 activation was reduced in the kidneys of both mouse strains, as indicated by decreased phosphorylated protein levels in western blotting (Figure 7A). The expression of HO-1 and NAD(P)H dehydrogenase (quinone)-1 (Nqo1), an anti-oxidant system activated in response to acute kidney injury[40], was decreased in both mouse strains exposed to hemolysate and treated with CORM-401 compared to vehicle-treated animals

(Figure 7B). Noteworthy, VCAM-1 and ICAM-1 were up-regulated in the kidney of SS mice exposed to hemolysate (Supplementary Figure 16S) and CORM-401 prevented the increased expression of ICAM-1 but not of VCAM-1. A similar pattern was observed for VCAM-1 and ICAM-1 in AA animals exposed to hemolysate with or without CORM-401 (Supplementary Figure 16S).

CORM-401 protects against hemolysate-induced inflammatory vasculopathy in SS mice

Finally, we evaluated the impact of CORM-401 on the progression of SCD-mediated inflammatory vasculopathy induced by hemolysate. CORM-401 reduced P-Selectin and VCAM-1 protein expression in aortas isolated from SS mice exposed to hemolysate (Figure 7C), indicating that the CO-releasing agent might prevent severe vascular dysfunction and organ damage observed in DHTR onset in SCD subjects.

DISCUSSION

Acute hyper-hemolysis such as that observed during DHTR, represents life threatening complications in patients with SCD.[3,4,6,41] Here, we established a fluidic model of HUVECs exposed to hemolysate, closely mimicking the early phase of acute hyper-hemolysis when RBC components, such as Hb, RBC membrane-derived particles and microparticles,[42] are released in plasma. Indeed, our model explores hyper-hemolysis in the early stage before heme release due to Hb oxidation, which occurs later in the clinical progression of acute hyper-hemolysis and can be mimicked by hemin.[24,32,43] We show that hemolysate affects vascular endothelial cells leading to inflammatory vasculopathy,[44] stimulating NF-kB pathways and acute phase pro-inflammatory and pro-adhesion responses as observed in acute sickle cell related clinical manifestations.[45–47] This effect was associated with HUVECs damage, leading to their detachment after PS exposure and favoring platelet aggregation in a GPIIbIIIa-dependent manner. Since the slides were only coated with fibronectin, PS could come from the internal side of the membrane of detached HUVECs or from the RBC membranes in hemolysate. In our model, aggregation and activation of platelets might be enhanced by plasma Hb and ADP released from RBCs and damaged endothelial cells.[48] These preliminary findings support a link between hemolysis and hemostasis in SCD in the early phase of acute hyper-hemolysis.

Different formulations of CO have been tested in mouse models of SCD with promising results. Intermittent inhalation of CO gas (three times/week) over 8-10 weeks reduced leukocytosis in peripheral blood and increased anti-inflammatory pathways in liver tissue of SCD mice.[32] In addition, modified human hemoglobin saturated with CO (MP4CO) inhibited microvascular stasis in sickle mice exposed to hypoxia/reoxygenation.[43] More recently, oral administration of a CO-saturated liquid

(HBI-002) improved Hb and Hct levels, RBC counts and vaso-occlusion in SCD mice.[22] Interestingly, COHbS cannot polymerize,[49] thus decreasing the intracellular concentration of HbS available for polymer synthesis. It is important to note that in these and other studies, mice were exposed to hemin infusion, mimicking the advanced phase of acute hemolysis.[24,32,43] Here the CO-releasing molecule CORM-401 was tested in our *in vitro* cell-based model of early phase of acute hyper-hemolysis. First, we found that the detrimental effect of hemolysate on HUVECs is prevented by CORM-401. The molecule significantly reduced inflammation, VCAM-1, ICAM-1 expression, and the acute response signaling, including the NF-kB pathways. Our data corroborate previous findings showing that other CO carriers, CORM-A1 and CORM-3, markedly reduced the adhesion of sickle RBCs to human and bovine aortic endothelial cells cultured in static conditions *in vitro*.[50] Importantly, humanized SCD (SS) mice exposed to acute hemolysate and treated with CORM-401 confirmed the therapeutic effects of CO on organ damage, particularly the lung, kidney, and liver, which are mainly affected during DHTR. The protective effect of CORM-401 might accelerate resolution of inflammation, preventing NF-kB and Nrf2 activation and modulating pro-inflammatory, pro-adhesive (VCAM-1, ICAM-1, ET-1, TBX) and antioxidants (HO-1, Gpx1) systems. Noteworthy, in SCD mice, the beneficial action of CORM-401 appears to be most effective during acute hyper-hemolysis. However, the reduction of ICAM-1 expression in lung and kidney from steady state SCD mice treated with CORM-401 suggests a possible local modulation of vascular tone by CO. This is supported by previous observation that ICAM-1 gene but not of VCAM-1 gene contains a shear-stress responsive element in the promoter region.[51,52] Indeed, Walpola et al. have reported that low shear stress down-regulates ICAM-1 expression but up-regulates VCAM-1, while the expression of both molecules is

increased in presence of an intense shear stress.[51] Collectively, our data agree with a report by Chiang N et al. showing that inhaled CO empowers endogenous pro-resolvin mechanisms in an inflammatory model of murine peritonitis.[53] Indeed, in CORM-401 treated SCD mice exposed to hemolysate, reduction of Nrf2 activation and downregulation of HO-1 expression resemble more the effect of exogenous *Rev-D17* infusion in the same SCD mouse strain[54] compared to other strategies to increase CO bioavailability in SCD mice.[22,32,43] This might be related to different delivery modes of CO by the various molecules and/or the times chosen for analysis (e.g. 1-29 hours after the stress *vs* 5 hours after hemolysate infusion in our study).[32,43] In addition, the pivotal action of CORM-401 is its ability to deliver CO not only to blood, as indicated by increased COHb levels, but also to the liver (and most likely in other organs as well) in association with a protective effect against hemolysate-induced hepatic damage. Indeed, we have data showing that liver, kidney, cecum and colon accumulate considerable amounts of CO following CORM-401 treatment in mice (Motterlini, Foresti et al., submitted article). These results indicate that CO efficiently delivered by orally administered CORM-401 is protecting remote organs from oxidative stress, inflammation and damage inflicted by heme/hemolysate. Therefore, key markers of these parameters such as Nrf2 and HO-1 were attenuated by CORM-401 treatment in SCD mice.

