Method Article:

An *in vitro* blood flow model to advance the study of platelet adhesion utilizing a damaged endothelium

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Abstract

In vitro flow assays utilizing microfluidic devices are often used to study human platelets as an alternative to the costly animal models of hemostasis and thrombosis that may not accurately represent human platelet behavior *in vivo*. Here, we present a tunable *in vitro* model to study platelet behavior in human whole blood flow that includes both an inflamed, damaged endothelium and exposed extracellular matrix. We demonstrate that the model is adaptable across various anticoagulants, shear rates, and proteins for endothelial cell culture without the need for a complicated, custom-designed device. Further, we verified the ability of this 'damaged endothelium' model as a screening method for potential anti-platelet or anti-thrombotic compounds using a $P2Y_{12}$ receptor antagonist (ticagrelor), a pan-selectin inhibitor (Bimosiamose), and a histamine receptor antagonist (Cimetidine). These compounds significantly decreased platelet adhesion to the damaged endothelium, highlighting that this model can successfully screen anti-platelet compounds that target platelets directly or the endothelium indirectly.

Keywords: platelets; *in vitro* blood flow; endothelium; extracellular matrix proteins; platelet adhesiveness

Introduction

Platelets must maintain a balancing act *in vivo* due to their contributions to both health and disease. A low platelet count or underperform ing platelets can disrupt hemostasis, leading to bleeding risk.[1] In contrast, excess or overactive platelets can contribute to thrombosis,[2] cardiovascular events,[2,3] and inflammation[4] in various diseases. The adhesion of platelets to the site of vascular injury is a crucial early step in both hemostasis and thrombosis, involving multiple points of binding between glycoproteins (GP) on platelets and the damaged vasculature. Thus, there is great interest in understanding dynamic contributions of various receptors and signal molecules that tip the balance from hemostasis to thrombosis.

Murine models are ubiquitously employed to study hemostasis and thrombosis; yet, major differences between animal and human blood physiology and disease development can make translating findings from animal models to humans complicated or inaccurate.[5] Thus, many researchers turn to *in vitro* flow assays with human platelets or whole blood, including the use of microfluidic devices, to study platelet adhesion and behavior contributing to hemostasis and thrombosis. These *in vitro* assays typically fall under one of two categories: platelets under flow conditions adhere to either patterned extracellular matrix (ECM) proteins[6] or an inflamed, intact endothelium that supports platelet adhesion.[7,8] Utilizing ECM proteins or other factors alone allows for tight control of platelet adhesion to one or more specific binding motifs but neglects the essential role the damaged endothelium plays in regulating thrombosis and hemostasis.[9]

A damaged or activated endothelium contributes to the coagulation cascade through upregulation of tissue factor[10] and suppression of thrombomodulin expression,[11] among others. When the endothelium is activated, it releases Weibel-Palade bodies containing ultra-long vWF,[12] which directly bind platelets from flowing

55 blood. Critically, the link between high or dysregulated vWF levels and thrombotic
56 diseases has been well-established using data from both clinical trials and animal
57 models.[13] In addition to vWF multimers, Weibel-Palade bodies also contain P-selectin
58 that can directly bind both platelets and leukocytes upon release, recruiting them to the
59 growing thrombus.[14] Moreover, a prolonged endothelial inflammation leads to
60 expression of other cell adhesion molecules, e.g., ICAM-1 and E-selectin, to further
61 recruit circulating leukocytes,[15] which, when bound to the endothelium, can bind to
62 platelets to further promote thrombosis.[14] Overall, the ability of the endothelium to
63 secrete vWF and other soluble factors while expressing cell adhesion molecules all
64 highlight the importance of including the endothelium in an *in vitro* model designed to
65 study platelet adhesion and resolution.
66 Indeed, endothelialized microfluidic models for studying platelet behavior have
67 gained recent attention in the literature.[7,8] For example, researchers recently developed a
68 novel, biologically patterned 3D hydrogel that supports endothelial cell growth[16] and
69 others established a 3D endothelial cell-pericyte co-culture microfluidic 'blood vessel'
70 *in vitro*.[17] While these new models represent steps towards producing more
71 physiological, 3D blood vessels *in vitro*, they have not yet been utilized to study platelet
72 behavior. Moreover, a commonality between previous endothelialized microfluidic
73 models is the reliance on a completely confluent endothelial cell monolayer, i.e., an
74 absence of a vascular injury, which does not allow for a critical first step in platelet
75 aggregation – platelet adhesion to the underlying extracellular matrix (ECM).[18] One
76 recent work attempts to build in the ECM by examining platelet adhesion to a
77 deliberately non-confluent endothelium.[19] Unfortunately, this approach is likely to yield
78 a wide variation in ECM spacing between endothelial cells, i.e., where platelet adhesion
79 occurs. Such lack of tight control over the ECM spacing likely limits the consistency of

platelet adhesion observed across experiments. Further, the endothelial cells utilized were not stimulated, omitting the endothelium's role in platelet adhesion through the release of vWF and expression of cell adhesion molecules.

Overall, there is an unmet need for an improved and tightly controlled *in vitro* model that incorporates all components of a damaged vasculature *in vivo*. Here, we describe a 'damaged endothelium model' that combines a functional inflamed endothelium with a pronounced and consistent exposed ECM, i.e., injury area. This model is straightforward, highly tunable, and allows for platelet adhesion in whole blood, both to the underlying ECM and the adjacent injured endothelium, creating an opportunity to elucidate the complex dynamics of platelet functions in human health and disease. Further, we demonstrate that this model successfully screens potential anti-inflammatory and anti-thrombotic molecules that target either platelets or the endothelium.

