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# Factor XII contributes to thrombotic complications and vaso-occlusion in sickle cell disease

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**Abstract:**
A hypercoagulable state, chronic inflammation, and increased risk of venous thrombosis and stroke are prominent features in patients with sickle cell disease (SCD). Coagulation factor XII (FXII) triggers activation of the contact system that is known to be involved in both thrombosis and inflammation, but not in physiological hemostasis. Therefore, we investigated whether FXII contributes to the prothrombotic and inflammatory complications associated with SCD. We found that when compared to healthy controls, SCD patients exhibit increased circulating biomarkers of FXII activation that are associated with increased activation of the contact pathway. We also found that FXII, but not tissue factor, contributes to enhanced thrombin generation and systemic inflammation observed in sickle cell mice challenged with TNFα. Additionally, FXII inhibition significantly reduced experimental venous thrombosis, congestion, and microvascular stasis in a mouse model of SCD. Moreover, inhibition of FXII attenuated brain damage and reduced neutrophil adhesion to the brain vasculature of sickle cell mice after ischemia/reperfusion induced by transient middle cerebral artery occlusion. Finally, we found higher FXII, uPAR and αMβ2 integrin expression in SCD patient neutrophils compared to healthy controls. Our data indicate that targeting FXII effectively reduces experimental thromboinflammation and vascular complications in a mouse model of SCD, suggesting that FXII inhibition may provide a safe approach for interference with inflammation, thrombotic complications, and vaso-occlusion in SCD patients.

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**KEY POINTS**

- FXII-mediated activation of the contact pathway is increased in mice and patients with SCD at steady state.
- FXII contributes to thrombin generation, inflammation, vascular stasis, venous thrombosis and ischemic brain injury in SCD mice.

**ABSTRACT**

A hypercoagulable state, chronic inflammation, and increased risk of venous thrombosis and stroke are prominent features in patients with sickle cell disease (SCD). Coagulation factor XII (FXII) triggers activation of the contact system that is known to be involved in both thrombosis and inflammation, but not in physiological hemostasis. Therefore, we investigated whether FXII contributes to the prothrombotic and inflammatory complications associated with SCD. We found that when compared to healthy controls, SCD patients exhibit increased circulating biomarkers of FXII activation that are associated with increased activation of the contact pathway. We also found that FXII, but not tissue factor, contributes to enhanced thrombin generation and systemic inflammation observed in sickle cell mice challenged with TNFα. Additionally, FXII inhibition significantly reduced experimental venous thrombosis, congestion, and microvascular stasis in a mouse model of SCD. Moreover, inhibition of FXII attenuated brain damage and reduced neutrophil adhesion to the brain vasculature of sickle cell mice after ischemia/reperfusion induced by transient middle cerebral artery occlusion. Finally, we found higher FXII, uPAR and αMβ2 integrin expression in SCD patient neutrophils compared to healthy controls. Our data indicate that targeting FXII effectively reduces experimental thromboinflammation and vascular complications in a mouse model of SCD, suggesting that

FXII inhibition may provide a safe approach for interference with inflammation, thrombotic complications, and vaso-occlusion in SCD patients.

## INTRODUCTION

Sickle cell disease (SCD), the most common inherited hemoglobinopathy, is caused by a single nucleotide mutation of the β-globin gene that results in the formation of misshapen rigid ('sickle') red blood cells (RBCs) under hypoxic conditions[1-4]. Although hemolytic anemia and vaso-occlusive crisis (VOC) are two primary pathologies caused by sickling of RBCs, SCD is also associated with chronic vascular inflammation and activation of coagulation[1,4]. It is now well-established that SCD patients are at increased risk of venous and arterial thrombosis (including ischemic stroke and silent cerebral infarction) that are associated with increased morbidity and mortality[5-7].

Tissue factor (TF)-initiated activation of coagulation contributes to systemic thrombin generation and inflammation[8,9], cardiovascular dysfunction[10], end-organ damage[11], and vascular stasis[12] in mouse models of SCD. There is growing interest in targeting the FXII(a)-initiated intrinsic pathway to prevent thrombosis, because unlike components of the extrinsic or common coagulation pathways, FXII is not required for hemostasis[13]. FXII is primarily produced and secreted by hepatocytes; however, neutrophils have recently been identified as an additional source of FXII[14,15]. In addition to its well-established role in initiation of the contact system that triggers the intrinsic coagulation and kallikrein-kinin-pathways[16,17], FXII plays an important role in leukocyte-mediated pro-inflammatory responses via interaction with its receptor, uPAR (urokinase plasminogen activator receptor, CD87)[15].

The role of FXII in the pathophysiology of SCD is largely unknown. Older reports demonstrated the reduction of plasma levels of FXII zymogen in SCD patients compared to

healthy controls[18-20], suggesting ongoing activation and consumption *in vivo*. Given the growing evidence that inhibiting zymogen FXII and its activated protease form (FXIIa) spares hemostasis while simultaneously reducing thrombosis and inflammation, we set out to determine if FXII/FXIIa contribute to the heightened thrombotic complications and VOC associated with SCD.

## MATERIALS and METHODS

### Patient sample collection

Outpatients with SCD (N=53) and healthy race-matched controls (N=23) were recruited for studies on *in vivo* contact pathway activation (patient characteristics are shown in Table 1). Inclusion criteria were steady state disease (> 1 month from last pain crisis), no transfusion within previous 3 months, and no current use of oral contraceptives, anticoagulants, or antiplatelet agents. Blood samples were obtained by clean venipuncture using a 21G butterfly needle. Platelet-poor plasma was prepared from blood drawn into 3.2% sodium citrate (ratio of blood to anticoagulant 9:1) and stored at −80°C until analysis. The study was approved by the University of North Carolina's Institutional Review Board for human subjects (UNC IRB# 13-1906), and written informed consent was obtained.

Neutrophils were isolated from whole blood (WB) obtained from healthy individuals and SCD patients in accordance with a University Hospitals Cleveland Medical Center approved protocol. Inclusion criteria were ≥18 years, male or female, not on medications (immunosuppressive or nonsteroidal anti-inflammatory agents), and without a diagnosis of an acute illness in the past 4 weeks. Eligible SCD patients were at steady state disease (> 1 month from the last pain crisis), had not received transfusion within 30 days, and were not on

anticoagulation, oral contraceptives, or antiplatelet therapy. WB was drawn by venipuncture into 3.2% sodium citrate (9:1).

**Mouse model of SCD**

The Townes knock-in model of SCD (hα/hα, hγ/hγ, $h\beta^A/h\beta^S$) was used for these studies[21,22]. Mice were bred by inter-crossing HbAS parents to generate mice that exclusively express human globins α, γ, and either $\beta^S$ (HbSS, sickle) or $\beta^A$ (HbAA, wild type). Studies used 4 – 5-month-old mice, with males and females in equal proportions. Mice were phenotyped by hemoglobin electrophoresis (Helena Laboratories, Texas). All studies were in accordance with the Animal Care and Use Committees of University of North Carolina, Louisiana State University, and Brunel University London and complied with Animal Research: Reporting In Vivo Experiments guidelines.

**Experimental procedures**

The dorsal skinfold chamber (DSFC) model of vascular stasis, femoral vein electrolytic injury venous thrombosis model, transient middle cerebral artery occlusion (MCAO) stroke model, bone marrow (BM) transplantation, isolation of mouse and human neutrophils, immunofluorescence and flow cytometry studies, FXIIa and TF activity assays, collection of plasma and tissue samples, histological analysis of liver and kidney paraffin sections and microscale thermophoresis are described in detail in the Supplemental Materials.

**Data Sharing Statement**

Original data can be obtained by contacting the corresponding author.

## RESULTS

***Markers of contact and intrinsic coagulation pathway activation are elevated in SCD patients during steady state.***

Previous studies reported decreased plasma levels of zymogen FXII, plasma prekallikrein (PK) and high molecular weight kininogen (HK) in SCD patients[18-20], suggesting that these proteins may be consumed due to chronic activation. We obtained plasma samples from matched controls and SCD patients (Table 1)[23]. SCD patients had lower hematocrit and hemoglobin levels, increased platelet and white blood cell counts, and significantly elevated thrombin antithrombin (TAT) complexes and D-dimer levels compared to controls (Table 1). To determine if components of the contact and intrinsic pathways are activated in SCD patients, we used ELISAs to detect FXIIa, PKa, FXIa, or FIXa, bound to their endogenous plasma inhibitors C1 esterase inhibitor (C1INH; for FXIIa, FXIa, and PKa) or antithrombin (for FIXa)[24,25]. We found that SCD patients had significantly elevated plasma levels of FXIIa:C1INH, FXIa:C1INH, PKa:C1INH and FIXa:AT complexes compared to healthy controls (Figure 1A-D) none of which correlated with RBC count, hematocrit, or hemoglobin levels in SCD patients (Figure S1). In the SCD group, we observed highly significant correlations between FXIIa:C1INH and FXIa:C1INH with FIXa:AT complexes (Figure 1E-F). Interestingly, a significant correlation was also observed between plasma levels of PKa:C1INH and FIXa:AT complexes (Figure 1G). These data suggest that in SCD patients, FXIIa activates both FXI and PK. Furthermore, correlation analyses suggest that activation of FIX is not only mediated by FXIa, but also in part by PKa. Consistent with increased plasma levels of FXIIa:C1INH complexes in SCD patients, immunoblot analysis demonstrated an increase in FXIIa in HbSS mouse plasma compared to HbAA controls (Figure S2).

***FXII contributes to thrombin generation and inflammation in HbSS mice during steady state.***

Given the above evidence suggesting chronic in vivo activation of FXII in SCD patients and mice, we next determined whether activation of FXII contributes to thromboinflammation at

steady state. BM from HbAA and HbSS mice was transplanted into irradiated wild type ($FXII^{+/+}$) and FXII-deficient ($FXII^{-/-}$) mice. This experimental approach generates HbAA and HbSS mice with either normal levels of FXII (HbAA/$FXII^{+/+}$ and HbSS/$FXII^{+/+}$) or with FXII deficiency only in non-hematopoietic cells (HbAA/$FXII^{-/-}$ and HbSS/ $FXII^{-/-}$). Four months after BM transplantation, plasma levels of TAT, IL-6, and sVCAM-1 were elevated in HbSS/$FXII^{+/+}$ over HbAA/$FXII^{+/+}$ mice (Figure 2A-C). Deficiency of FXII in non-hematopoietic cells completely attenuated TAT and IL-6 in HbSS mice to the same level observed in HbAA/$FXII^{+/+}$ controls but had no effect on sVCAM-1 (Figure 2A-C). Using the same BM transplantation approach, we generated HbSS mice lacking FXI or PK in all non-hematopoietic cells. In contrast to what we observed in HbSS/$FXII^{-/-}$ mice, plasma TAT levels in HbSS/$FXI^{-/-}$ and HbSS/$PK^{-/-}$ mice were only modestly reduced (Figure S3), suggesting that eliminating only one of the FXIIa-dependent pathways is not sufficient to significantly attenuate systemic thrombin generation.

***Tissue factor does not contribute to enhanced thromboinflammation observed in HbSS mice after TNFα challenge.***

Challenging HbSS mice with low-dose TNFα induces neutrophil activation, increases levels of circulating cell free (CF)-DNA, and promotes vaso-occlusion[26,27]. Consistent with these data, we also observed that TNFα challenge reduced the number of circulating neutrophils and dramatically increased plasma levels of CF-DNA in HbSS mice (Figure S4A-B). Moreover, TNFα challenge further enhanced the plasma levels of TAT and IL-6 in HbSS mice (Figure S4C-D), without affecting levels of sVCAM-1 (Figure S4E).

We previously demonstrated that inhibition of TF, the initiator of the extrinsic coagulation pathway, attenuates thrombin generation and vascular inflammation in HbSS mice at steady state[9,10]. To determine if TF also contributes to the enhanced thromboinflammation after

TNFα challenge, HbAA and HbSS mice received an inhibitory anti-mouse TF antibody 1H1[9,28] (generously provided by Dr. Daniel Kirchhoffer, Genentech, CA) or control IgG (25mg/kg, i.p.) 1 hour before TNFα administration. TF inhibition had no effect on TAT or IL-6 levels in TNFα-challenged HbSS mice and did not reverse the TNFα-mediated reduction in circulating neutrophils (Figure S5A-C). This lack of effect occurred despite sufficiently high plasma levels of 1H1 to completely attenuate TF-mediated procoagulant activity in an *in vitro* cell-based assay (Figure S5D). These data indicate that TF does not significantly contribute to thromboinflammation in TNFα-challenged HbSS mice.

***Deficiency of FXII in non-hematopoietic cells attenuates thromboinflammation in HbSS mice after TNFα challenge.***

We sought to determine if the intrinsic coagulation pathway is activated in HbSS mice. Although we were unable to develop mouse equivalents for all assays used on clinical samples, we successfully developed an ELISA to measure murine FIXa:AT complexes in plasma. HbSS mice had a modest increase in FIXa:AT complexes compared to HbAA controls, and TNFα challenge significantly increased FIXa:AT plasma levels in HbSS mice (Figure S4F). We also observed a strong positive correlation between FIXa:AT and TAT complexes in all HbSS mice ($r^2$= 0.62, $p<0.001$). We then evaluated whether FXII deficiency in non-hematopoietic cells affects these parameters in TNFα-challenged HbSS mice. FXII deficiency in non-hematopoietic cells resulted in partial but significant attenuation of plasma levels of TAT and IL-6 in TNFα-challenged HbSS/FXII$^{-/-}$ mice compared to HbSS/FXII$^{+/+}$ mice (Figure 2D-E). sVCAM-1 levels were not elevated by TNFα in HbSS mice and were unaffected by FXII deficiency (Figure 2F). These data indicate that non-hematopoietic FXII contributes to the increased levels of TAT and IL-6 observed in HbSS mice both at steady state and after TNFα challenge.