Based on its high capacity to release CO and exert anti-inflammatory activities in a model of endotoxin challenge,[36] as well as its efficacy after prolonged treatment in mice subjected to high fat diet, CORM-401 might be a concrete alternative mode of CO delivery in SCD.[28] The efficacy of this “solid form of CO” is complemented by practical advantages including an easy synthesis process, solubility in aqueous solutions and great stability once stored at -20°C. Controlled CO delivery can be

safe provided that COHb levels do not exceed 12%.[25] Our previous and present reports clearly shows that at 30 mg/kg CORM-401, COHb levels in mice are far below this threshold (around 5% in normal mice and less than 8% in SCD mice).[28] The toxic effects of CO related to an impairment of oxygen delivery to tissues by Hb are known to occur at much higher levels of COHb.[55]

In conclusion, our study highlights two important outcomes: 1) the development and characterization of a new *in vitro* cell-based model to mimic the early phase of acute hyper-hemolysis encountered in DHTR; 2) the protective effects of CORM-401 on endothelial dysfunction and organ damage induced by acute hemolysis in both *in vitro* and *in vivo* models of SCD. Our data support the emerging evidence that CO delivery might be therapeutically effective to counteract the early phase of acute intravascular hemolysis occurring in DHTR.[4] CORM 401 could be considered in situations where hyper-hemolysis is likely to occur, before it occurs, to limit its catastrophic effects.

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CONFLICT OF INTEREST DISCLOSURES

The authors have declared that no conflict of interest exists.

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Table 1. Effects of CORM01 on lung, liver and kidney pathology of sickle cell (SS) mice exposed to hemolysate.

	SS mice treated with saline buffer	SS mice Exposed to hemolysate and treated with vehicle	SS mice Exposed to hemolysate and treated with CORM-401
Lung	(*n*=6)	(*n*=6)	(*n*=4)
Inflammatory cell infiltrates	7.6±0.4	84.1±2.9*	26.4±3.0*#
Thrombi	0	+ (1/6)	0
Kidney	(*n*=6)	(*n*=6)	(*n*=4)
Inflammatory cell infiltrates	0	6.62±0.51*	1.2±0.014*
Thrombi	0	0	0
Fibrosis	0	0	0
Liver	(*n*=6)	(*n*=6)	(*n*=4)
Inflammatory cell infiltrates	9.2±1.4	96±2.8*	44.1±3.7*#
Thrombi	0	+ (1/6)	0
Score	2-2-2-2-1-1	3-3-2-3-3-3	2-2-3-2

Lungs

Score for inflammatory cell infiltrate. Quantification of inflammatory cell infiltrates was expressed as the mean of cells per field at magnification of X250, as resulting by the analysis of at least 10 different fields on each hematoxylin-eosin-stained whole lung section (see also refs: 28 and 29). Data are expressed as mean ± SEM; * p<0.05 compared to normoxia; #p<0.05 compared to vehicle.

Kidney

Score for inflammatory cell infiltrate. Quantification of inflammatory cell infiltration in renal cortex of kidney was determined in hematoxylin-eosin-stained sections using a 0 to 4 scale based on the percentage of cell infiltrates occupied area in each filed. 0 (no sign of infiltration); 1 (1-10% of the area with cell infiltration); 2 (11-25%); 3 (26-50%); 4 (50%). The mean of 15 randomly selected field were analyzed at magnification X400 (see also refs: 22, 46). Data are expressed as mean ± SEM; * p<0.05 compared to normoxia; #p<0.05 compared to vehicle.

Liver score

0: no hepatocellular damage; **1**: *mild* injury characterized by cytoplasmic vacuolization and focal nuclear pyknosis; **2**: *moderate* injury with dilated sinusoids, cytosolic vacuolization, and blurring of intercellular borders; **3**: *moderate to severe* injury with con coagulative necrosis, abundant sinusoidal dilatation, red blood extravasation into hepatic chords, hypereosinophilia and migration of neutrophils; **4**: *severe necrosis* with loss of hepatic architecture, disintegration of hepatic chords, haemorrhage and neutrophils infiltration.

Table Statistical analysis: Non-parametric Pairwise Wilcoxon Rank Sum Tests) was used. Each group was balanced in sex (50% males-50%female). We repeated the comparisons for males and females separately with equivalent results. Regarding the gender, our data did not show evidence of differences between males and females.

FIGURES LEGENDS

Figure 1. Impact of hemolysate on HUVECs in a fluidic model mimicking intracellular hemolysis

(A) Three-step fluidic model reproducing intracellular hemolysis. HUVECs cultured in a flow system (step 1, flow culture of HUVECs) were exposed to either AA hemolysate, TNFα or serum for 4 hours (step 2, HUVEC preconditioning). Step 3, functional assays were performed by infusing heparinized WB from either AA donors or SS patient on hemolysate-preconditioned HUVECs from step 2 for 10 min. The infusion flow rate in the fluidic model was adjusted to reach a shear stress 1 dyne.cm−2.

(B) Hemolysate induced activation and damage of HUVECs after preconditioning:

HUVECs cultured in flow system were exposed to either AA hemolysate, TNFα or serum for 4 hours then stained for activation and apoptosis markers by PECAM-1 Alexa Fluor 647 (red), ICAM-1 PE (orange), VCAM-1 Alexa Fluor 488 (green), Hoescht 34442 (blue) and Annexin V Alexa Fluor 594 (purple) staining. Images representative of 5 different experiments. MFI of PECAM-1, ICAM-1, VCAM-1 and Annexin V markers were quantified by ImageJ. Data represent mean ± SEM (n=5), Kruskal-Wallis test with p-value < 0.05 (*) and <0.001(***).

(C) Hemolysate up-regulates inflammation and acute response genes on HUVECs. HUVECs cultured in a flow system were exposed to either serum or hemolysate for 4 hours. Cells were then recuperated to analyze gene expression profiles by mRNA sequencing as described in supplementary data. Example of genes with global fold change > 1.5 and FDR p-value < 0.05 induced by hemolysate vs serum from 7 AA donors.

Figure 2. Hemolysate induced HUVECs dysfunction

HUVECs cultured in a flow system were exposed to either AA hemolysate, TNFα or serum for 4 hours. Functional assays were performed by infusing heparinized WB on hemolysate-preconditioned HUVECs for 10 min. The infusion flow rate in the fluidic model was adjusted to reach a shear stress 1 dyne.cm−2.

(A) RBC adhesion after infusion of heparinized WB from AA donors on either AA hemolysate, TNFα or serum-preconditioned HUVECs for 10 min at shear stress 1 dyne.cm−2. Data represent mean ± SEM (n=8), Kruskal-Wallis test with p-value < 0.05 (*) and < 0.001 (***).

(B-C) Platelet aggregation and activation at endothelial injury sites after infusion of heparinized WB from AA donors on either AA hemolysate, TNFα or serum-preconditioned HUVECs for 10 min at shear stress 1 dyne.cm−2. (**C**) Fixed cells were stained with PECAM-1 FITC (green), CD41a PE (orange) and CD62P (mouse-anti human primary Ab and Alexa Fluor 647 goat-anti mouse secondary Ab, red), Data represent mean ± SEM (n=5), Kruskal-Wallis test with p-value < 0.05 (*) and < 0.001 (***).

(D) Inhibitory effect of integrilin on platelets aggregation. HUVECs cultured in a flow system were exposed to AA hemolysate for 4 hours. AA heparinized WB was pretreated or not with intergrilin, a GPIIbIIIa antagonist at 10µg/mL for 30 min before infusion on hemolysate-preconditioned HUVECs for 10 min. The infusion flow rate in the fluidic model was adjusted to reach a shear stress 1 dyne.cm−2. Following the washing step, fixed cells were stained with PECAM-1 FITC (Green) and CD41a PE (orange). Images representative of 5 different experiments, data represent mean ± SEM (n=5) (**) paired t test with p-value < 0.01.