Methods

Materials

Anti-CD41/61 PE was purchased from Biolegend (359806), and anti-vWF FITC was purchased from Novus Biologicals (NB120-8822). Type I rat tail collagen was purchased from Corning (354236). Adenosine diphosphate (ADP) was purchased from MP Biomedicals (100056). Histamine was purchased from Acros Organics (411710050). #1 ½ 30 mm round glass coverslips were purchased from Warner Instruments (64-1499). The parallel plate flow chamber (PPFC) and silicone gaskets were purchased from Glycotech (31-001). Cimetidine was purchased from Alfa Aesar (J62825). Bimosiamose was purchased from MedChemExpress (HY-106139). Ticagrelor was purchased from Cayman Chemical Company (15425). All other reagents and chemicals were purchased from Sigma Aldrich or Fisher Scientific.

Blood draw and preparation for flow

Human whole blood was obtained via venipuncture and drawn into acid citrate dextrose (ACD), 3.2% sodium citrate, or heparin. Informed, written consent was obtained by all donors according to a protocol approved by the University of Michigan Internal Review Board (IRB-MED). Unless otherwise stated, the hematocrit of the anticoagulated blood was adjusted to 40% using packed RBCs or extra plasma obtained from the same donor to normalize for the known impact of hematocrit on platelet accumulation *in vivo* and *in vitro*.[20,21]

To isolate platelets, platelet-rich plasma (PRP) was first obtained through whole blood centrifugation at 200g for 20 minutes. An additional 10% ACD and 1 μL apyrase per 2.5 mL PRP was added to prevent platelet activation during a 10 minute, 1000g centrifugation. Isolated platelets were resuspended in a ‘flow buffer’ solution of

phosphate buffered saline containing calcium and magnesium ions (DPBS $^{+/+}$; pH 7.4; Thermo Fisher cat# 14080-055) with 1% BSA added. Packed RBCs were obtained through a centrifugation of the whole blood at 2250g for 10 minutes and washed with PBS – "washed RBCs". Isolated platelets were combined with washed RBCs at a 40% hematocrit for experiments utilizing platelets with RBCs in flow buffer.

Isolated platelets or platelets in whole blood were stained with 5 μL/mL anti-CD41/61 PE for one hour immediately prior to blood flow experiments and where stated, activated with 20 μM ADP for one hour to induce platelet activation and aggregation similar to prior publications.[22–25]

Platelet adhesion to damaged monolayer under flow

Umbilical cords (Mott Children's Hospital, Ann Arbor) were acquired under an IRB-MED approved human transfer protocol. Human umbilical vein endothelial cells (HUVEC) were obtained using a modified collagenase perfusion technique,[26] and HUVEC from three or more umbilical cords were pooled per isolation. For flow assays, HUVEC were cultured at 37°C and 5% CO_2 until confluent, then trypsinized and seeded onto glutaraldehyde-crosslinked gelatin coverslips at least 36 hours before use. Glutaraldehyde-crosslinked gelatin coverslips were achieved via coating glass coverslips with 1% w/v gelatin crosslinked with 0.5% glutaraldehyde, which was quenched with 0.1 M glycine and washed with PBS.[27]

In flow experiments utilizing an endothelium, HUVEC were activated for 2 minutes with 100 μM histamine in media. After 2 minutes, a scalpel was used to scratch the coverslip with parallel lines to induce an acute vascular injury and expose the underlying gelatin. The coverslip was attached to a PPFC fitted with a silicone gasket (2 cm x 0.25 cm x 127 μm in height) via vacuum pump and the outlet was attached to a

syringe pump. The blood or blood components were perfused over the damaged HUVEC perpendicular to the scalpel scores for 5 minutes at a fixed wall shear rate described by Equation 1:

$$\gamma_w = \frac{6Q}{h^2 w} \quad (1)$$

Where Q is the volumetric flow rate (mL/s), h is the channel height (0.0127 cm), and w is the channel width (0.25 cm). After 5 minutes of laminar flow, the channel was rinsed with flow buffer and 10 fluorescent images of platelets bound along the scalpel scores were taken.

In experiments testing different agonists to induce vWF secretion, HUVEC on coverslips were activated by calcium ionophore (10 μM) for 10 minutes, or histamine (100 μM) for 2 minutes with or without mechanical injury with a scalpel before being attached to a PPFC. To visualize vWF multimer strands, 5 μg/mL anti-vWF-FITC was flowed over the endothelium for 5 minutes and rinsed with flow buffer for 5 minutes. 10 fluorescent images of vWF multimers around the scalpel scores were taken.

Where stated, whole blood was treated with 50 μM Bimosiamose or with 10 μM ticagrelor in other experiments for 1 hour prior to use in flow experiments. In other experiments, HUVEC were pretreated with 1 or 50 μM Cimetidine for 5 hours prior to use in flow experiments.

Platelet adhesion to collagen and gelatin-coated coverslips under flow

Glass coverslips were cleaned with Piranha solution (3:1 concentrated sulfuric acid: 30% hydrogen peroxide) and silanated with 2% 3-(trimethoxysilyl)propyl methacrylate (MPS) by volume in 95% ethanol for 30 minutes. The cleaned coverslips were coated with 1 mg/mL collagen or gelatin for 2 hours at room temperature; the entire coverslip

surface was coated with collagen or gelatin. After the 2-hour incubation, the coverslips were rinsed with PBS, then attached to the PFFC and perfused with blood as described above.

Statistics

Ten fluorescent images of adherent platelets were acquired for each experiment, with n ≥ 3 for each data point presented. ImageJ was utilized to determine the % area of the 'damaged endothelium' covered by platelets, which combined adhesion to the intact endothelium and the exposed extracellular matrix. In short, the background fluorescence for each image was subtracted and threshold set. ImageJ then calculated the surface area coverage of each image using the threshold. Data are plotted with standard error bars. Student's unpaired t-tests and one- and two-way ANOVA in GraphPad Prism software were used to analyze statistical differences between samples. Asterisks indicate p values of * < 0.05, ** < 0.01, *** < 0.001, and **** < 0.0001. A lack of asterisks indicates a lack of significance.