Degradation of CF-DNA with DNase improves survival of HbSS mice after TNFα challenge[26]. Since CF-DNA and NETs are thought to activate FXII, we set out to determine if DNAse treatment attenuates systemic thrombin generation. Surprisingly, DNase had no effect on plasma levels of TAT in HbSS mice after TNFα challenge (Figure S6), undermining the contribution of CF-DNA and NETs to systemic thrombin generation in this model.

***FXII contributes to vascular stasis in HbSS mice.***

To determine whether FXII contributes to VOC, we used the well-established dorsal skinfold chamber model [29,30]. HbSS mice were infused with control IgG or 15D10 antibodies (10 mg/kg, IV) 30 minutes before hemoglobin infusion (1 μmol/kg, IV). 15D10 is a mouse anti-FXII antibody that reacts with FXII from multiple species, including mouse and human, and significantly attenuates FXIIa formation and FXIIa-driven coagulation by binding to the heavy chain of FXII[31]. In our studies, intraperitoneal or intravenous dosing of 15D10 prolonged the activated partial thromboplastin time (aPTT) in C57 mice (Figure S7A-B). In IgG-treated HbSS mice, hemoglobin induced stasis in 29.8 ± 3.0%, 26.2 ± 2.5%, 23.9 ± 3.6%, 19.1 ± 2.2% (mean ± SEM) of pre-selected microvessels at 1, 2, 3 and 4 hours after infusion, respectively. This was significantly reduced in HbSS mice pre-treated with 15D10 (Figure 3A) at all time points. In addition, pathologic scoring of liver and kidney sections revealed a significant increase in vascular congestion in HbSS/FXII$^{+/+}$ mice compared to HbAA/FXII$^{+/+}$ controls; this difference was attenuated by FXII deficiency in non-hematopoietic cells (Figure 3B-C). Further histological analysis of kidney sections revealed modest pathological changes in HbSS/FXII$^{+/+}$ mice that were either FXII dependent (glomerular sclerosis, interstitial fibrosis, mesangial expansion, medullary congestion; Figure S8 A-D) or independent (brush border loss; Figure S8 E).

***FXII inhibition reduces fibrin and platelet accumulation in venous clots of HbAA and HbSS mice***

Using a model of venous thrombosis induced by electrolytic injury, we previously showed that HbSS mice have enhanced fibrin deposition in thrombi formed in the femoral vein[32]. Since FXII contributes to thrombin generation both at steady state and after TNFα challenge, and that FXII contributes to experimental venous thrombosis in animal models[17,33-35], we investigated the role of FXII in venous thrombosis in HbSS mice. HbAA and HbSS mice received control IgGκ1 or 15D10 (5 mg/kg, IV); 30 minutes later, electrolytic injury was applied to the femoral vein. The intensity of fibrin and platelet deposition was measured by intravital microscopy over time within a defined length of the femoral vein (Figure S9). Consistent with previously published data[32], mean fluorescent fibrin intensity and total fibrin deposition (measured by area under the curve, AUC) were significantly increased in HbSS mice compared to HbAA mice treated with IgGκ1. Importantly, 15D10 treatment significantly reduced these endpoints in both HbAA and HbSS mice (Figure 4A-B). FXII inhibition with 15D10 also blocked mean fluorescent platelet intensity and total platelet deposition (Figure 4C-D). Representative images of thrombi from each treatment group are shown in Figure 5E. Analysis of time-lapse videos from both HbAA and HbSS mice that received control IgG demonstrated that initial platelet deposition occurred at the site of injury and was followed by fibrin accumulation, additional platelet recruitment, and clot formation in the direction of blood flow (Supplemental Movies 1 and 2). Inhibition of FXII autoactivation by 15D10 retarded both initial platelet accumulation and fibrin formation. Thus, targeting FXII interferes with initial clot formation resulting in reduction in overall clot size in both HbAA and HbSS mice (Supplemental Movies 3 and 4).

Consistent with these results, 15D10 significantly prolonged aPTT in HbAA and HbSS mice (Figure S7 C).

***Inhibition of FXII generation reduces the volume of venous clots formed in HbAA and HbSS mice***

The relatively small size of the thrombi that develop in the femoral vein prevents accurate clot weight quantification. Therefore, we harvested the femoral vein with the entire clot *in situ* for histomorphometric reconstruction of total clot volume (Figure S9). Unexpectedly, despite a higher mean fluorescent fibrin intensity, the clot volumes in HbSS mice were significantly smaller compared to clots in HbAA mice (Figure 4F). Importantly, clot volume was largely reduced in both HbAA and HbSS mice treated with 15D10 (Figure 4F). Histological analysis of HbAA and HbSS clots revealed interesting differences in clot structure and cellular composition. Thrombi formed in the femoral veins of HbAA mice demonstrated a classical pattern with well-defined red blood cell- and fibrin/platelet-rich areas (Figure 5). In contrast, sickle clots were characterized by increased neutrophil incorporation at the interface of clot and endothelium, and within the core of the clot. Clot structure was also less organized and less compact in the red cell-rich areas with multiple acellular spaces observed in HbSS mice compared to HbAA controls.

***FXII contributes to enhanced neuronal damage and neurologic deficit observed in HbSS mice subjected to brain ischemia reperfusion injury***

Ischemic stroke and silent cerebral infarction frequently occur in SCD, leading to neurological deficits and cognitive impairment[7]. HbAA and HbSS mice were infused with control IgG or 15D10 antibodies (10 mg/kg, IV) 30 minutes before tMCAO (1-hour transient occlusion) and 6 hours into reperfusion. After 24 hours of reperfusion, brain injury and

neurologic deficit were assessed in HbAA/IgG, HbSS/IgG, and HbSS/15D10 mice. The mean stroke severity score and proportion of mice at highest severity scores were increased in HbSS/IgG compared to HbAA/IgG mice; both parameters were significantly decreased in the HbSS/15D10 group (Figure 6A-B). Infarct volume was also increased in HbSS/IgG mice compared to HbAA/IgG counterparts, and it was significantly decreased by 15D10 treatment (Figure 6C). Intravital microscopy showed significantly more rolling leukocytes in the cerebral microcirculation in HbSS/IgG versus HbAA/IgG mice, likely due to neutrophilia in HbSS mice; this was not affected by 15D10 pre-treatment (Figure 6D). In contrast, enhanced leukocyte adhesion to the endothelium observed within cerebral pial vessels of HbSS/IgG mice was significantly reduced by 15D10 administration (Figure 6E), suggesting that FXII contributes to leukocyte adhesion after MCAO. We previously showed that FXII signals in isolated neutrophils through uPAR and thereby upregulates the surface expression of $\alpha M\beta 2$ (CD11b-CD18) integrin[15], a critical regulator of neutrophil firm adhesion to the vessel wall. Therefore, we investigated whether the binding kinetics of FXII and uPAR differed in the presence of 15D10. We utilized microscale thermophoresis (MST), an assay that captures binding-induced changes in thermophoretic mobility[36,37]. For these studies, murine His-tagged FXII was fluorescently labelled with RED-tris-nitriloacetic acid and subsequently incubated with serially diluted murine uPAR (final concentrations: $6.85 \times 10^{-9}$ M to $2.6 \times 10^{-6}$ M), in the absence or presence of 1 µM 15D10 (Figure 6F). The fluorescent intensity of labeled FXII is monitored before, during, and after infrared laser excitation and the initial fluorescent intensity prior to heating is used to normalize fluorescence changes ($\Delta$Fnorm representing the bound fraction). Based on MST measurements, a binding constant (Kd) of $0.89 \times 10^{-7}$ M was determined for FXII and uPAR. In

the presence of 15D10, Kd rose to ~ 3 x $10^{-7}$ M suggesting that 15D10 interferes with the FXII-uPAR interaction (Figure 6F, Figure S10).

***Basal FXII activity is elevated in neutrophils from SCD patients and HbSS mice compared to controls***

Hepatocytes are the primary source of circulating FXII[38]. We performed western blot analysis on plasma from $FXII^{+/+}$ and $FXII^{-/-}$ mice that were transplanted with BM from FXII-expressing HbAA and HbSS mice. There was no detectable zymogen FXII in the plasma of either HbAA/$FXII^{-/-}$ or HbSS/$FXII^{-/-}$ mice (Figure S11), indicating that neutrophil-derived FXII does not significantly contribute to the pool of circulating FXII. However, we previously showed that FXII becomes activated on the surface of primed neutrophils[15,39]. Here, we examined if neutrophil derived FXII can raise local FXIIa generation. Healthy human neutrophils (final count 30,000 cells/well) were co-incubated with normal human plasma or FXII-deficient plasma and 200 μM of FXIIa chromogenic substrate S-2302. Where indicated, reactions were supplemented with 15 μM $Zn^{2+}$ or aPTT reagent (positive control) and optical density (OD) at 405 nm was monitored. These studies demonstrated that adding neutrophils to normal human plasma in the presence of $Zn^{2+}$, which supports FXII cell binding, resulted in robust FXIIa generation even in the absence of aPTT reagent (Figure 7A). Importantly, when healthy neutrophils were incubated with FXII-deficient plasma, there was detectable chromogenic substrate conversion to ~40% of that observed when cells were mixed with normal human plasma (Figure 7A). These data support that neutrophils are a source of functional FXII and can partially sustain FXII procoagulant responses in settings of heightened neutrophil activation.

Given the prominent role of neutrophils in SCD pathology, we used immunofluorescence to detect FXII in peripheral blood neutrophils isolated from healthy individuals or SCD patients

at steady state. Neutrophils from individuals with SCD exhibited higher surface exposure of FXII than cells from healthy controls (Figure 7B). We also found that neutrophil derived FXIIa activity was significantly higher in neutrophils isolated from HbSS mice compared to HbAA controls (Figure 7C). Additionally, when the reaction rate was adjusted to the number of neutrophils in each sample, HbSS neutrophils exhibited a significant, near 7-fold increase in FXIIa activity compared to HbAA neutrophils (Figure 7D).

Next, we set out to determine if the enhanced FXII expression in SCD neutrophils confers these cells with upregulated CD11b expression and consequently, an increased adhesive potential. Neutrophils isolated from healthy individuals and SCD patients were purified and either left untreated or stimulated with 200 nM FXII and 15μM $ZnCl_2$. As assessed by flow cytometry, constitutive surface expression of uPAR, total and active CD11b were significantly higher in neutrophils of SCD patients compared to healthy controls (Figure 7E-G). Stimulation of control neutrophils with FXII modestly increased uPAR expression and significantly enhanced total and active CD11b surface expression (Figure E-G), whereas FXII stimulation of neutrophils from SCD patients did not result in a further increase in surface expression of uPAR or CD11b integrin (Figure 7E-G). We posit that heightened basal neutrophil activation in SCD, in part mediated by FXII, leads to saturable expression of adhesive receptors.

## DISCUSSION

Using genetic and pharmacologic approaches coupled with clinical samples and murine models, we demonstrated a role for FXII in several vascular complications associated with SCD. Specifically, this is the first report to: (1) directly evaluate and demonstrate increased activity of the contact pathway in SCD patients; (2) show that FXII, but not TF, contributes to enhanced thrombin generation and inflammation in HbSS mice after TNFα-challenge; (3) reveal that

inhibition of FXII attenuates venous thrombosis, microvascular stasis and ischemic brain injury in a mouse model of SCD; and (4) show that SCD neutrophils have higher levels of FXII, uPAR and activated αMβ2 integrin that confers these cells with enhanced adhesive properties.

Previous reports found a reduction in circulating levels of FXII, PK, and HK in SCD patients suggesting the possibility of zymogen consumption due to chronic activation[18,19]. The present study is the first to demonstrate activation of these proteases by measuring complexes of FXIIa, FXIa, and PKa bound to their principal serpins[24,25] in the plasma of SCD patients. Our correlation analyses suggest that FXIIa contributes to activation of both the intrinsic coagulation (FXIa/FIXa) and contact (PKa) pathways in SCD. In addition to the canonical activation of FIX through FXIa, we and others have reported that PKa can directly activate FIX, independent of FXIa[40-42]. Our data show that PKa:C1INH levels correlate with FIXa:AT complexes, suggesting that this pathway of FIX activation may be operative in SCD. These observations, together with the data showing a significant reduction of plasma TAT levels in HbSS/FXII$^{-/-}$ but only partial attenuation of this marker in HbSS/FXI$^{-/-}$ and HbSS/PK$^{-/-}$ mice, strongly suggest that both FXII-dependent activation of FXI and PK contribute to the systemic thrombin generation in SCD.

Challenging sickle cell mice with TNFα[26,43,44] or hemoglobin[29,30,45] are the two commonly used models mimicking vascular pathologies induced by either acute inflammatory or hemolytic events, respectively. We revealed that hemoglobin-induced microvascular stasis in HbSS mice can be attenuated by blocking TF[9] as well as FXII (Figure 3A). In contrast, FXII deficiency but not TF inhibition attenuated the thromboinflammatory state in TNFα-challenged HbSS mice. These observations suggest that inhibition of FXII/FXIIa may be more effective than blocking TF to attenuate vascular pathologies in SCD. Future studies will focus on identifying the cellular and molecular triggers of FXII activation in SCD, such as the unique properties of

sickle RBCs and/or their microparticles[41,46,47], cell free DNA[48], or mast cell-derived glycosaminoglycans[49,50]. It will also be important to determine the selective contribution of FXII cell signalling activities and FXIIa procoagulant functions to the pathology of SCD.