Figure 3. Hemolysate-induced pro-inflammatory and activation of HUVECs is prevented by CORM-401

HUVECs cultured in a flow system were pre-treated or not with either CORM-401 or iCORM at a different concentration for 1h before being exposed to either hemolysate alone or hemolysate plus either CORM-401 or iCORM (recapitulated in following table). Non-pretreated HUVECs exposed to hemolysate alone served as control group. The infusion flow rate was adjusted to reach a shear stress 1 dyne.cm−2.

Group (n=5 for each group)	Pre-treatment during 1H	Exposition during 4H
Non-treated hemolysate (red graph)	-	Hemolysate
Hemolysate+iCORM 100 µM (green)	iCORM 100 µM	Hemolysate +iCORM 100 µM
Hemolysate+CORM-401 50µM (purple)	CORM-401 50 µM	Hemolysate+CORM-401 50 µM
Hemolysate+CORM-401 100 µM (blue)	CORM-401 100 µM	Hemolysate+CORM-401 100 µM

(A) Immunofluorescence staining of membrane markers on HUVECs: PECAM-1 Alexa Fluor 647 (red), ICAM-1 PE (orange), VCAM-1 Alexa Fluor 488 (green), Annexin V Alexa Fluor 594 (purple), Phalloidin Alexa Fluor 488 (green), and Hoescht 33442 (blue). Images representative of 5 different experiments. ICAM-1 and VCAM-1 MFI was quantified by ImageJ. Data represent mean ± SEM (n=5), Kruskal-Wallis test with p-value < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****).

(B) CORM-401 prevented RBC and platelet recruitment on HUVECs preconditioned with hemolysate. Heparinized WB from AA donors was perfused on preconditioned HUVECs for 10 min at shear stress 1 dyne.cm−1. Fixed cells were stained with PECAM-1 FITC (Green) and CD41a PE (orange). Images representative of 5 different experiments. Data represent mean ± SEM (n=5), Kruskal-Wallis test with p-value < 0.05 (*), < 0.01 (**).

Figure 4. CORM-401 prevented hemolysate-induced up-regulation of pro-inflammatory pathways in endothelial cells.

HUVECs cultured in a flow system were pre-treated or not with 100 µM CORM-401 for 1h before being exposed to either serum only, hemolysate alone or hemolysate plus 100 µM CORM-401(recapitulated in following table). The infusion flow rate was adjusted to reach a shear stress 1 dyne.cm−2. Cells in indicated conditions were harvested and gene expression profiles were analyzed by mRNA sequencing, as described in Supplementary data.

Group	Pre-treatment during 1H	Exposition during 4H
Serum only (n=7)	-	Serum
Hemolysate (n=9)	-	Hemolysate
Hemolysate+CORM-401 (n=6)	CORM-401 100 µM	Hemolysate+CORM-401 100 µM

(A) Heatmaps of normalized read counts of differentially expressed genes in endothelial cells exposed to hemolysate alone compared to hemolysate plus CORM-401 treatment (only those having fold change > 1.5 or < -1.5, and FDR p-value < 0.05 are represented).

(B) Main canonical pathways (z-score) in hemolysate- vs. serum-treated HUVECs (red dots) and in cells exposed to hemolysate plus CORM-401 treatment vs. hemolysate alone (green dots). Large red dots: z-score>2, large green dots: z-score <-2: considered significant to predict up-regulation or down-regulation pathway respectively.

Figure 5. CORM-401 prevents hemolysate-induced lung damage and counteracts pulmonary inflammatory vasculopathy in humanized SCD (SS) mice.

(A) Representative micro picture of hematoxylin and eosin-stained (H&E) sections and Perls'-stained sections of lung at 200x magnification from SCD (SS) mice exposed to sterile buffer (SB) or hemolysate (respectively AA hemolysate in AA

recipient and SS hemolysate in SS recipient) and treated with either vehicle or CORM-401 (scale bar: 50 mm) (see also Table 1).

(B) Western blot analysis with specific antibodies against phosphorylated (p)Nrf2 and Nrf2 in lung from AA and SS mice infused with sterile buffer or hemolysate and treated with either vehicle or CORM-401. GAPDH is used as protein loading control. One representative gel from 5 with similar results is shown. Densitometric analysis of immunoblots (DU: Densitometric Unit) is shown on the right. Data represent mean ± SEM (n=5); ^ $p<0.05$ compared to vehicle-treated animals; * $p<0.05$ compared to AA sterile buffer (SB)-treated animals; $p<0.05$ compared to SS sterile buffer (SB)-treated animals by one-way ANOVA.

(C) Western blot analysis with specific antibodies against heme oxygenase 1 (HO-1) and thromboxane synthase (TBXS) in lung from AA and SS mice infused with sterile buffer or hemolysate and treated with either vehicle or CORM-401. GAPDH is used as protein loading control. One representative gel from 5 with similar results is shown. Densitometric analysis of immunoblots (DU: Densitometric Unit) is shown in the lower panel. Data represent mean ± SEM (n=5); ^ $p<0.05$ compared to vehicle-treated animals; * $p<0.05$ compared to AA mice treated with sterile buffer (SB)-; # $p<0.05$ compared to SS mice treated with sterile buffer (SB) by one-way ANOVA.

Figure 6. CORM-401 attenuates liver injury in SCD (SS) mice exposed to hemolysate.

(A) Representative micro picture of Hematoxylin and Eosin-stained (H&E) sections and Perls'-stained sections of liver at 200x magnification from SCD (SS) mice exposed to sterile buffer (SB) or hemolysate and treated with either vehicle or CORM-401 (scale bar: 50 mm) (see also Table 1).

(B) Quantification of CO content in the liver of AA and SS mice treated with either vehicle or CORM-401 (30 mg/kg) by oral gavage. Accumulation of CO in hepatic tissue was measured spectrophotometrically using the hemoCD1 assay. Data are presented as mean ± SEM; *p<0.02 compared to vehicle treated animals.

(C) Western blot analysis with specific antibodies against phosphorylated (p)-Nrf2, Nrf2, p-NF-kB and NF-kB, in liver from AA and SS mice infused with sterile buffer or hemolysate and treated with either vehicle or CORM-401. GAPDH is used as protein loading control. One representative gel from 5 with similar results is shown. Densitometric analysis of the immunoblots (DU: Densitometric Unit) is shown in the bottom panel. Data are presented as mean ± SEM (n=5); ^ p<0.05 compared to vehicle-treated animals; * p<0.05 compared to AA animals; # p<0.05 compared to SS sterile buffer (SB)-treated animals by one-way ANOVA analysis.