Results and Discussion

Development of an in vitro flow model utilizing an endothelium

Our first goal in developing an *in vitro* model to better represent platelet adhesion *in vivo* was to determine the agonists that would result in a consistent release of vWF multimers from a damaged endothelium. We subjected a monolayer of human umbilical vein endothelial cells (HUVECs) to either a scalpel score mechanical injury, chemical stimulation (histamine or calcium ionophore), or a combination of mechanical injury and chemical stimulation. Chemical stimulation via histamine alone increases vWF secretion by HUVEC (Fig. 1A), which is further enhanced with the addition of a mechanical injury. Mechanical injury induces platelet adhesion in two separate ways; it increases vWF secretion by HUVEC, a primary first step in hemostasis (Fig. 1A), while also exposing the underlying ECM proteins. Manual damage via scalpel scores resulted in a 'gap' in the confluent HUVEC monolayer of approximately 157 ± 3 μm; a representative brightfield image of the damaged HUVEC, with an EC-free gap visible, overlaid with fluorescent imaging of fluorescently-labeled platelets is shown in Supplementary Figure 1. Figure 1B demonstrates that the 'damaged endothelium' model facilitates the adhesion of ADP-activated human platelets to both tethered vWF multimers and the underlying ECM proteins. Overall, the injury condition in which both histamine stimulation and mechanical injury are present resulted in the largest increase in vWF secretion compared to the other conditions (Fig. 1A) and so was utilized for all other assays.

Previous works examining whole blood platelet adhesion in fluidic devices observed differing platelet adhesion behavior across varying shear rates. These discrepancies may be due to studies utilizing different flow channels, microfluidic surface coatings for platelet adhesion, and anticoagulants. As such, we examined the

207 impact of shear on platelet adhesion after establishing the damaged endothelium flow
208 model. Blood flow is represented here using shear rates as opposed to volumetric flow
209 rates due to the latter's known direct impact on convective transport of soluble factors
210 as well as cell-surface interactions.[28] We chose a range of shear rates representative of
211 blood flow in different vessels *in vivo*. Specifically, we utilized 100s^{-1} (representing
212 blood flow in veins), 500s^{-1} (representing large arteries), and 1000s^{-1} (representing
213 arterioles and capillaries).[7] Activation of platelets in whole blood with 20 μM ADP led
214 to increased platelet adhesion across all shear rates tested (100, 500, and 1000s^{-1}; Fig.
215 1C) in comparison to flow experiments utilizing resting platelets. This increase in
216 adhesion highlights the known ability of ADP to stimulate platelets, an essential step in
217 platelet adhesion at shear rates <1000s^{-1}.[29] Further, activated platelet adhesion at low
218 shear rates (100s^{-1}) was higher than that at higher shear rates (500 and 1000s^{-1}). This
219 observation aligns with other works that observed maximal platelet adhesion at low
220 shear rates in whole blood – an impact the authors hypothesized was due to the lag time
221 in plasma protein adsorption onto the microfluidic surface at high shear rates.[30]
222 To further explore the binding mechanism and the role of different components
223 of blood in this model, we examined activated platelet adhesion in buffer alone or buffer
224 containing human RBCs at 40% hematocrit. Platelet adhesion in buffer flow was
225 greatest at high shear rates (1000s^{-1}; Fig. 1D). In the absence of plasma, high shear
226 allows for ultra-long vWF multimers released from activated endothelial cells to
227 elongate and expose adhesion sites without cleavage by plasma ADAMTS-13,
228 enhancing platelet binding.[31,32] Previous works determined that HUVEC in flow do not
229 release sufficient quantities of ADAMTS-13 to cleave ultra-long vWF multimers
230 tethered to their surface without the presence of healthy plasma or purified ADAMTS-
231 13.[12,31,33] Therefore, the high level of platelet adhesion in buffer at high shear rates

suggests that vWF plays an important role in this model. As expected, the addition of RBCs to platelets in buffer increased platelet flow adhesion at all shear rates (Fig. 1D), highlighting the importance of RBCs to facilitate margination of platelets towards the vascular wall and increasing binding to the damaged endothelium.[34] Representative images of activated platelet adhesion at $1000s^{-1}$ in whole blood, isolated in buffer, and isolated in buffer with the addition of RBCs are shown in Fig. 1E. For all conditions, platelet adhesion predominates at and around the scalpel score and adjacent endothelium area, i.e., the injury region, including adhesion to long vWF strings attached to the endothelium at high shear rates.

We confirmed that our model supports platelet adhesion across various anticoagulants as reported in Supplementary Figure 2A for adhesion at $1000s^{-1}$ of platelets in blood anticoagulated with ACD, heparin, and sodium citrate. We find the highest platelet adhesion occurred when sodium citrate (3.2%) is used as the anticoagulant, leading to a significant increase in platelet coverage compared to ACD-anticoagulated blood. Adherent platelets are primarily localized to the area with and near the scalp score for all anticoagulant types, as illustrated in Supplementary Figure 2B. Importantly, the presence of the endothelium in our model does not preclude its utility for testing anti-platelet compounds in blood flow. Specifically, we find that addition of ticagrelor, a $P2Y_{12}$ receptor binding antagonist, to blood led to a significant decrease in activated platelet adhesion relative to adhesion in untreated blood, which was consistent across both ACD and heparin anticoagulated whole blood (Supplementary Figure 3).