SCD is associated with an increased risk of venous thrombosis that is associated with a high risk of recurrence and mortality[6,51]. In SCD, there is a modestly higher incidence of pulmonary embolism than deep vein thrombosis[52]. We previously demonstrated that *ex vivo* clots from HbSS mice are more heterogeneous in structure than clots from HbAA mice due to the presence of sickled RBCs[32]. Moreover, despite the higher abundance of fibrin deposition within the clots formed in the femoral veins after electrolytic injury[32], clots from HbSS mice had a significantly smaller clot volume compared to HbAA controls. Since the number of RBCs affects the overall size and volume of venous clots[53-55], the reduced clot volume observed in HbSS mice may reflect the reduced hematocrit in these animals. However, reduced clot volume could also be a result of decreased clot stability and resultant embolization. Data from our group and others suggest that morphological properties of sickle RBCs may lead to the formation of less stable clots that are prone to embolization[32,56] and this hypothesis may be further supported by morphologic differences in clot structure between HbAA and HbSS clots presented in Figure 5. Moreover, the decreased clot volume, despite the greater number of neutrophils in HbSS clots, suggests that the cellular mechanisms of venous thrombosis vary depending on disease state.

The standard treatment for SCD patients with venous thromboembolism (VTE) is systemic anticoagulation for a minimum of three months[51]. However, a recent retrospective study of over 6400 subjects with SCD highlights the fact that these patients have an inherently elevated risk of bleeding (particularly from the upper GI tract), amounting to a 21% cumulative incidence of bleeding by age 40, and an associated 2-fold increased risk of death[57]. Furthermore, a recent

history of ischemic stroke or VTE was an independent risk factor for bleeding that was presumed to relate to antithrombotic agent usage[57]. Thus, it is clear that safe alternative anticoagulant agents are needed to prevent recurrent thrombosis in SCD patients. One candidate target molecule is FXII/FXIIa. FXII deficiency attenuates experimental thrombosis in animal models, including the electrolytic injury femoral vein model[17,33-35,58-61]. In the current study, we found that pre-treatment with 15D10 antibody attenuated fibrin and platelet deposition, and reduced clot volume in HbSS mice. The intravital microscopy time-lapse videos suggest that FXII inhibition blocks the initiation of fibrin deposition and platelet accumulation at the injury site, however, it is possible that FXII inhibition will also affect clot stability. Indeed, FXII is known to directly interact with fibrinogen and fibrin to increase clot stability[62,63]. Thus, more studies will be required to determine whether reduced clot size seen with FXII inhibition increases embolic risk.

Stroke is a common and severe complication of SCD[64,65]. Others have demonstrated that FXII plays a role in mouse models of ischemic stroke[35–66]. Therefore, we investigated whether FXII inhibition also attenuates neurologic damage in HbSS mice. Consistent with our previous studies[67,68], we found that after tMCAO, brain injury was more severe in HbSS mice than in HbAA controls. Importantly, pre-treating HbSS mice with 15D10 significantly attenuated neuronal damage and improved behavioural deficits caused by brain I/R injury. These protective effects may be attributed to the suppression of procoagulant responses occurring after brain I/R injury[35,66,69-71]. However, given that significantly lower numbers of adherent leukocytes were observed in brain pial vessels of HbS mice treated with 15D10, and since neutrophils have previously been shown to enhance brain injury following tMCAO[68,72], an additional hypothesis is that FXII regulates the brain inflammatory milieu following ischemic injury. In this respect,

we previously showed that FXII-uPAR signalling in neutrophils upregulates αMβ2 integrin to promote neutrophil adhesion[15,39]. Here, we found constitutively higher expression of FXII and uPAR in neutrophils from subjects with SCD which correlated with higher basal αMβ2 integrin expression and activity. Finally, a microscale thermophoresis analysis revealed that the presence of 15D10 antibody interferes with the FXII -uPAR interaction. Although additional investigations are required, these data suggest that the signalling properties of FXII may also contribute brain injury in SCD.

Although clinical trials of FXII/FXIIa inhibitors are presently focused on hereditary angioedema and thrombosis of extra-corporeal medical devices (NCT 04278885, NCT04653766, NCT04934891), the unique features of SCD-related thrombosis[32] and the evidence presented in this communication suggest that FXII(a) inhibition may be a rational and safe antithrombotic and anti-adhesive strategy in SCD.

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## AUTHORSHIP CONTRIBUTIONS

EMS: designed the research, performed experiments, analyzed data, wrote the manuscript. MWH, MM, CMA, AI, FT, NR, VS, DB, KLB, MP, CC, MK and BC: performed experiments, analyzed data. MW, TR, AG: provided valuable reagents; DG: performed experiments and provided valuable reagents. NSK and GMV: provided critical guidance on experimental procedures and revised the manuscript. FEG, JDB, and EXS: performed experiments, analyzed data, critically reviewed the manuscript. RP: designed the research, analyzed data, wrote the manuscript.

## DISCLOSURES

RP and EMS have research funding from CSL. AG and MW are employees of Aronora, Inc.
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## FIGURE LEGENDS

**Table 1: Demographic and descriptive data of participating subjects.**
*p<0.05, **p<0.01, ****p<0.0001 vs. control samples by unpaired Student's *t*-test. These cohorts were previously described[23]. Hgb – haemoglobin; TAT – thrombin-antithrombin complexes.

| Parameter | Controls (n=23) | SCD (n=53) |
|---|---|---|
| Age: Median (range) | 28 (19-54) | 33.3 (19.1 – 59.4) |
| Gender | 16 Female, 7 Male | 27 Female, 26 Male |
| Hematocrit (%) | 39 (34-45) | 27.5 (19.5 – 34.4)**** |
| Hgb (g/dL) | 13 (10.8 – 14.8) | 9.4 (6.5 – 11.5)**** |
| MCV (fL) | 89 (80.8 – 97.0) | 98.5 (69 – 122)* |
| Platelet ($10^9$/µL) | 253 (167 – 352) | 329 (191 – 695)** |
| White Blood Cells ($10^3$/µL) | 5.4 (3.2 – 8.5) | 8.75 (7.1 – 11.9)**** |
| TAT (ng/mL) | 1.44 (0.02 – 8.80) | 8.13 (0.16 – 32.00) *** |
| D-dimer (ng/mL) | 226.2 (43.7 – 1347.0) | 1865 (213.5 – 7659.0) **** |
| HU% | 0 | 79 (42/53) |

**Figure 1: Markers of contact and intrinsic pathway activation are elevated in SCD patients compared to healthy controls.** Plasma from healthy controls (N=23) and SCD (N=53) patients was assayed for (**A**) FXIIa-C1 esterase inhibitor (C1INH), (**B**) FXIa:C1INH, (**C**) PKa:C1INH and (**D**) FIXa-Antithrombin (AT) complexes. Data are presented as mean ± SEM and analyzed by Student's T-test. *P <0.05; **P<0.01. Correlations between (**E**) FXIIa:C1INH, (**F**) FXIa:C1INH, and (**G**) PKa:C1INH with FIXa-AT were calculated and analyzed by linear regression. Mean r with range and p value are reported on graphs.

**Figure 2: Non-hematopoietic FXII contributes to increased thrombin generation and inflammation in HbSS mice at steady state and after TNFα challenge.** $FXII^{-/-}$ and $FXII^{+/+}$ mice were irradiated and transplanted with HbAA or HbSS bone marrow. Four months later, plasma was collected for analysis of (**A**) TAT, (**B**) IL-6, and (**C**) sVCAM-1. In a separate study, all mice were treated with TNFα (2 µg/kg, ip). Five hours later, plasma was collected for analysis of (**D**) TAT, (**E**) IL-6, and (**F**) sVCAM-1. Data are represented as mean ± SEM, N=10–26 per group. * p<0.05, ** p<0.01, and ****p<0.0001 by Two Way ANOVA and Tukey's post-hoc test. Asterisks above bars indicate significance within the same FXII genotype. Asterisks above lines indicate difference between Hb genotype within FXII genotype.

**Figure 3: FXII contributes to vascular stasis and congestion in HbSS mice. (A)** Townes HbSS mice were implanted with dorsal skinfold chambers. Using intravital microscopy, 20-25 subcutaneous venules were selected and mapped. Mice were treated with IgG or 15D10 (10 mg/kg, IV) 30 minutes prior to challenge with stroma free hemoglobin (1 µmol/kg, IV). The preselected venules were marked as flowing or static at 1, 2, 3 and 4 hours after hemoglobin infusion, and the percent static venules was calculated. N=4 mice per group, data represent mean

± SEM. ****$p<0.0001$ vs IgG/SS at each time point by Two Way ANOVA. Paraffin sections of livers and kidneys from AA/FXII$^{+/+}$, AA/FXII$^{-/-}$, SS/FXII$^{+/+}$, and SS/FXII$^{-/-}$ mice four months after bone marrow transplantation were stained with H&E. (**B**) Sinusoidal congestion and (**C**) glomerular congestion were scored by blinded pathologists. Representative images are shown. N=4-8 mice per group, data represent mean ± SEM. **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by Two Way ANOVA and Tukey's post-hoc test. Asterisks above bar represent difference from HbAA, asterisks above line indicate difference between FXII genotype. Red staining on representative images showed in panels B and C demonstrates presence of RBC within congested vessels.

**Figure 4: FXII(a) inhibition attenuates femoral vein thrombosis induced by electrolytic injury.** Townes HbAA and HbSS mice were treated with IgG or 15D10 (5 mg/kg, IV) 30 minutes prior to electrolytic injury to the femoral vein. Quantification of (**A**) relative fibrin intensity over time and (**B**) the total fibrin fluorescence measured by area under curve (AUC). Quantification of (**C**) relative platelet intensity over time and (**D**) the total platelet fluorescence measured by AUC. Data represent mean ± SEM, N = 6-10 mice per group. (**E**) Representative images of fibrin (red) and platelet (green) accumulation taken 45 minutes after electrolytic injury. (**F**) Histomorphometric quantification of clot volume. Data represent mean ± SEM of clot volume ($mm^3$), N = 6-10 mice per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ by Two Way ANOVA and Tukey's post-hoc test.

**Figure 5: SCD affects morphology of venous clots.** Representative, H&E-stained images of femoral vein clots harvested from HbAA and HbSS mice 1 hour after electrolytic injury, (original magnification = 4X). In the clots, areas rich in RBCs stain dark red whereas areas of fibrin and platelets stain light pink. In the enlarged images on the right, asterisks denote acellular empty spaces within the clots; dark blue staining denotes inflammatory cells within the clots.

**Figure 6: FXII inhibition attenuates stroke severity in HbSS mice after transient middle cerebral artery occlusion (tMCAO).** Quantification of (**A**) stroke score, (**B**) proportion of stroke severity, (**C**) and brain infarct in HbAA and HbSS mice subjected to brain ischemia/reperfusion injury after treatment with IgG or 15D10 (10 mg/kg, IV) antibodies. Intravital microscopy analysis was performed to assess the number of (**D**) rolling and (**E**) adherent leukocytes. Data represent as mean ± SEM, N = 6 mice per group. * $p<0.05$, **$p<0.01$, ***$p<0.001$ by One Way ANOVA and Tukey's post-hoc test. (**F**) Microscale thermophoresis was performed to measure direct binding between uPAR and FXII, in the absence (blue line) or presence (red line) of 15D10. Recombinant His-tagged murine FXII was fluorescently labeled with RED-tris-nitrilotriacetic acid and subsequently incubated with 15 μM $Zn^{2+}$ and serially diluted murine uPAR. Where indicated, 1 μM of 15D10 was added to the reaction mixture. Initial fluorescent intensity of RED-FXII was used to normalize fluorescence changes (ΔFnorm representing the bound fraction). Binding constants (Kd) over time were determined for FXII-uPAR based on triplicate measurements of n=3 individual experiments.

**Figure 7: Neutrophil-derived FXII contributes to local FXIIa formation and enhanced neutrophil integrin activation in SCD.**

(**A**) Normal human plasma (NHP) or FXII-deficient plasma (*F12*$^{-/-}$ plasma) were incubated with aPTT reagent (aPTT-R) or neutrophils supplemented with 15 μM $Zn^{2+}$. The generation of FXIIa

was determined by monitoring the cleavage of S2302 (200 µM) over time. NHP incubated with aPTT reagent (aPTT-R) was used as positive control. N=3 individual experiments run in triplicate. (**B**) Visualization of FXII (green) and DAPI (blue) in neutrophils isolated from healthy controls (AA) and SCD patients. Images shown are representative of 3 individual experiments. Images shown at 20X magnification, scale:10 µm. Mouse HbAA and HbSS neutrophils (N = 3-4 run in duplicate) were assessed for FXIIa activity (S2302 cleavage) over 4 hours. Reaction rate of FXIIa activity was calculated in pM/s (**C**) per 250,000 neutrophils (**D**) or multiplied by the total number of circulating neutrophils in each individual mouse prior to isolation (pM/s x PMN). Data represent mean ± SEM, *P<0.05 vs. HbAA by Student's *t*-test. Flow cytometry analysis of (**E**) uPAR, (**F**) total αMβ2 integrin and (**G**) active αMβ2 integrin surface expression on untreated (Veh) and FXII/$Zn^{2+}$–stimulated neutrophils isolated from healthy controls (Con) and SCD patients. Data represent mean ± SEM, n=4-9; *p<0.05, **p<0.01,***p<0.001 by Kruskal-Wallis one-way analysis of variance.