(D) Western blot analysis with specific antibodies against heme oxygenase 1 (HO-1), glutathione peroxidase-1 (Gpx1) and thromboxane synthase (TBXS) in liver from AA and SS mice infused with sterile buffer or hemolysate and treated with either vehicle or CORM-401. GAPDH is used as protein loading control. One representative gel from 5 with similar results is shown. Densitometric analysis of the immunoblots (DU: Densitometric Unit) is shown in the bottom panel. Data are presented as mean ± SEM (n=5); ^ p<0.05 compared to vehicle-treated animals; * p<0.05 compared to AA

animals; # $p<0.05$ compared to SS sterile buffer (SB)-treated animals by one-way ANOVA analysis.

Figure 7. CORM-401 modulates the hemolysate-induced activation of pro-oxidation and pro-inflammatory pathways in kidney SS mice.

Western blot analysis with specific antibodies against phosphorylated (p)-Nrf2 and Nrf2 in kidney from AA and SS mice in steady state and exposed to hemolysate, treated with either vehicle or CORM-401. GAPDH is used as protein loading control. 75 µgr/µl of protein loaded on an 8% T, 2.5%C polyacrylamide gel. One representative gel from 5 with similar results is shown. Densitometric analysis of immunoblots (DU: Densitometric Unit) is shown on the right. Data represent mean ± SEM (n=5); ^ $p<0.05$ compared to vehicle-treated animals; * $p<0.05$ compared to AA animals; # $p<0.05$ compared to sterile buffer (SB)-treated animals by one-way ANOVA analysis. (B) Western blot analysis with specific antibodies against heme oxygenase-1(HO-1) and NAD(P)H dehydrogenase (quinone)-1 (Nqo1) in kidney from AA and SS mice in steady state and exposed to hemolysate, treated with either vehicle or CORM-401. GAPDH is used as protein loading control. 75 µgr/µl of protein loaded on an 11% T, 2.5%C polyacrylamide gel. One representative gel from 5 with similar results is shown. Densitometric analysis of immunoblots (DU: Densitometric Unit) is shown on the right. Data represent mean ± SEM (n=5); ^ $p<0.05$ compared to vehicle-treated animals; * $p<0.05$ compared to AA animals; # $p<0.05$ compared to sterile buffer (SB)-treated animals by one-way ANOVA analysis. **(C)** Western blot analysis with specific antibodies against VCAM-1 and ICAM-1, in kidney from AA and SS mice exposed to hemolysate, treated with either vehicle or CORM-401. GAPDH is used as protein loading control. 75 µgr/µl of protein loaded on an 11% T, 2.5%C polyacrylamide gel. One representative gel from 5 with similar results is shown. Densitometric analysis of immunoblots (DU: Densitometric Unit) is shown on the

right. Data represent mean ± SEM (n=5); ^ $p<0.05$ compared to vehicle treated animals by one-way ANOVA analysis.

Efficacy of CORM-401, an Oral Carbon Monoxide-Releasing Molecule, Against Acute Hyper-Hemolysis in a Humanized Mouse Model of Sickle Cell Disease

Context of Research

• Acute hyper-hemolysis is a severe life-threatening complication in patients with sickle cell disease (SCD)

• Its pathogenesis is poorly understood and limited therapeutic options are available

• Carbon monoxide (CO), a byproduct of heme metabolism, might have beneficial therapeutic effects

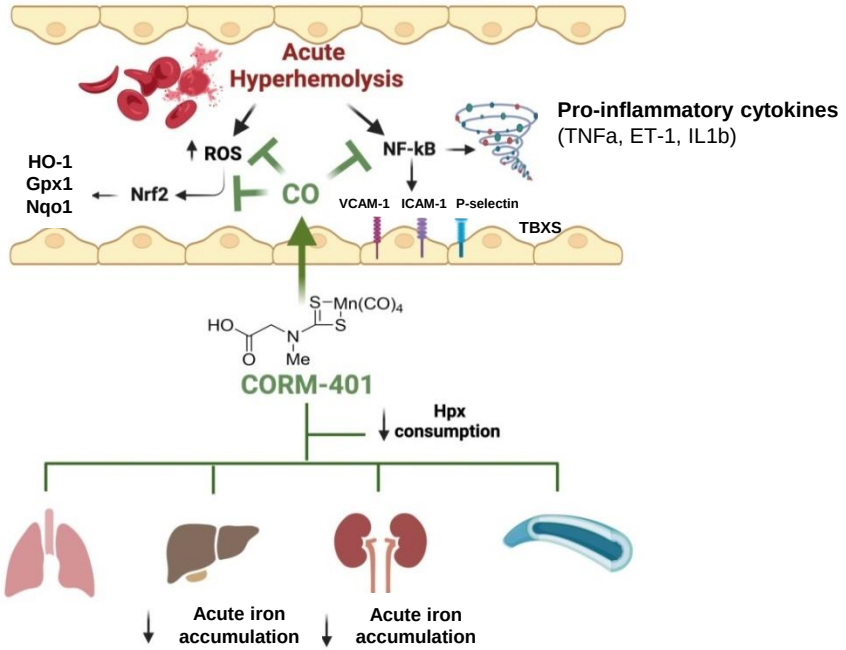
Methods

• CORM-401 is an orally active carbon monoxide-releasing molecule

• We studied the effects of CORM-401 during acute hyper-hemolysis *in vitro* and in a humanized mouse model of SCD

Main Findings

• RBC membrane-derived particles cause endothelial dysfunction



• CORM-401 blocks endothelial damage

Conclusions: 1) RBC membrane-derived particles cause endothelial dysfunction and organ damage during acute hyper-hemolysis in SCD mice. 2) CORM-401 protects against organ damage in these animals.