Platelet adhesion on damaged endothelium is similar to controls utilizing collagen and gelatin

A common method of examining platelet behavior in flow involves platelet adhesion to

patterned ECM proteins, especially collagen. To determine how platelet adhesion using our 'damaged endothelium' model compared to the standard, we examined platelet adhesion in whole blood flow to the damaged endothelium and collagen alone at 1 mg/mL.[30,35,36] We included another control of platelet adhesion to gelatin alone at the same concentration as collagen because crosslinked gelatin is used in our damaged endothelium model. Fig. 2 shows that when platelets in blood are activated, they adhere with the same frequency to the damaged HUVEC model as collagen alone; there was no difference between the magnitude of platelet adhesion to the damaged HUVEC model and collagen at any of the shear rates examined. Qualitatively, platelet adhesion to collagen was distributed randomly in small aggregates on the collagen surface. Conversely, platelet adhesion to the damaged endothelium was primarily concentrated on the exposed ECM between endothelial cells for all shear rates examined and on the long, vWF strands bound to the endothelium for higher shear rates examined. Adhesion to collagen alone follows the same trend of higher adhesion under low shear rate conditions ($100s^{-1}$) than higher shear conditions (500, $1000s^{-1}$). Platelet adhesion to gelatin followed similar trends, but adhesion at $100s^{-1}$ was significantly lower than platelet adhesion to collagen at the same shear rate, suggesting that gelatin alone at 1 mg/mL does not facilitate platelet adhesion as well as collagen. Conversely, when platelets were resting, there were no significant differences between adhesion to the damaged HUVEC, collagen, or gelatin at any of the shear rates tested (Supplementary Figure 4).

Treatment of whole blood with pan-selectin inhibitor decreases platelet adhesion to damaged endothelium

One benefit of utilizing the damaged endothelium model is the inclusion of multiple binding motifs for platelets, both the damaged endothelium itself and the underlying

extracellular matrix proteins. These multiple binding motifs make this model ideal for screening potential drug compounds that impact platelet adhesion in human whole blood flow. One compound of interest is Bimosiamose, a siayl Lewis^x mimetic and pan-selectin inhibitor.[37] Despite the ability of this antagonistic compound to target E-selectin, P-selectin, and L-selectin, Bimosiamose has primarily been studied for its ability to inhibit leukocyte binding and recruitment *in vitro*,[37] *in vivo*,[38,39] and in clinical trials.[40,41]

To determine if Bimosiamose's ability to inhibit selectins could impact platelet adhesion in the damaged endothelium model, we pretreated whole blood with 50 μM Bimosiamose for 1 hour prior to flow experiments. Like all previous experiments, we stimulated the endothelium with histamine with mechanical injury via scalpel, which does not facilitate the adhesion of leukocytes in blood flow; these conditions allow us to examine the impact of Bimosiamose on platelet adhesion alone. We treated blood with ADP to induce platelet activation at the same time as Bimosiamose. The Bimosiamose treatment led to a 40% reduction in activated platelet adhesion to the damaged endothelium model relative to its untreated counterparts (Fig. 3). In comparison, whole blood pretreated with Bimosiamose and flowed over collagen only coverslips did not impact platelet adhesion (Fig. 3). We attribute Bimosiamose's effectiveness in decreasing platelet adhesion on the damaged endothelium to the increased platelet binding motifs present that are not present on the collagen substrate. Specifically, we hypothesize that Bimosiamose in whole blood blocked the GPIb-IX-V receptor complex on platelets that binds to P-selectin on the endothelium.[18] The lack of P-selectin on collagen alone renders this blocking impact inconsequential. Further, excess Bimosiamose in blood may also have blocked P-selectin on the endothelial cell surface once blood flow through the chamber began. The capability of Bimosiamose to block

leukocyte adhesion via blocking of selectins has been well established.[37–41] However, we demonstrated that Bimosiamose further has the ability to directly modulate platelet behavior in the complete absence of leukocyte adhesion.

To confirm that the impact Bimosiamose has on ACD-anticoagulated platelets is translatable to other anticoagulants, we examined the impact of Bimosiamose on activated platelet adhesion in heparinized blood. Bimosiamose led to a significant decrease in platelet adhesion to the damaged endothelium using heparin as an anticoagulant (Supplementary Figure 5); further, there was no significant difference between the change in ACD-anticoagulated platelet adhesion and heparin-anticoagulated platelet adhesion when treated with Bimosiamose. We are thus able to confirm that results obtained using ACD-anticoagulated blood translate to other commonly used anticoagulants.

Treatment of damaged endothelium with Cimetidine decreases platelet adhesion

The inclusion of the endothelium in this model also allows for the testing of compounds that have a direct therapeutic impact on the endothelium instead of on platelets themselves. One potential candidate to screen for impact on platelet adhesion is Cimetidine, a histamine receptor antagonist marketed under the brand name 'Tagamet' to treat heartburn and peptic ulcers. More recently, Cimetidine has gained interest for its ability to inhibit the expression of selectins by endothelial cells. In particular, Cimetidine was found to decrease neutrophil adhesion to endothelial cells activated by high concentrations of glucose, decreasing the expression of cellular adhesion molecules P-selectin and ICAM-1 on the surface of HUVEC.[42] Further, Cimetidine has been repurposed in combination with other oncological therapeutics and pharmaceuticals in a variety of clinical trials for different cancers to capitalize on

several qualities of Cimetidine, including its ability to inhibit cancer cell adhesion to endothelial cells.[43]

To take advantage of Cimetidine's ability to decrease cellular adhesion molecule expression as well as its histamine receptor antagonism, we treated HUVEC with low (1 μM) or high (50 μM) concentrations of Cimetidine 5 hours prior to use in blood flow experiments. Similar to all previous experiments, we treated the endothelium only with histamine and mechanical injury to determine if Cimetidine has an effect on platelet adhesion in the absence of adherent leukocytes. Due to treating the endothelium itself with Cimetidine instead of whole blood, there was no corresponding collagen-only control for this experiment. Treatment with either low or high concentrations of Cimetidine significantly decreased activated platelet adhesion to approximately 67% of untreated HUVEC, demonstrating that even a low dosage of the compound was enough to significantly decrease platelet adhesion in this model. This knockdown in platelet adhesion occurred even in the absence of leukocyte adhesion, highlighting Cimetidine's effectiveness at modulating platelet adhesion behavior. Further, this experiment demonstrates that this damaged endothelium model can be used to screen potential therapeutics for impact on platelet adhesion, even if the target of the therapeutic is the endothelium instead of platelets themselves.