Figure 1

# Figure 2

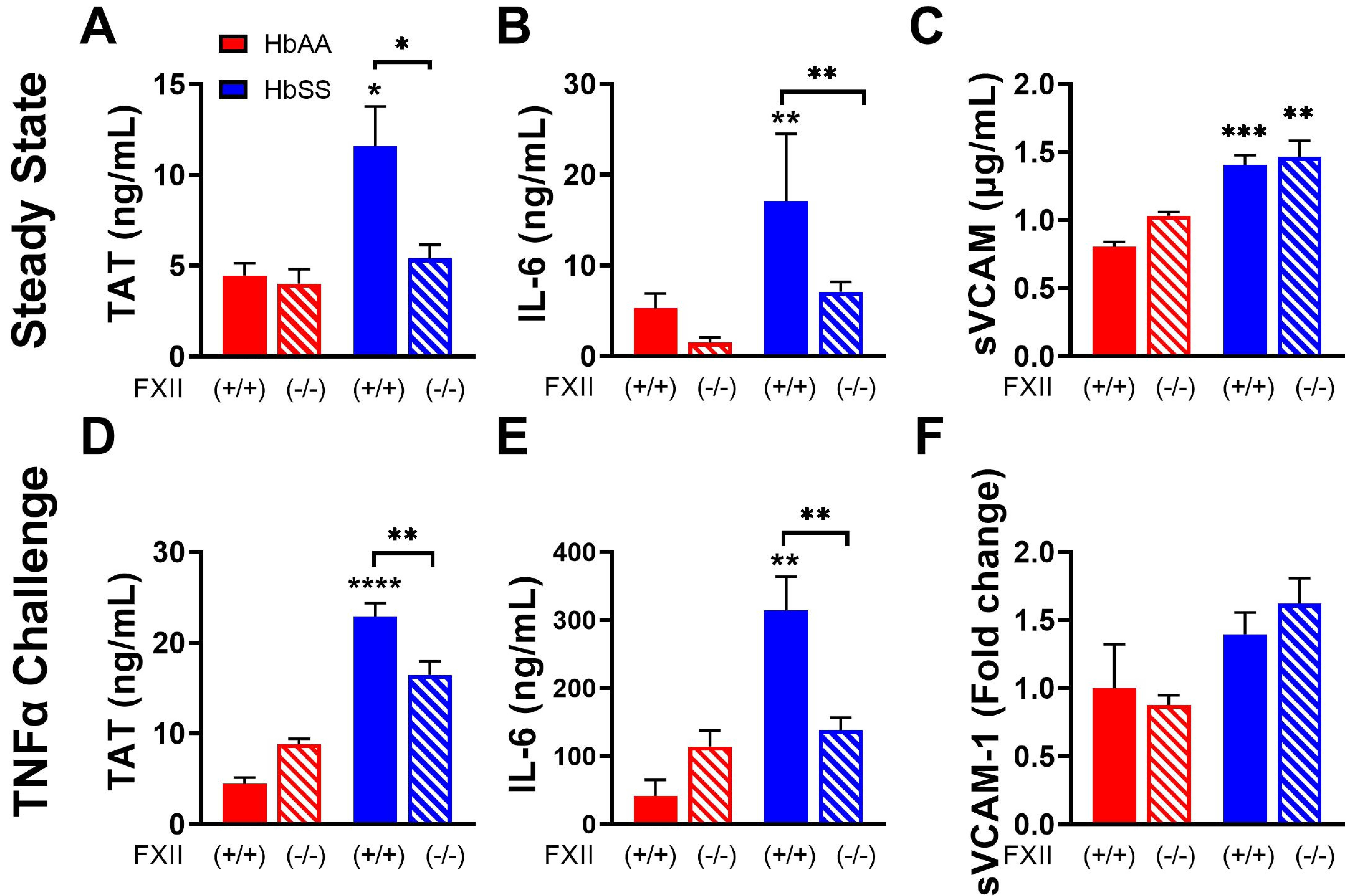
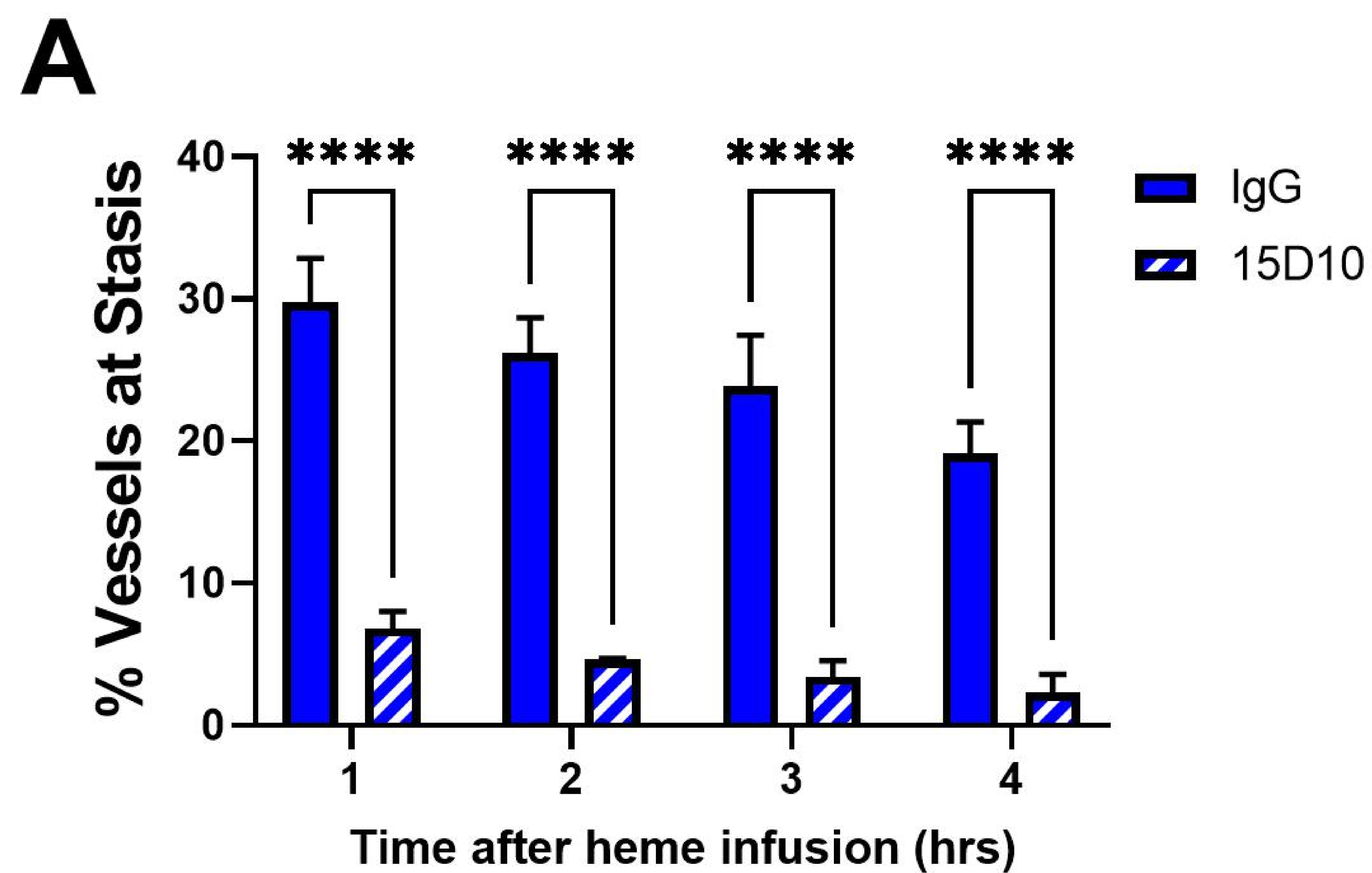
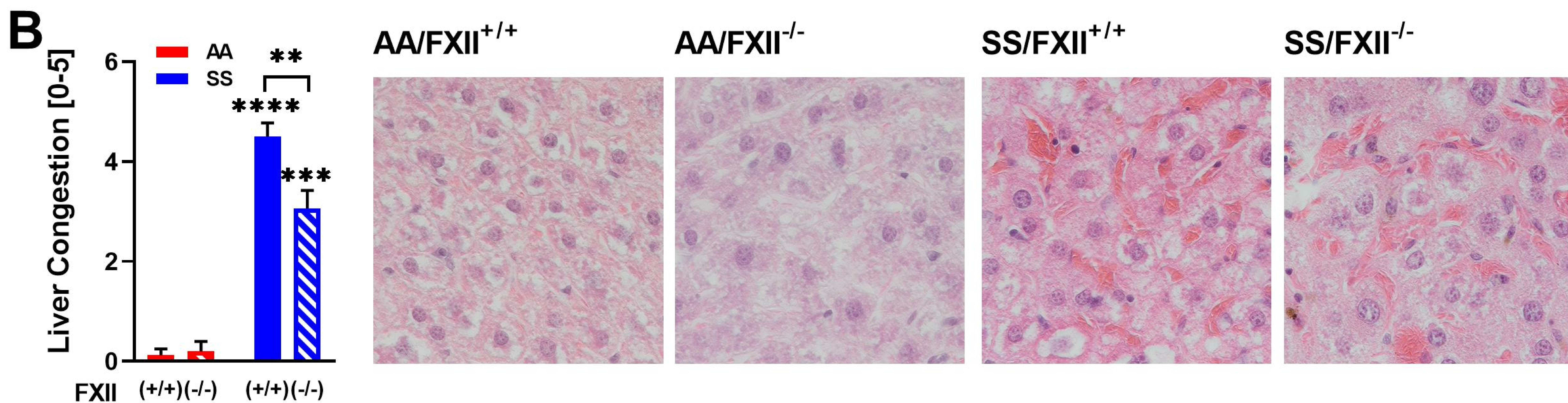
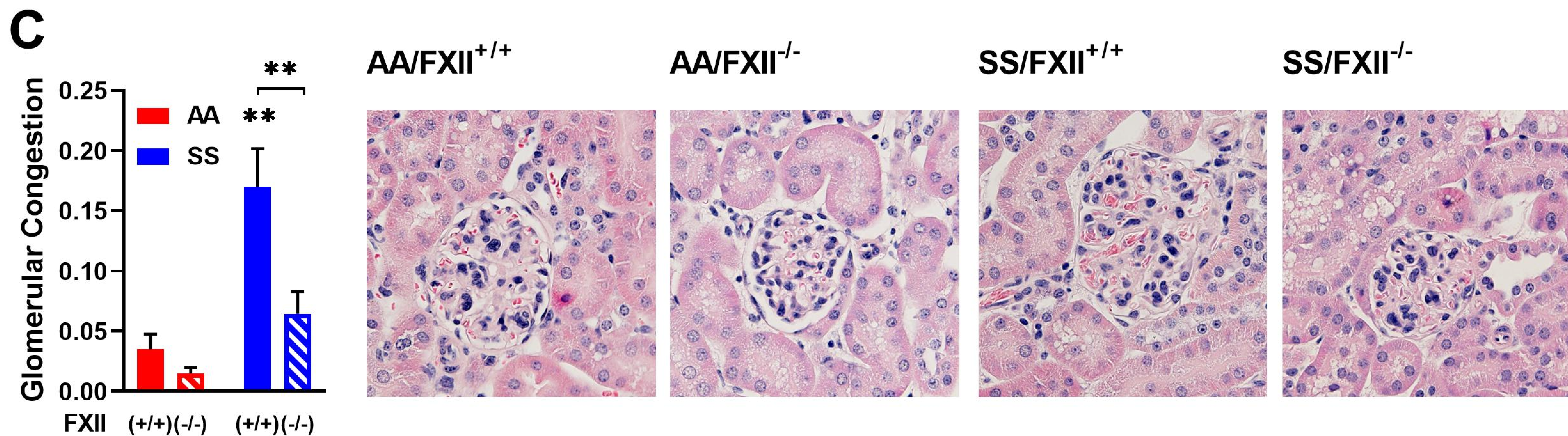