*Nguyen et al. DOI: 10.xxxx/**blood**.2024xxxxxx*

Blood Visual Abstract

Figure 1

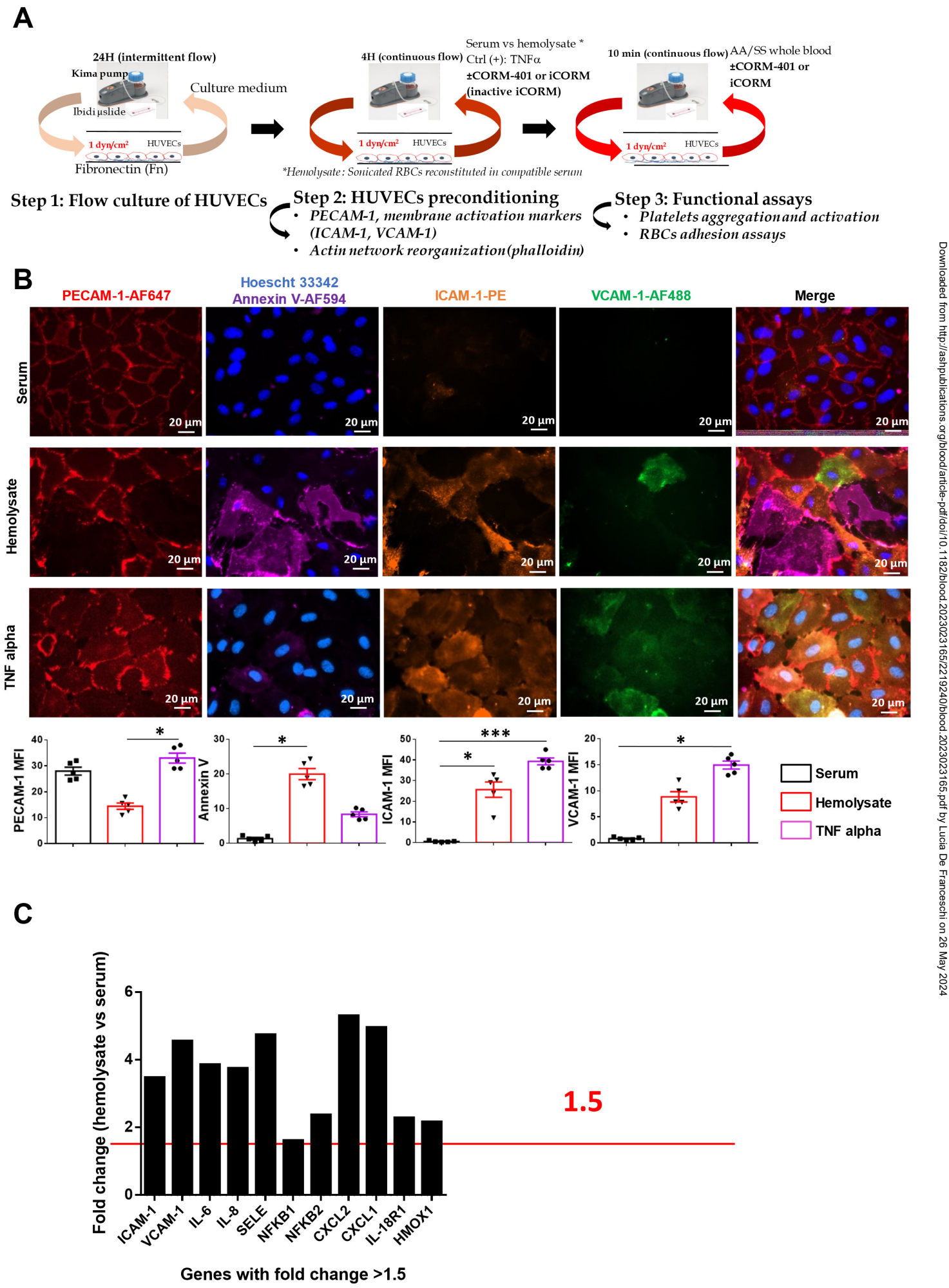
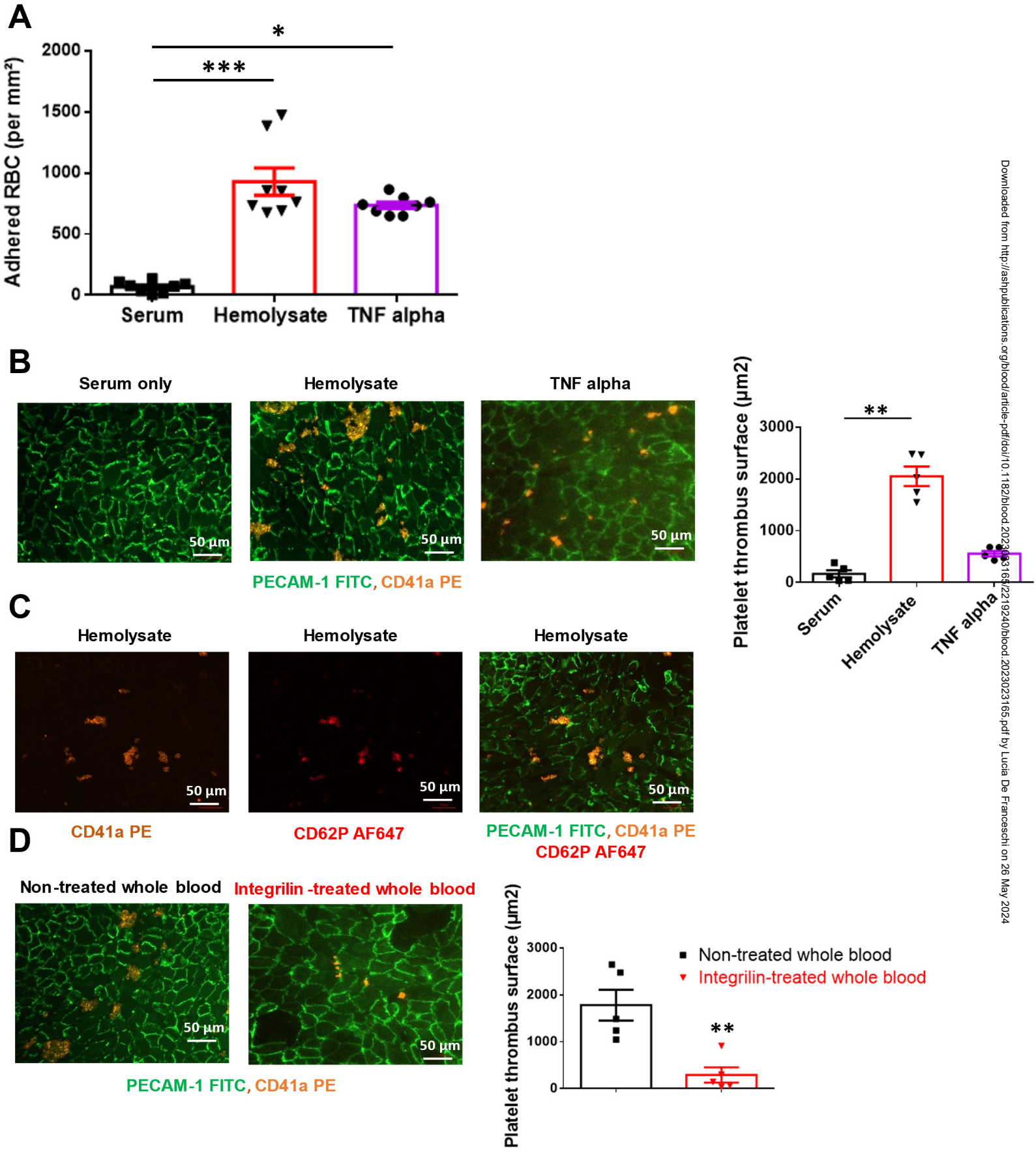