Conclusions

In vitro microfluidic models are an excellent way to study human platelet behavior in a laboratory setting to better understand hemostasis and thrombosis. However, most models utilize simple protein substrates patterned onto glass or plastic to study platelet behavior, which neglects the role of the endothelium in clotting and clot resolution. Alternatively, other models utilize confluent endothelial monolayers that neglect

platelet adhesion to exposed extracellular matrix proteins. Here, we described a novel, *in vitro* flow model utilizing a damaged endothelium that allows for the study of platelet binding to both the endothelium and underlying extracellular matrix proteins without requiring the design and production of custom microfluidic devices. Further, the model is tunable depending on the specific needs of the user. Platelet adhesion to the damaged endothelium occurs at various shear rates and across different anticoagulants. As a proof of concept, two potential therapeutics were screened for their ability to impact platelet adhesion; the pan-selectin inhibitor Bimosiamose significantly decreased activated platelet adhesion to the damaged endothelium, as did Cimetidine. The screening of these two compounds demonstrates the ability of this damaged endothelium model as an initial screening method of potential anti-thrombotic or anti-platelet compounds that can impact either platelets or the endothelium itself and represents a novel method to study future platelet-cell and platelet-drug interactions.

A limitation of this work is that only one specific type of protein was utilized for HUVEC culture (gelatin). However, since glutaraldehyde crosslinks proteins by reacting with their free primary amines[44], proteins of interest other than gelatin can be utilized for HUVEC culture with this method. Future work utilizing this adaptive model can explore how tuning the crosslinked protein or proteins can alter platelet behavior, in addition to elucidating the behavior of endothelial cells isolated from different vascular beds with shear rates corresponding to those vessels (i.e., studying saphenous vein endothelial cells at ~$100s^{-1}$ in contrast to coronary artery endothelial cells at ~$400s^{-1}$,[7]).

Availability of Data:

All data generated or analyzed during this study are included in this published article and supplementary material. The raw datasets in this current study are available from the corresponding author on reasonable request. Identity of study participants will not be shared.

Competing Interests:

The authors declare no competing interests.

Funding: This work was in part supported by an NSF Research Grant (CBET1854726) and Falk Medical Research Trust Catalyst Award to O.E.A. A.L.B was supported by NIH Cellular Biotechnology Training Grant.

Author Contributions: A.L.B and O.E-A. conceived and designed the experimental protocols. A.L.B. prepared all blood samples for measurement and analyzed all the collected data. A.L.B. and O. E-A. wrote the manuscript. All authors read and approved the final manuscript.

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569 **Figure Legend**

570 Figure 1: Development of in vitro flow model utilizing an endothelium. (A) Change in
571 median fluorescent intensity (MFI) of von Willebrand factor secreted by cultured
572 HUVEC in comparison to unstimulated controls, (B) Representative fluorescence image
573 of endothelium activated with histamine and physically disrupted with scalpel (scalpel
574 mark shown in white), allowing for adhesion of activated, isolated platelets in buffer
575 flow containing RBCs. Green represents von Willebrand factor multimers (labelled with
576 anti-vWF FITC) and red represents adherent platelets (labelled with anti-CD41/61 PE),
577 scale bar 200 μm., (C) Platelet coverage (as % of total surface area of photo) after 5
578 minutes of laminar flow in whole blood at a 40% hematocrit and $1000s^{-1}$ on histamine-
579 stimulated and mechanically disrupted HUVEC. Platelets were either quiet or activated
580 with 20 μM ADP, (D). Platelet coverage (%) on damaged endothelium of activated
581 platelets after 5 minutes of laminar flow. Activated platelets were anticoagulated with
582 ACD at varying shear rates either in whole blood at 40% hematocrit, isolated in flow
583 buffer, or in flow buffer with RBCs at 40% hematocrit, (E) Representative image of
584 adherent, activated platelets (red) on damaged endothelium after 5 minutes of laminar
585 flow at $1000s^{-1}$ in whole blood at 40% hematocrit, isolated platelets in flow buffer, or
586 isolated platelets and RBCs at 40% hematocrit in flow buffer, scale bar 100 μm.
587 Statistical analyses were performed using one-way ANOVA (A) or two-way ANOVA
588 (C, D) with Tukey's multiple comparisons test, where (*) indicates $p<0.05$, (**)
589 indicates $p<0.01$, (***) indicates $p<0.001$, and (****) indicates $p<0.0001$.