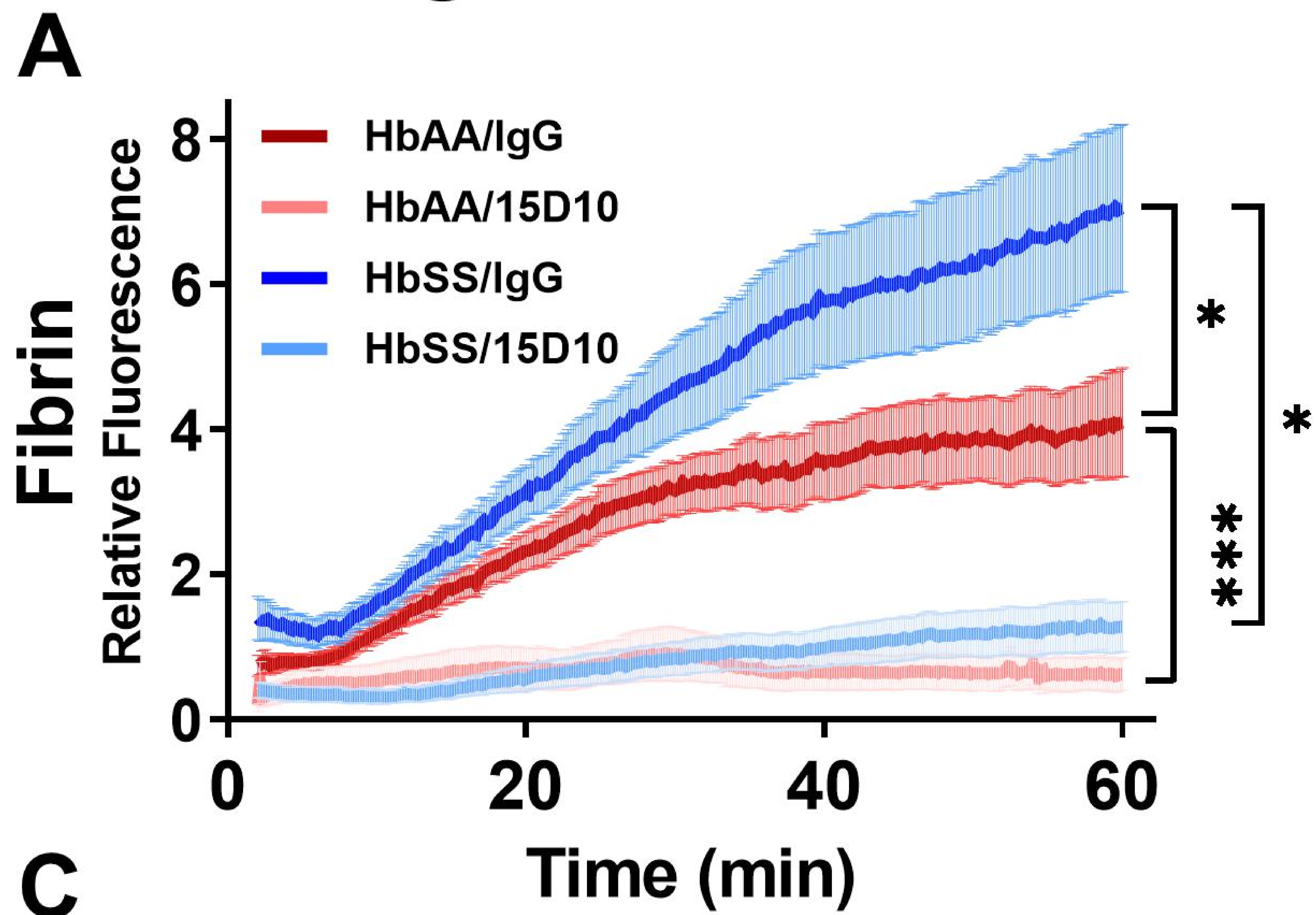
**Figure 3**

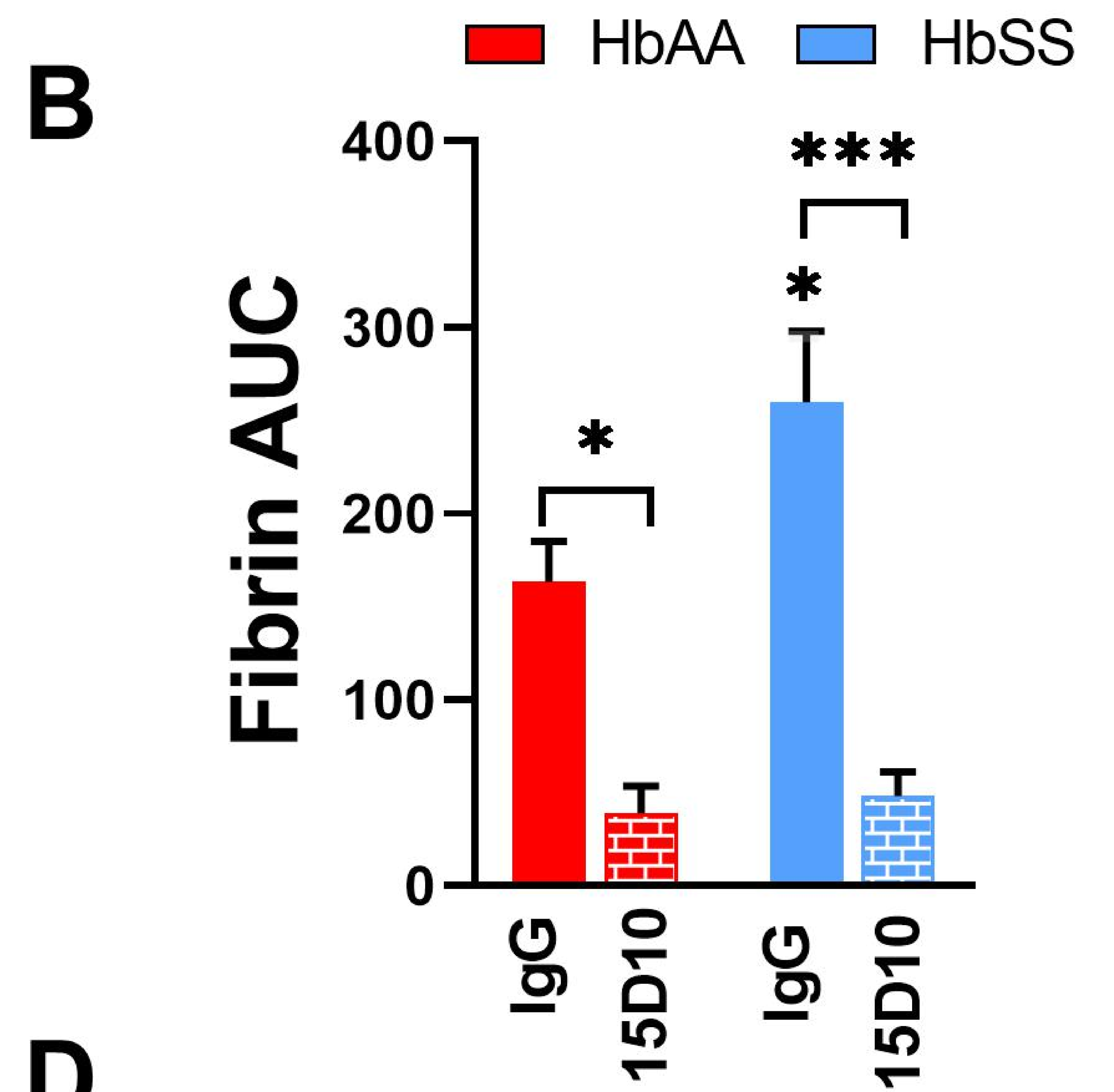


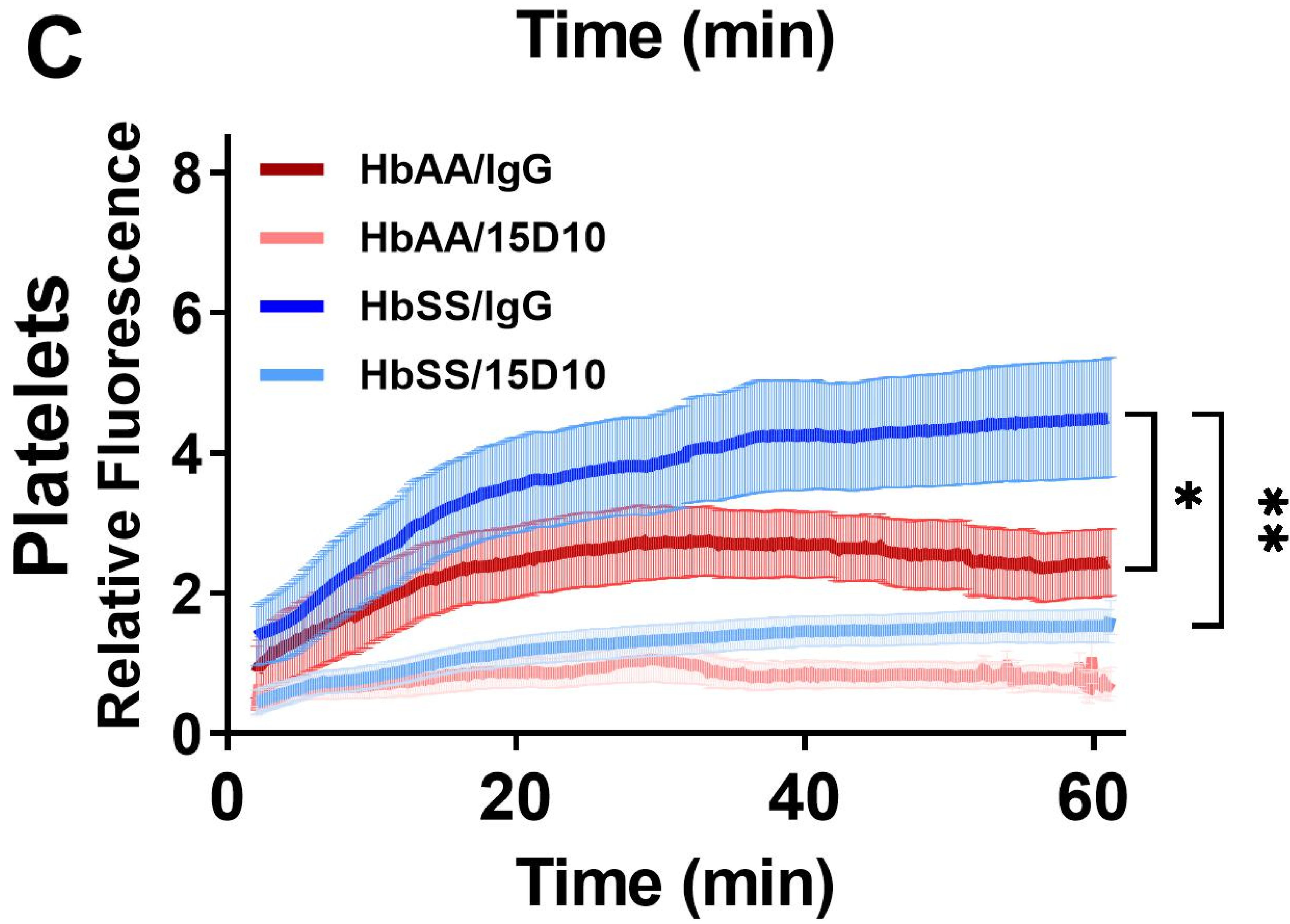



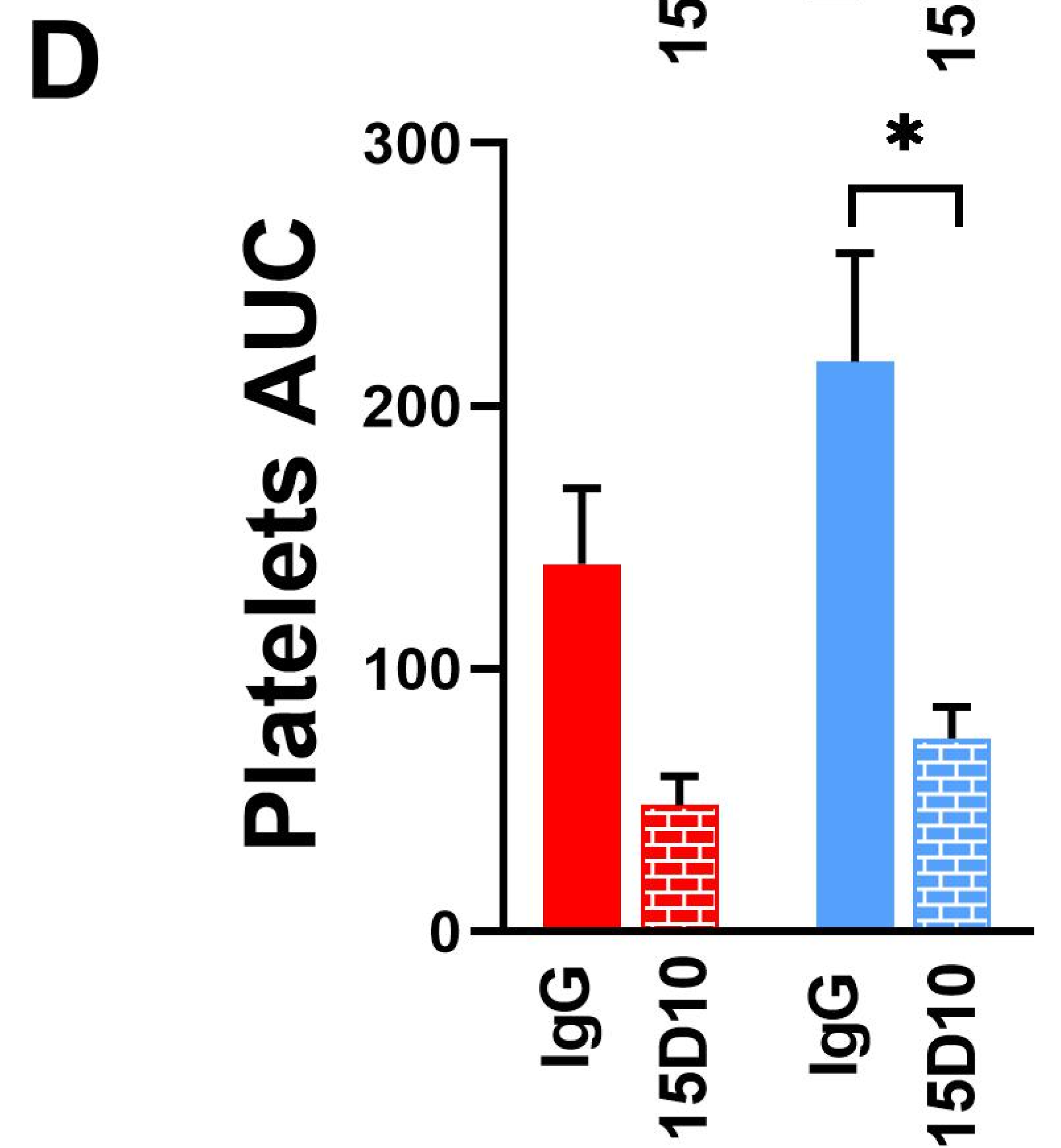



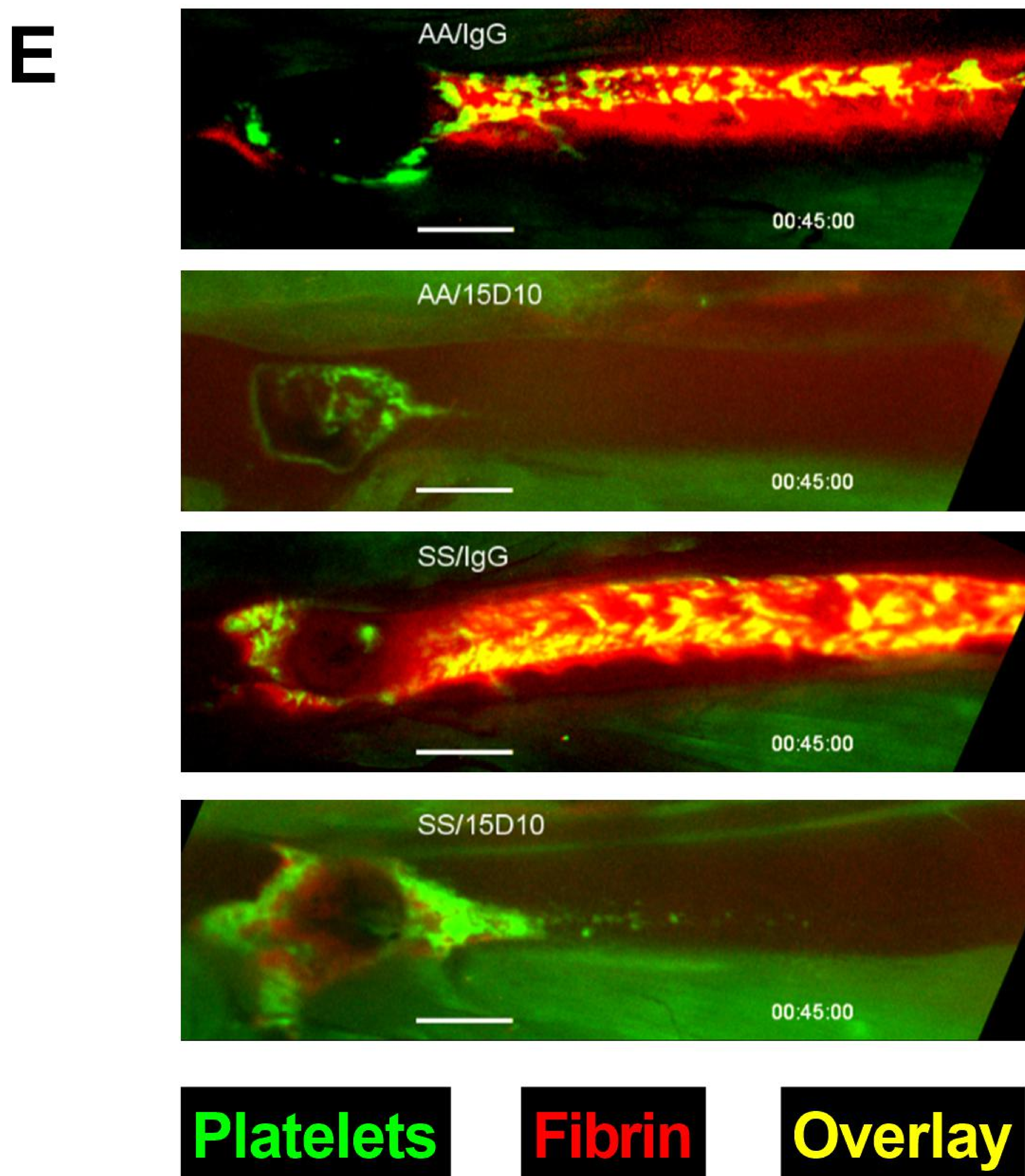