Figure 2



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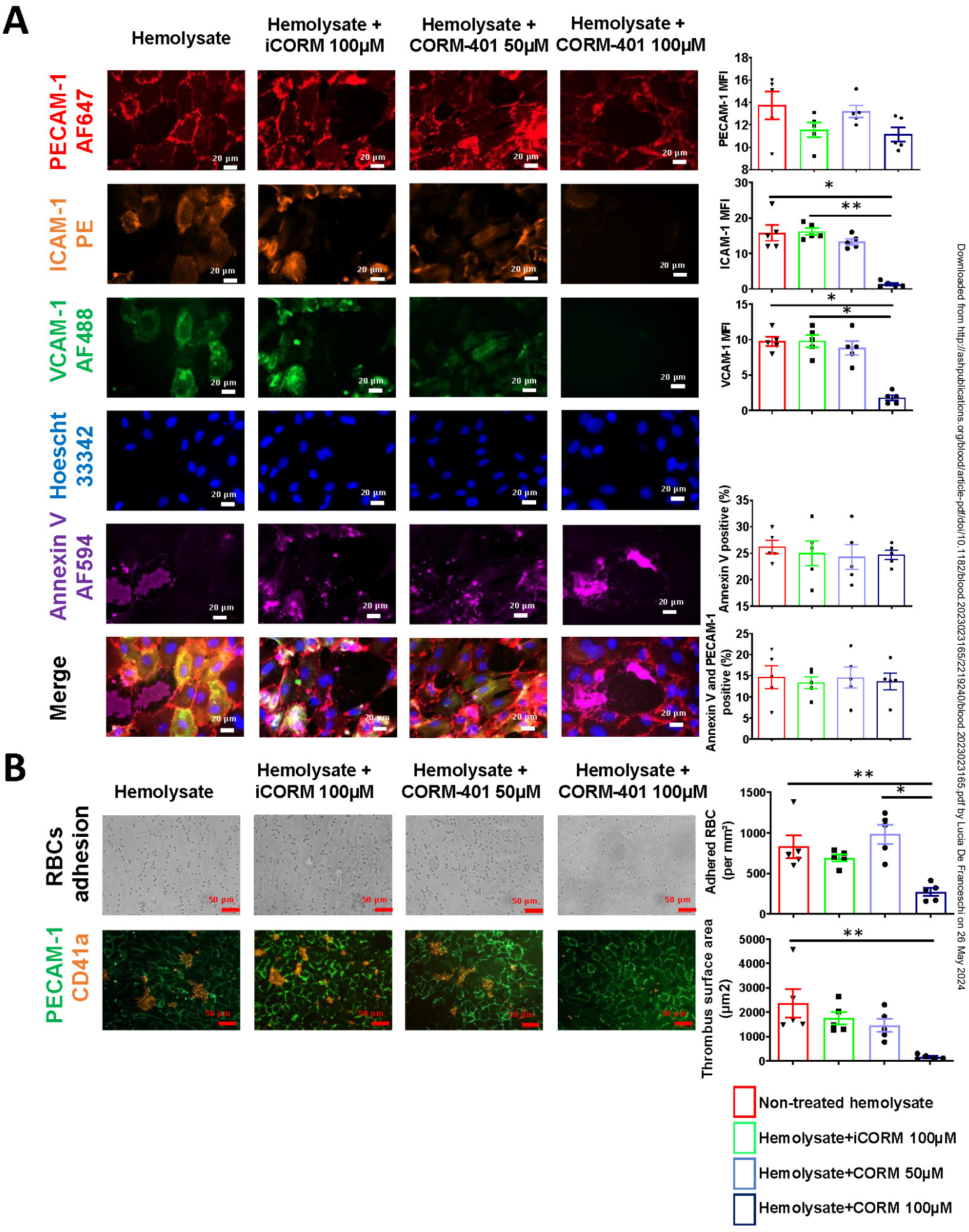