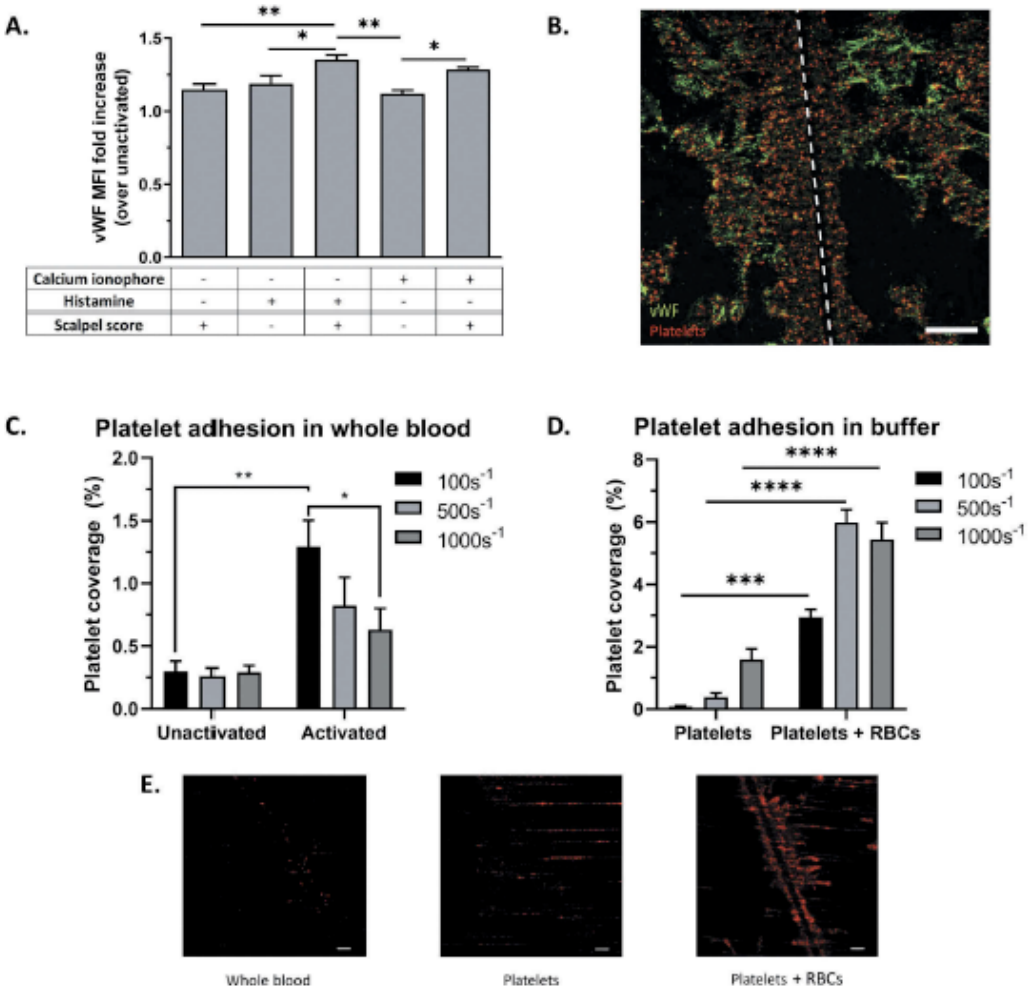
590 Figure 2: Platelet adhesion on damaged endothelium in comparison to controls ECM
591 proteins. Platelet coverage (as % of total surface area of photo) on damaged
592 endothelium, collagen (1 mg/mL), or gelatin (1 mg/mL) of activated platelets in whole
593 blood at 40% hematocrit after 5 minutes of laminar flow. Statistical analyses were
594 performed using two-way ANOVA with Tukey's multiple comparisons test, where (*)
595 indicates $p<0.05$.

596 Figure 3: Pan-selectin inhibitor decreases platelet adhesion to HUVEC. Activated
597 platelet adhesion fold change on damaged HUVEC or collagen alone of whole blood at
598 40% hematocrit at $1000s^{-1}$ for 5 minutes with the addition of 50 μM Bimosiamose to
599 whole blood 1 hour before flow experiments. Statistical analyses were performed using
600 Student's unpaired t-test where (***) indicates $p<0.001$.

Figure 4: Platelet adhesion to damaged HUVEC decreases with Cimetidine pretreatment of HUVEC. Activated platelet adhesion fold change on damaged HUVEC of whole blood at 40% hematocrit at $1000s^{-1}$ for 5 minutes with the addition of 1 μM (low) or 50 μM (high) Cimetidine to HUVEC 5 hours prior to blood flow experiments. Statistical analyses were performed using one-way ANOVA with Dunnett's multiple comparisons test where (*) indicates $p < 0.05$.

610 FIGURES

611 Figure 1



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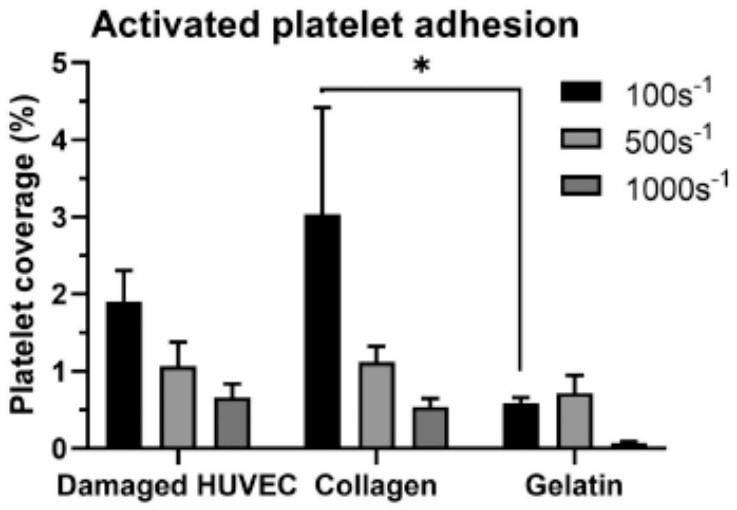
613 Figure 2