# Figure 4



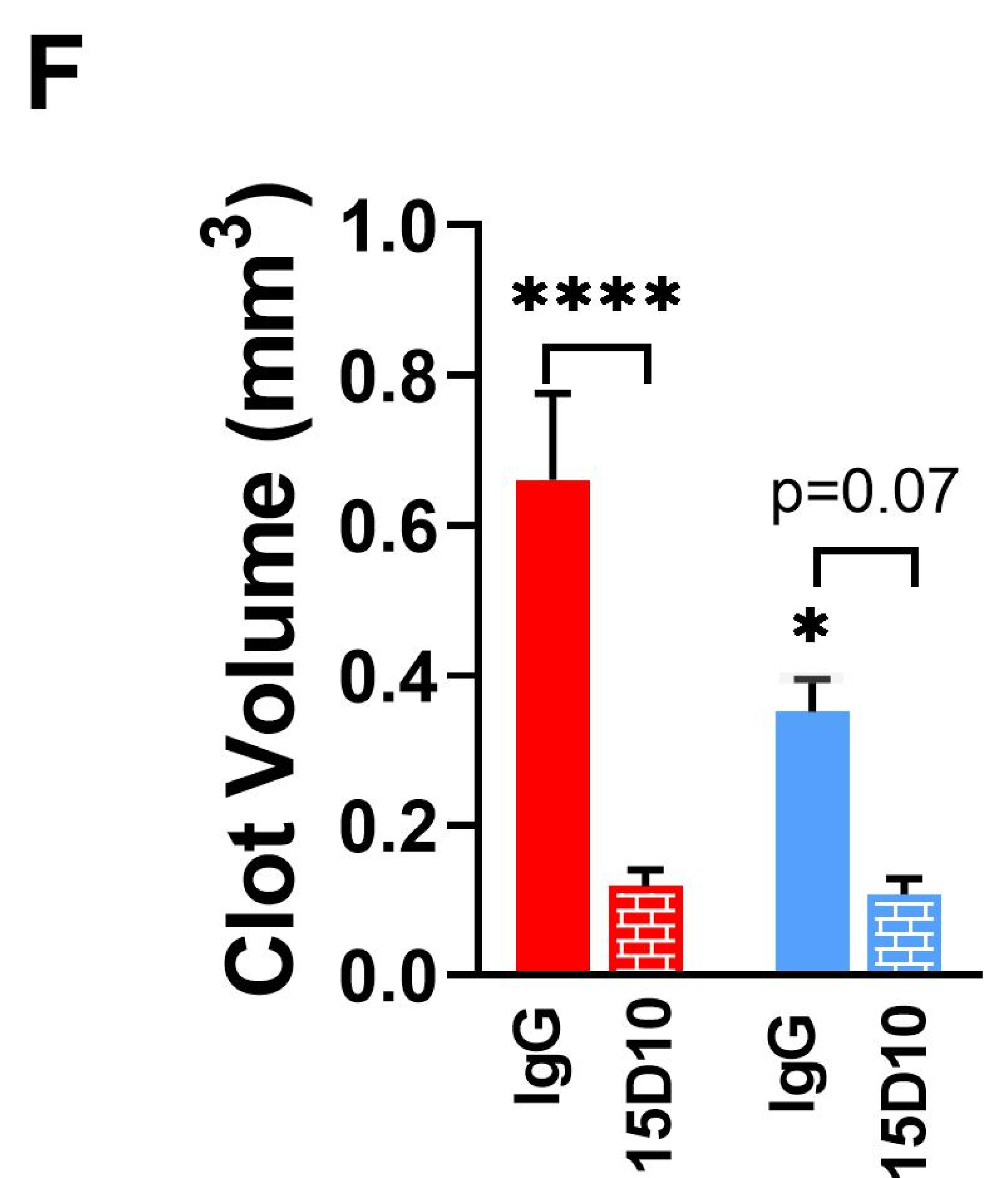
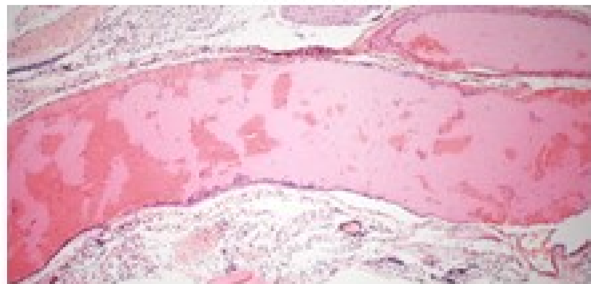
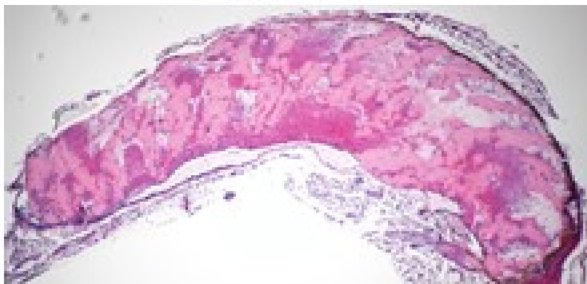
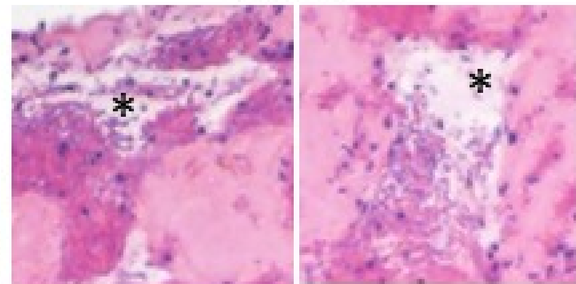
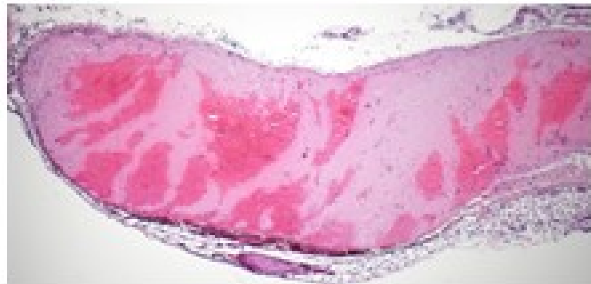