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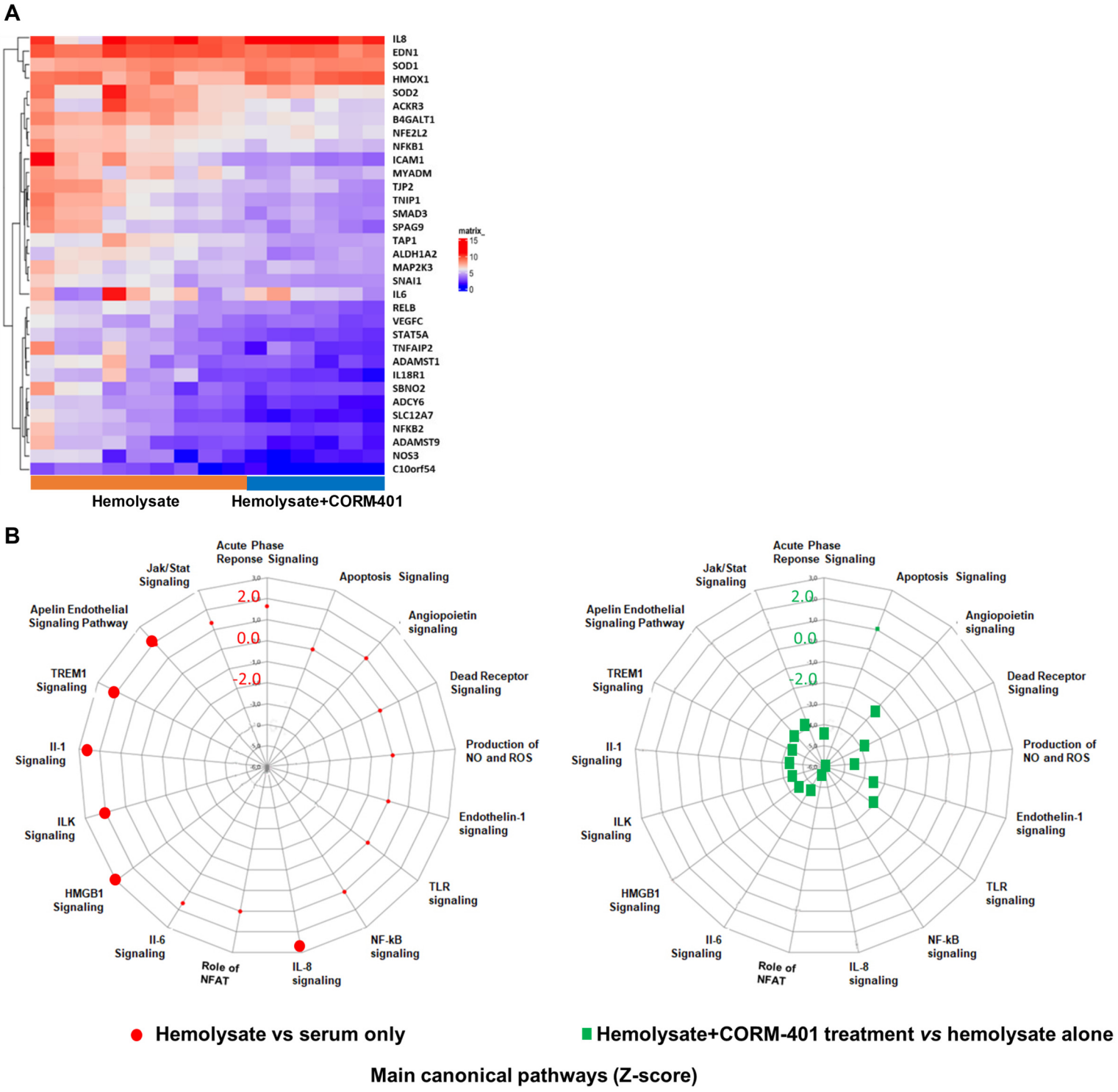
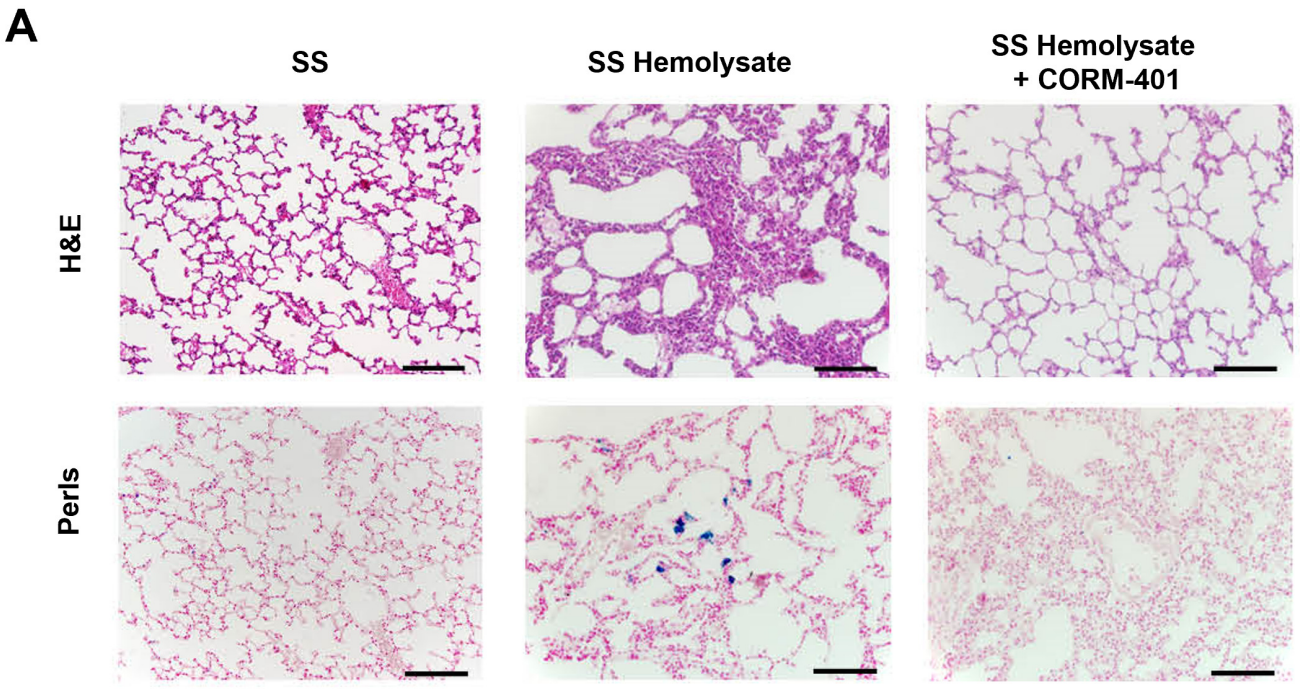
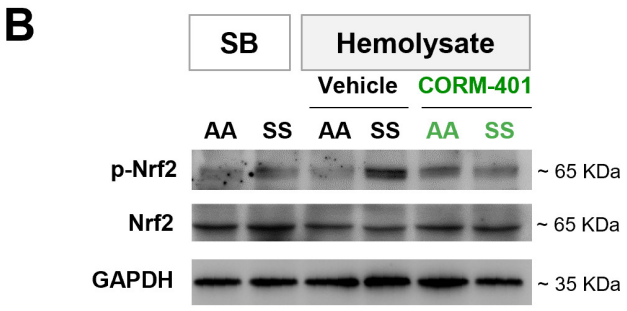
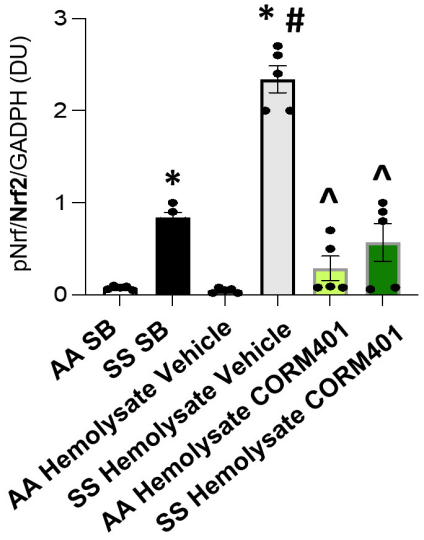
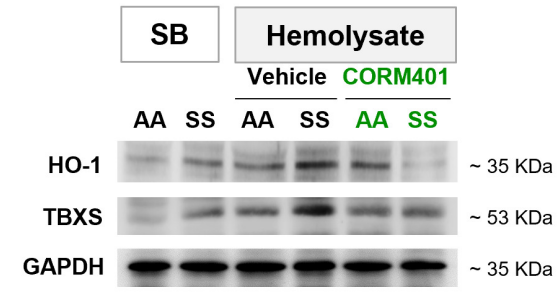