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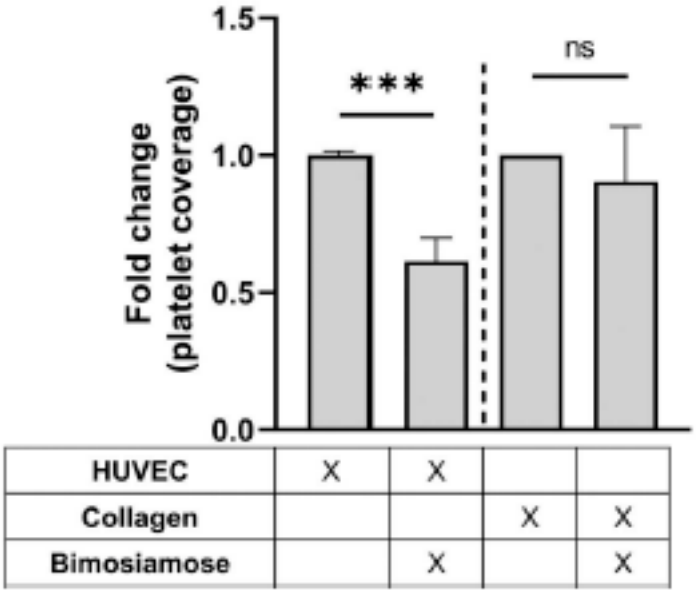
615 Figure 3



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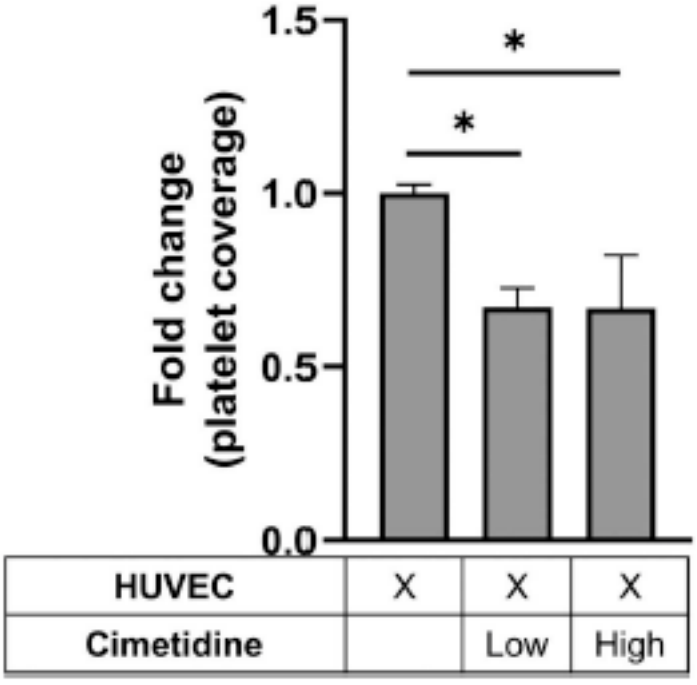

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Supplemental File

An *in vitro* blood flow model to advance the study of platelet adhesion utilizing a damaged endothelium

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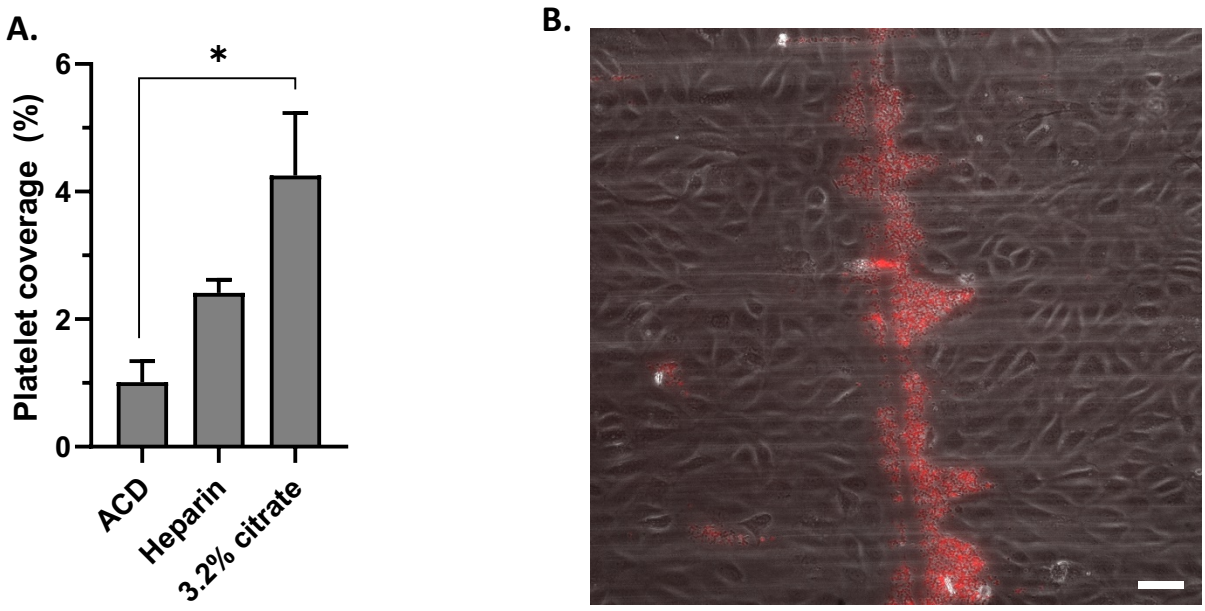

Fig. S1: Platelet adhesion on 'damaged' HUVEC with different anticoagulants. (A) Platelet adhesion (% of total surface area) of activated platelets after 5 minutes of laminar flow in whole blood at 40% hematocrit and 1000s^{-1} using different anticoagulants, (B) Platelet adhesion in heparinized blood of activated, anti-CD41/61 PE-stained platelets (red) overlaid on top of a HUVEC monolayer activated with histamine and manually damaged with scalpel. Scale bar, 100 µm. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test, where (*) indicates $p<0.05$.