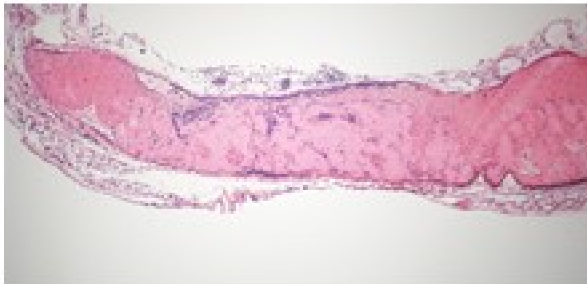
Figure 5

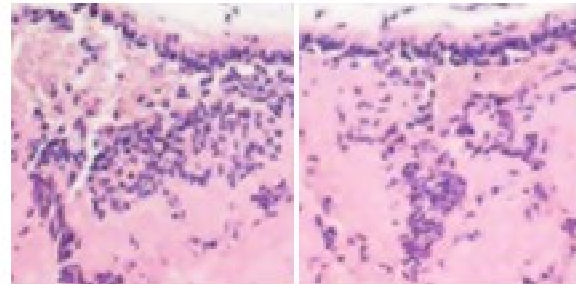


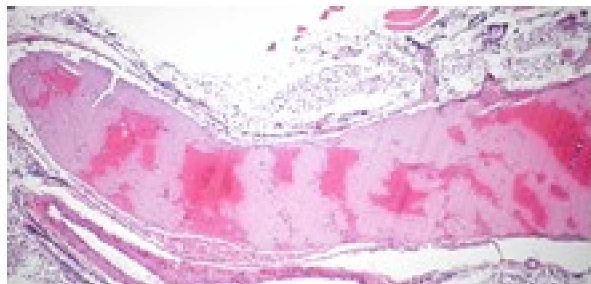



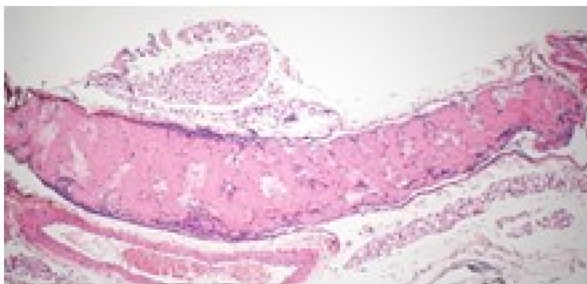



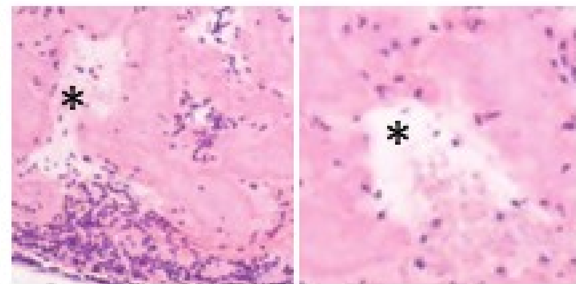





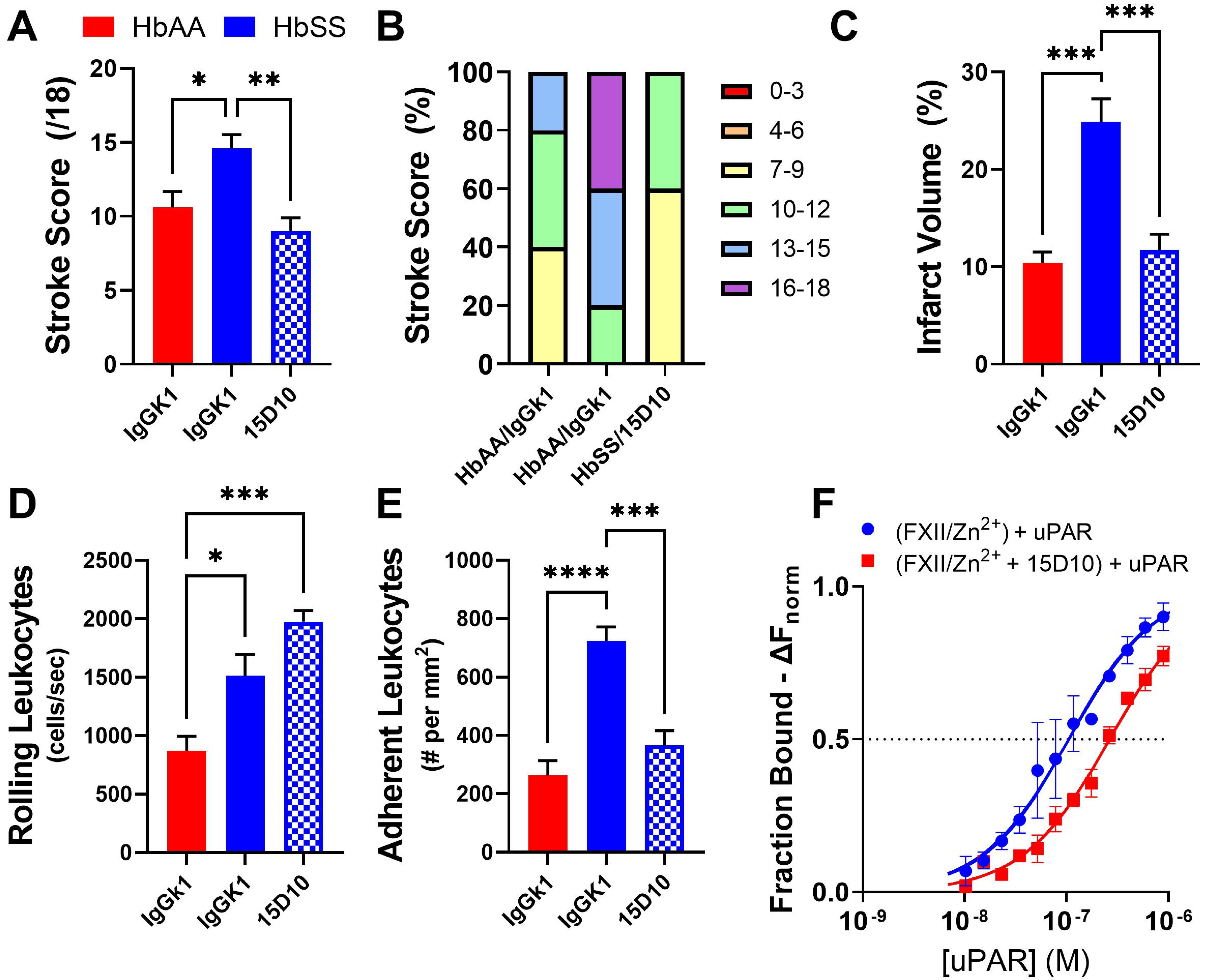


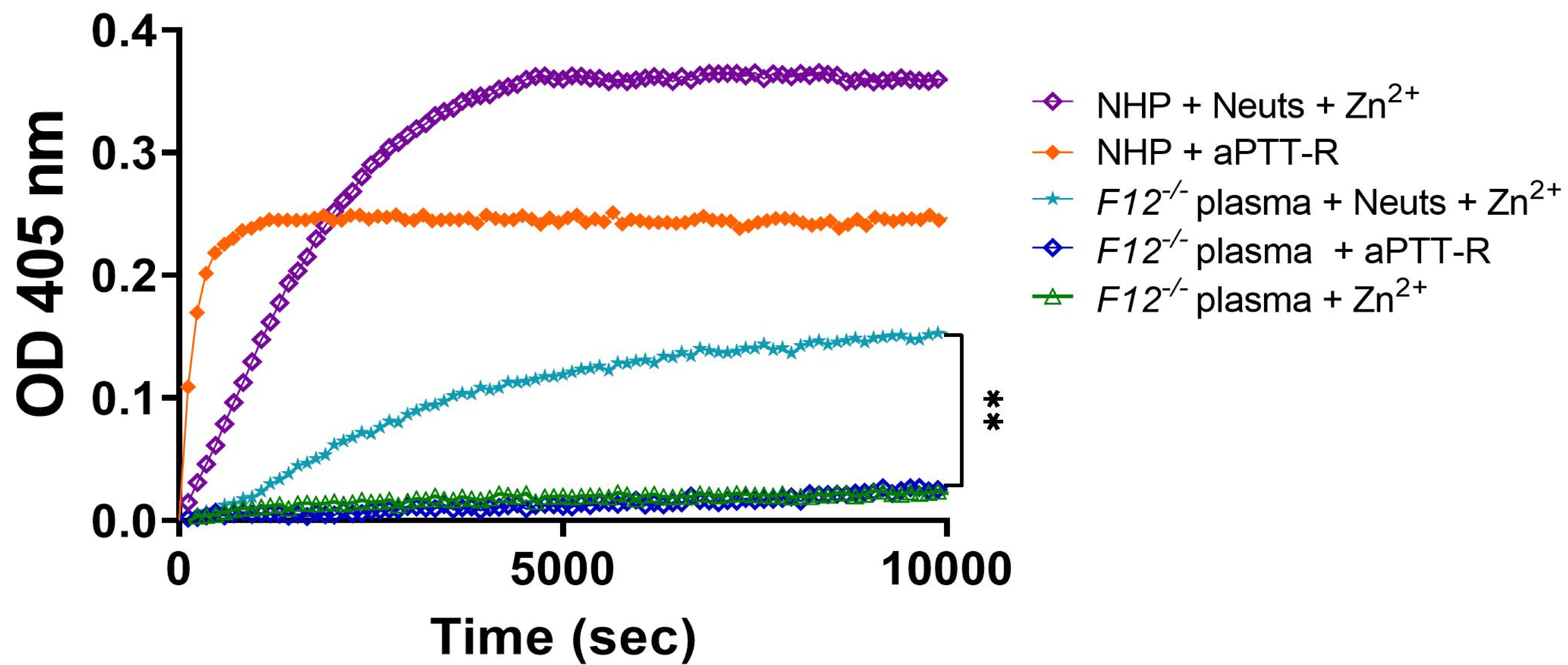


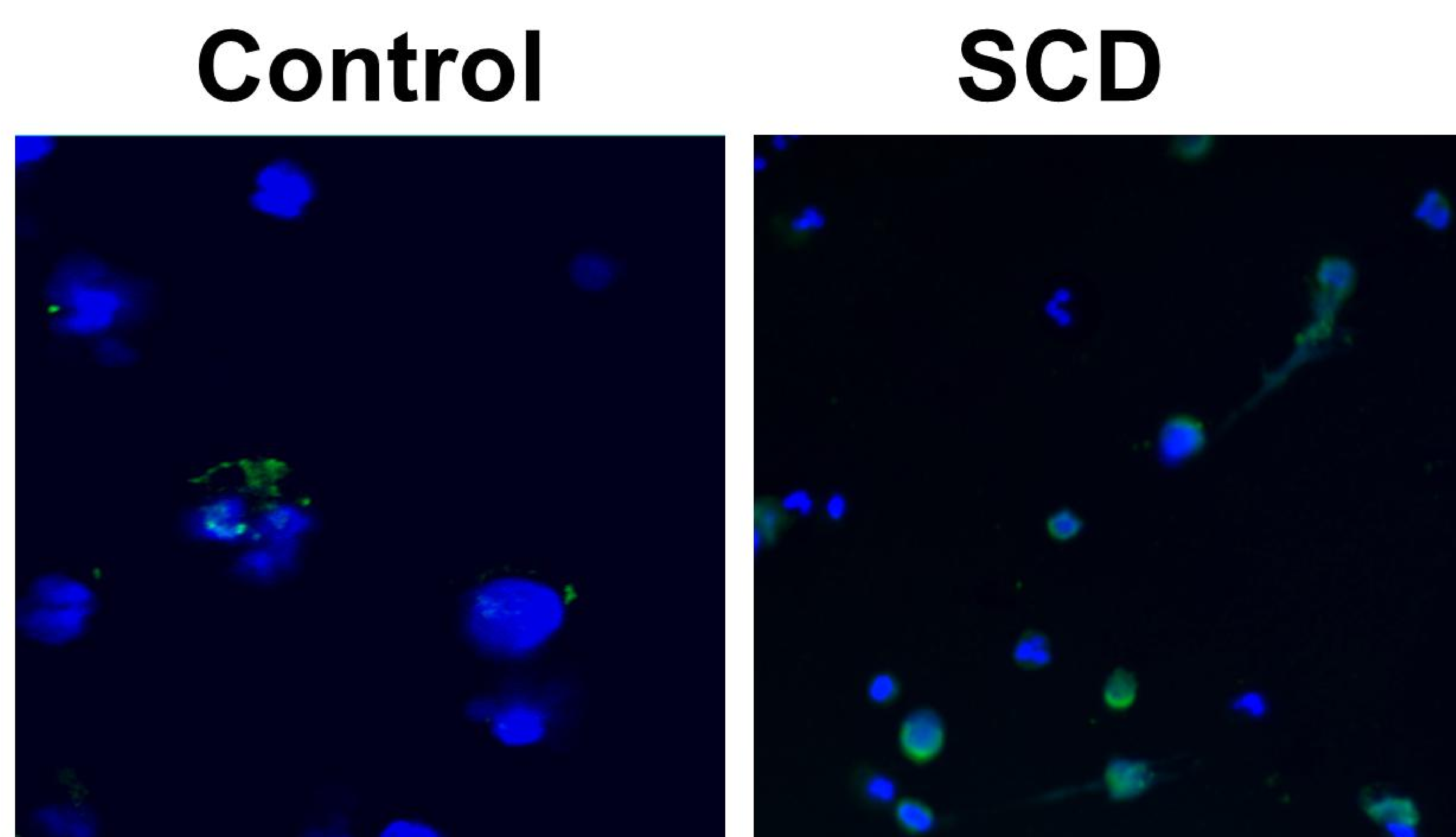


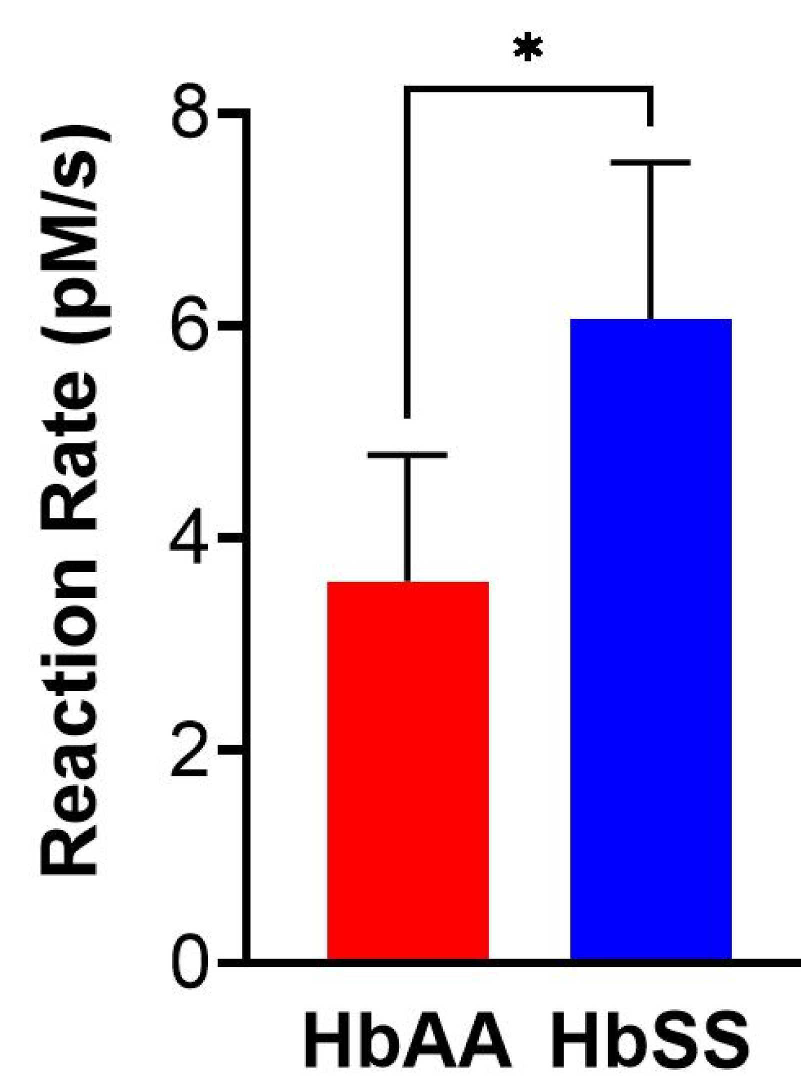


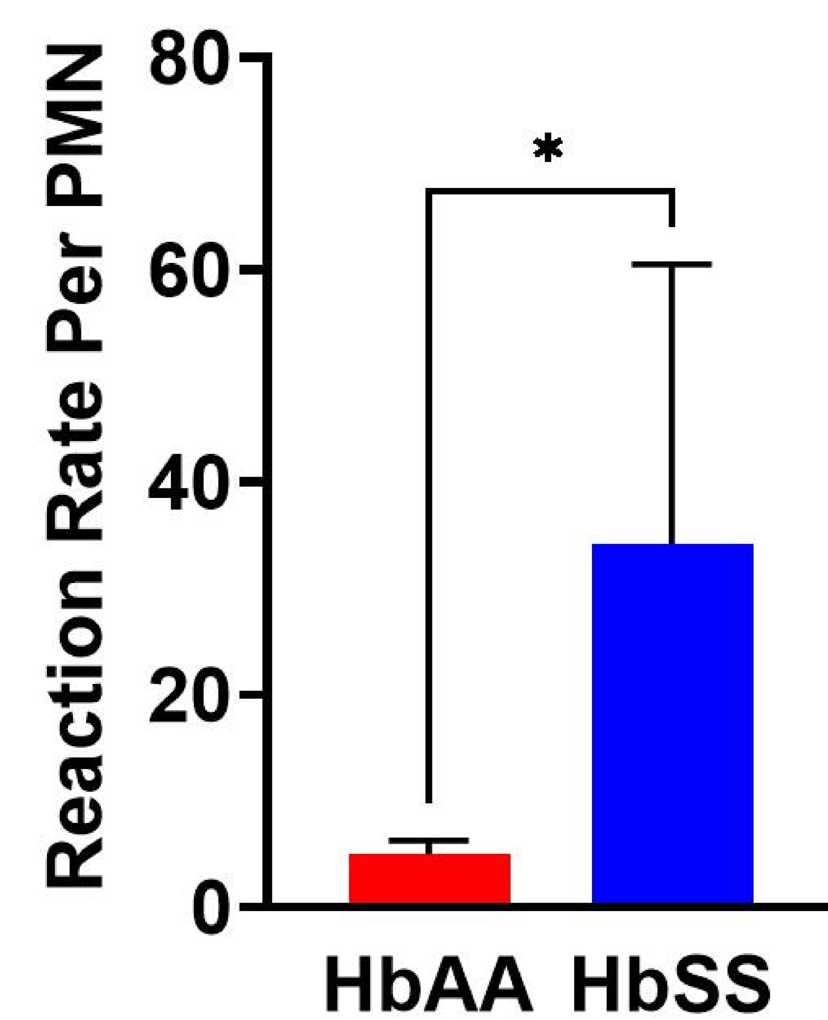
# Figure 6

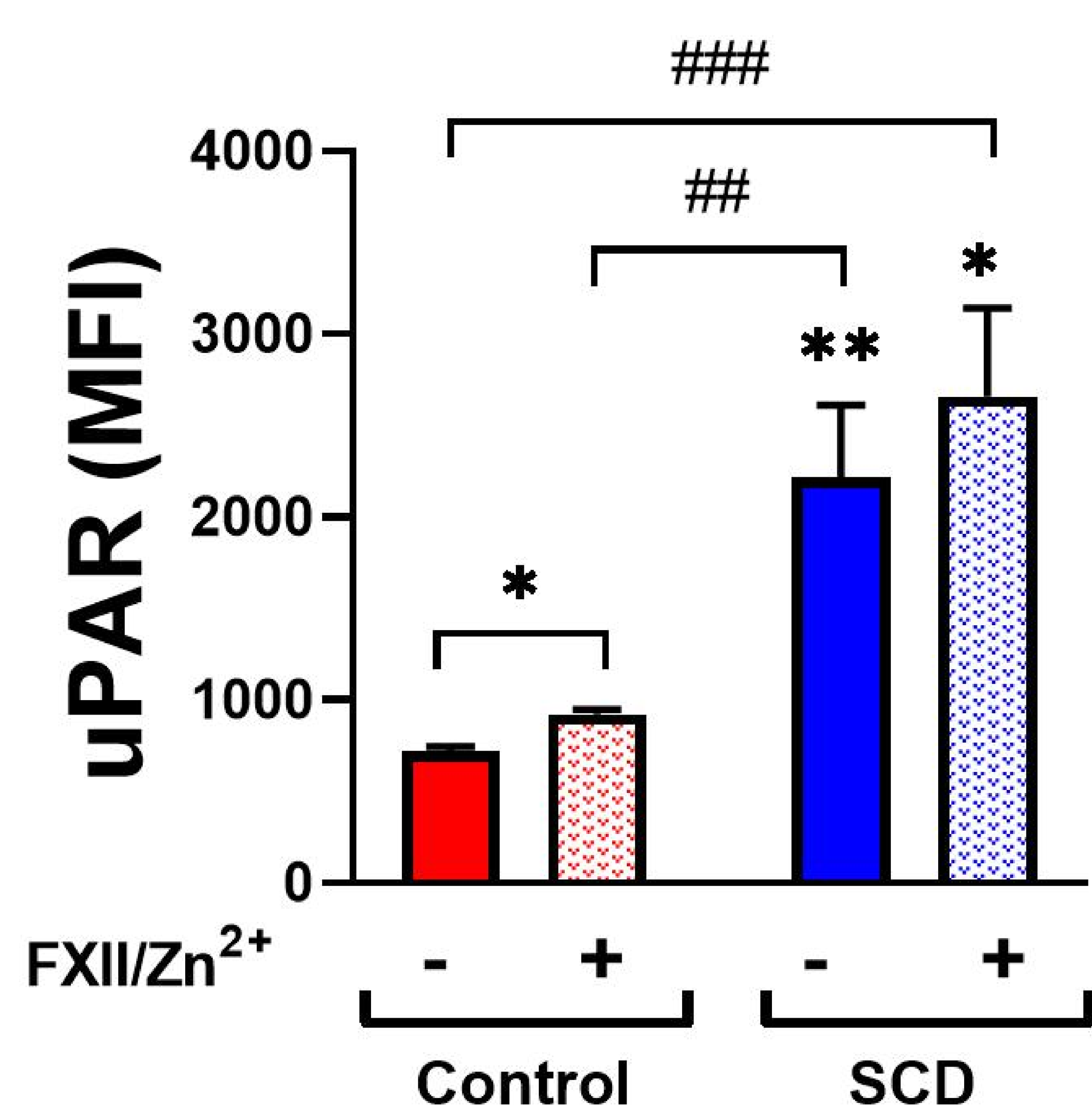


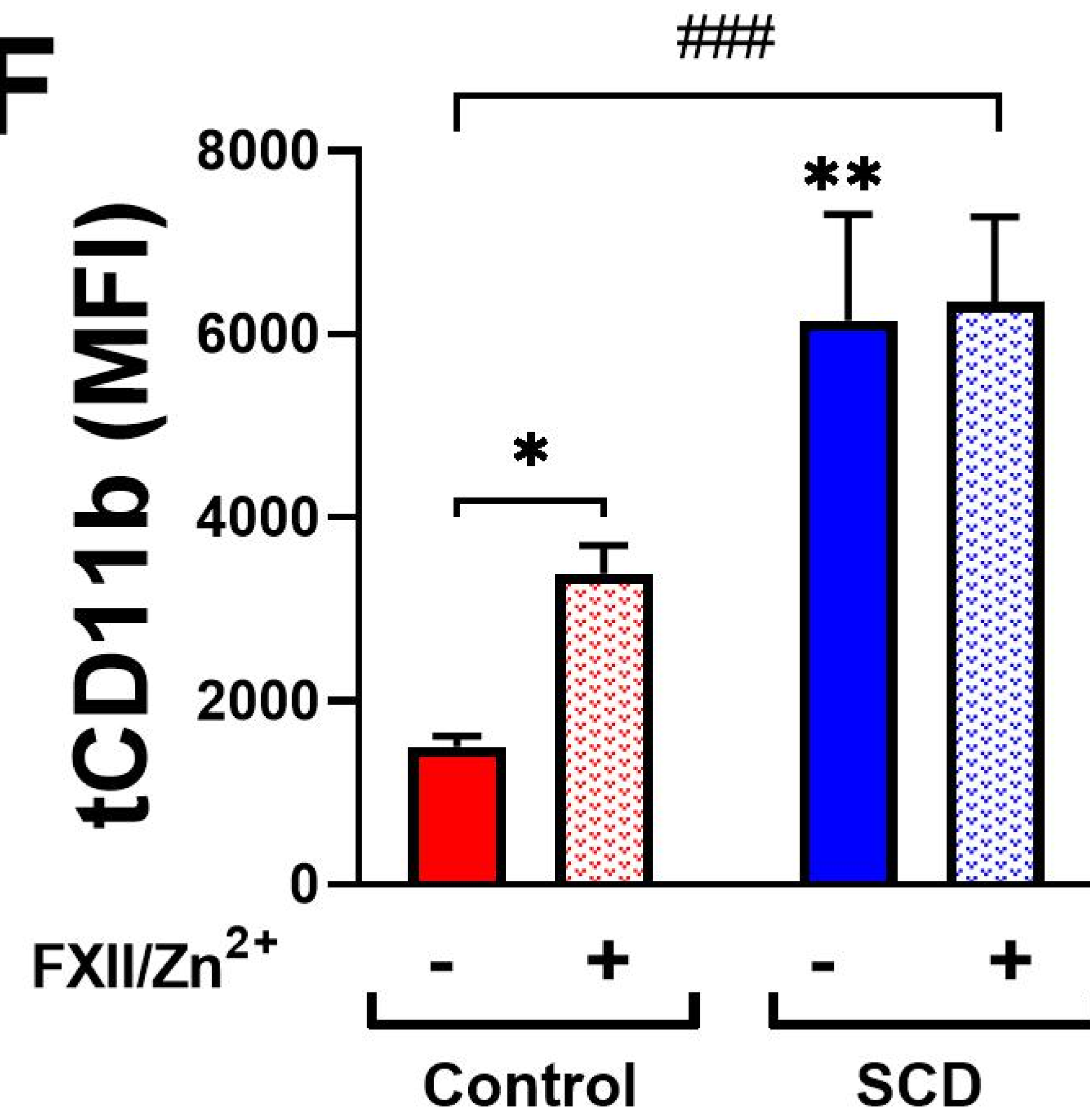

A
OD 405 nm