Figure 5









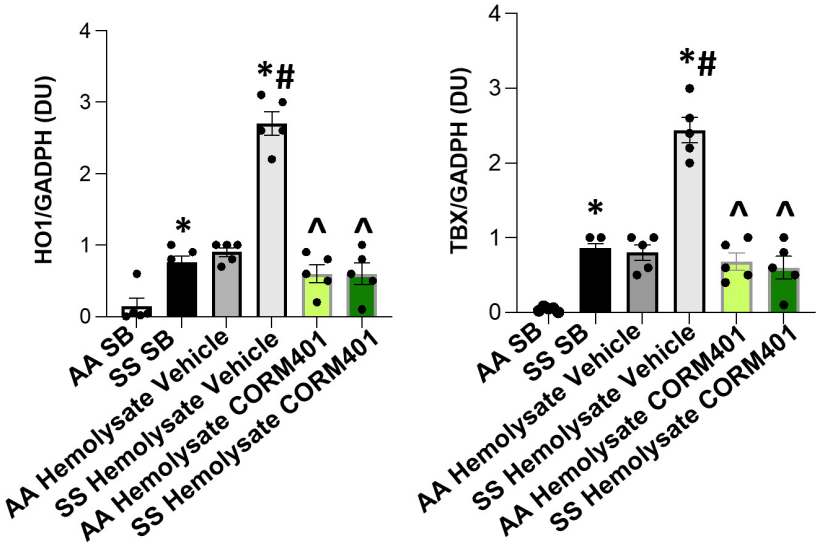
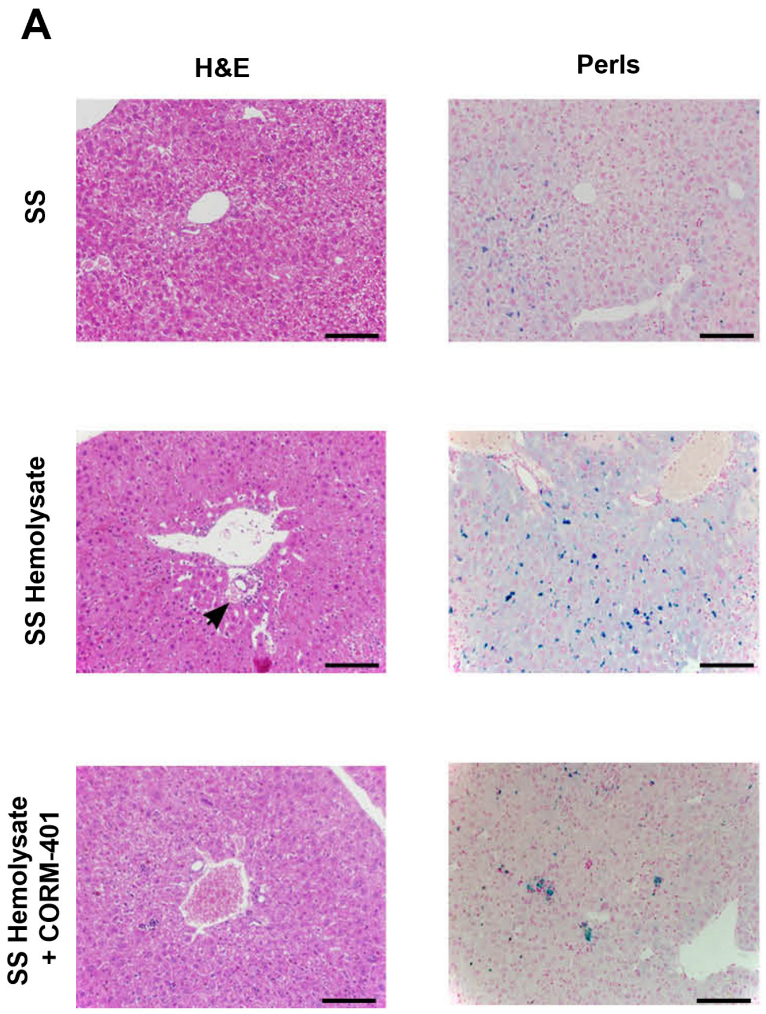
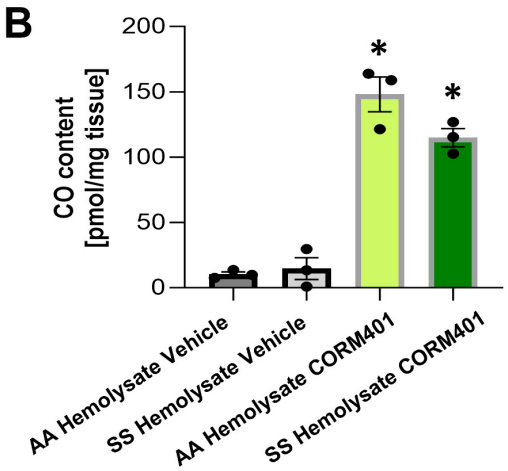
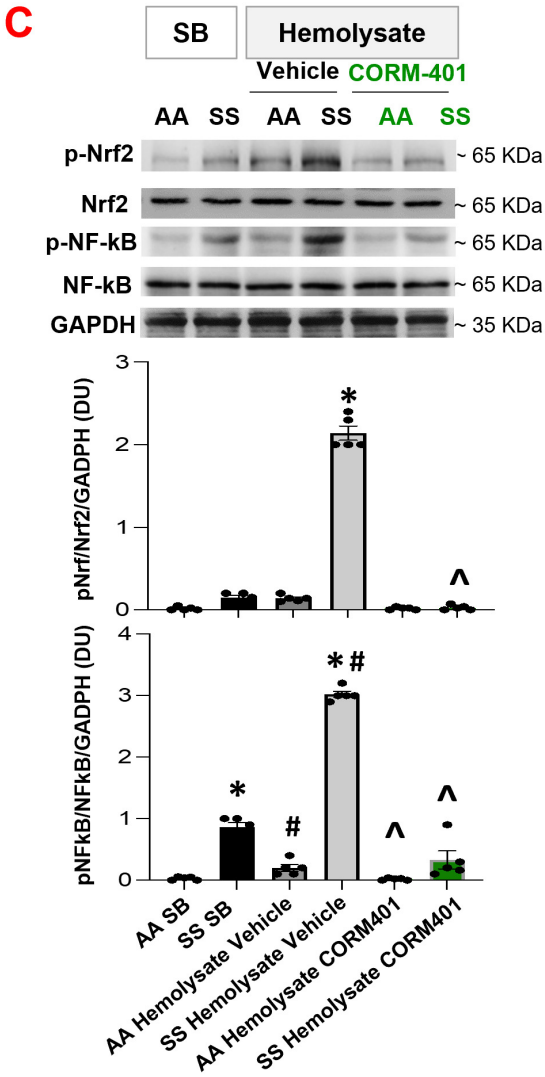
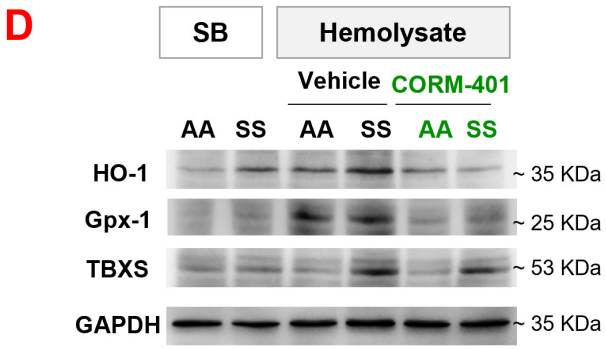


Figure 6









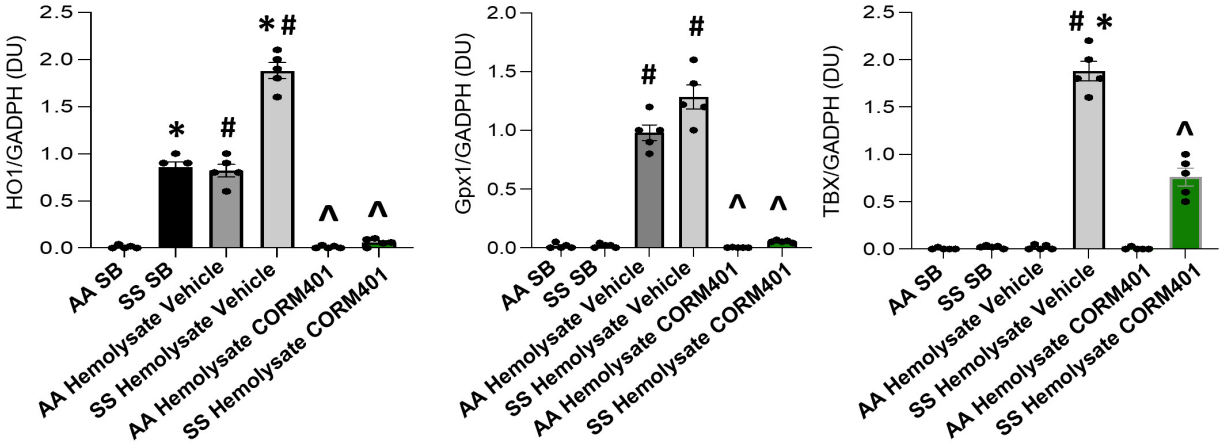


Figure 7