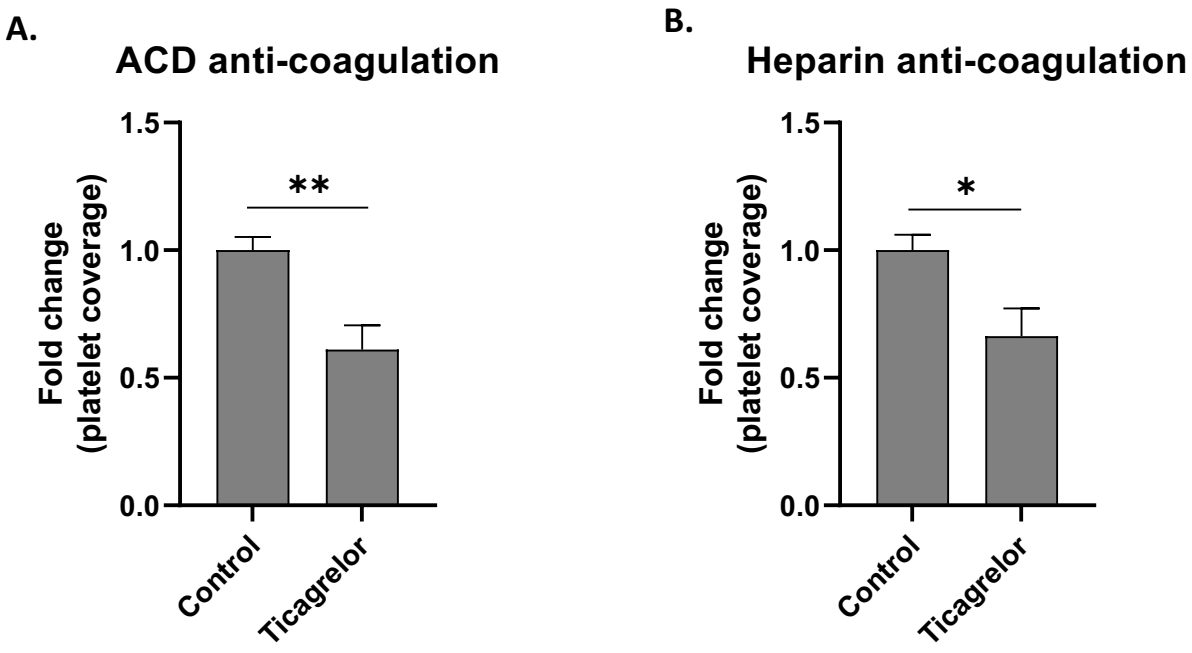

Fig. S2: Impact of Ticagrelor on platelet adhesion. Platelet adhesion (% of total surface area) of activated platelets after 1 hour treatment with 10 μM ticagrelor using (A) ACD or, (B) heparin-anticoagulated whole blood. Blood was perfused for 5 minutes of laminar flow at 40% hematocrit and $1000s^{-1}$ over an activated, damaged HUVEC monolayer. Statistical analysis was performed using an unpaired t-test, where (*) indicates $p<0.05$ and (**) indicates $p<0.01$.

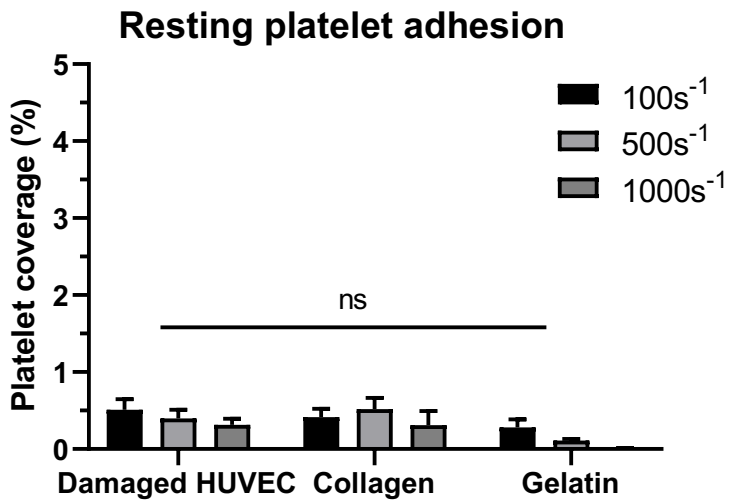

Fig. S3: Resting platelet adhesion on damaged endothelium compared to control utilizing collagen and gelatin. Platelet coverage (as % of total surface area of photo) on damaged endothelium, collagen (1 mg/mL), or gelatin (1 mg/mL) of resting platelets in whole blood at 40% hematocrit after 5 minutes of laminar flow. Statistical analyses were performed using two-way ANOVA with Tukey's multiple comparisons test.

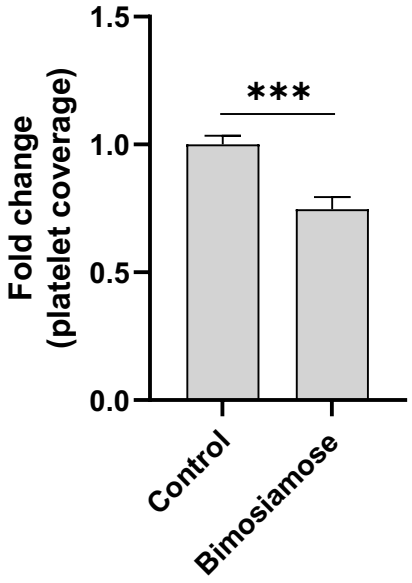

Fig. S4: Impact of Bimosiamose on heparinized platelet adhesion. Platelet adhesion (% of total surface area) of activated platelets after 1 hour treatment with 50 μM Bimosiamose using heparin-anticoagulated whole blood. Blood was perfused for 5 minutes of laminar flow at 40% hematocrit and $1000s^{-1}$ over an activated, damaged HUVEC monolayer. Statistical analysis was performed using an unpaired t-test, where (***) indicates $p<0.001$.