0.0
0.1
0.2
0.3
0.4
0
5000
10000
Time (sec)
NHP + Neuts + Zn2+
NHP + aPTT-R
F12-/- plasma + Neuts + Zn2+
F12-/- plasma + aPTT-R
F12-/- plasma + Zn2+
**

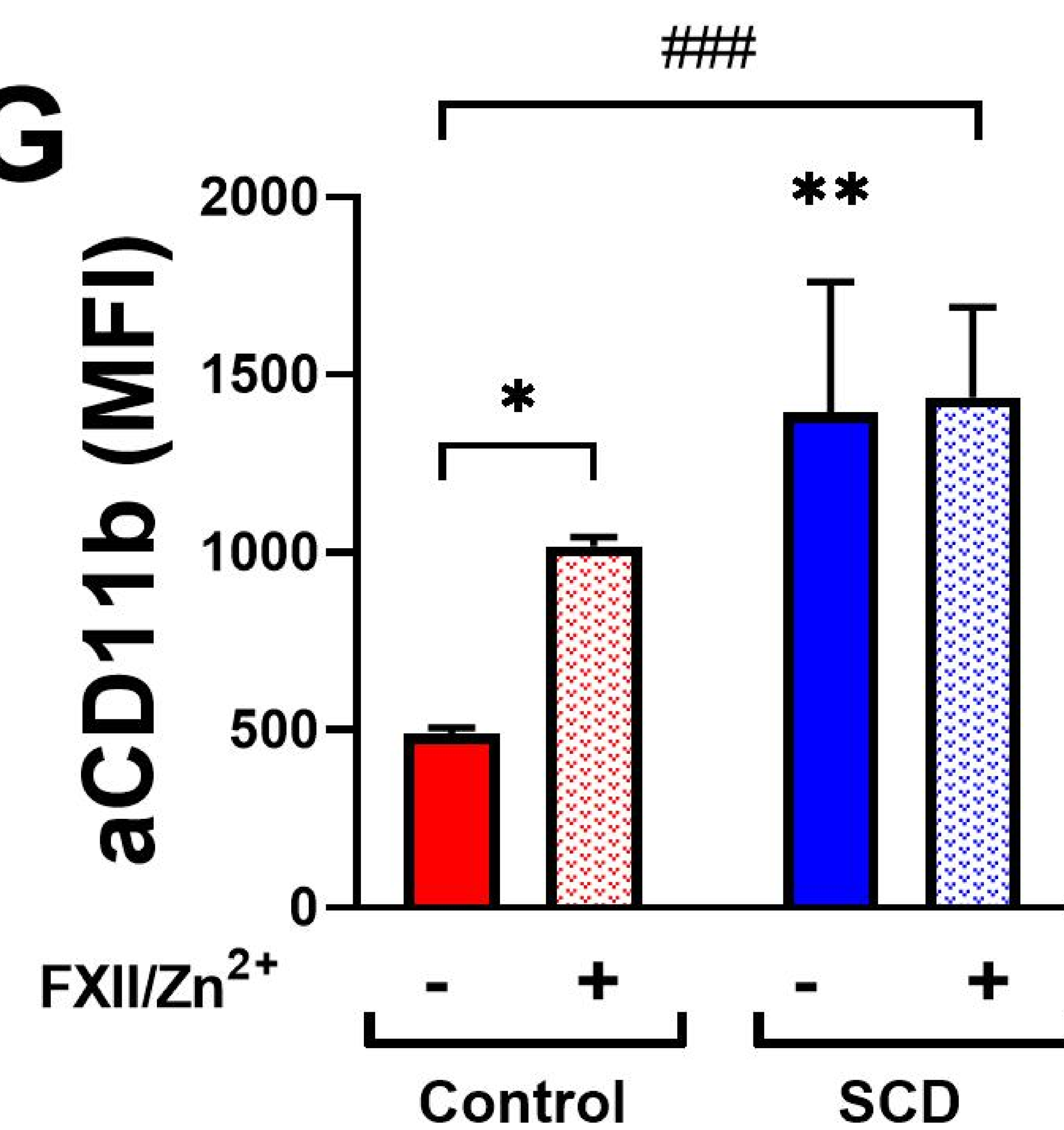

B
Control
SCD


C
Reaction Rate (pM/s)
0
2
4
6
8
*
HbAA
HbSS


D
Reaction Rate Per PMN
0
20
40
60
80
*
HbAA
HbSS


E
uPAR (MFI)
0
1000
2000
3000
4000
###
##
*
**
*
FXII/Zn2+
-
+
-
+
Control
SCD


F
tCD11b (MFI)
0
2000
4000
6000
8000
###
*
**
FXII/Zn2+
-
+
-
+
Control
SCD


G
aCD11b (MFI)
0
500
1000
1500
2000
###
*
**
FXII/Zn2+
-
+
-
+
Control
SCD