

Fibrin exposure triggers α IIb β 3-independent platelet aggregate formation, ADAM10 activity and glycoprotein VI shedding in a charge-dependent manner

SJ Montague,¹ SM Hicks,¹ C S-M Lee,¹ LA Coupland,¹ CR Parish,¹ WM Lee,^{1,2} RK Andrews,^{1,3} EE Gardiner.¹

¹ACRF Department of Cancer Biology and Therapeutics, The John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia

²Research School of Electrical, Energy and Materials Engineering, College of Engineering and Computer Science, The Australian National University, Canberra, ACT, Australia

³Australian Centre for Blood Diseases, Monash University, Melbourne, VIC, Australia

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Corresponding author:

Professor Elizabeth E. Gardiner,
ACRF Department of Cancer Biology and Therapeutics
John Curtin School of Medical Research
The Australian National University,
131 Garran Rd,

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Canberra, ACT, 2601, Australia

Email: elizabeth.gardiner@anu.edu.au

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Abstract

Collagen and fibrin engagement and activation of glycoprotein (GP) VI induces proteolytic cleavage of the GPVI ectodomain generating shed soluble GPVI (sGPVI). Collagen-mediated GPVI shedding requires intracellular signalling to release the sGPVI, mediated by α Disintegrin And Metalloproteinase 10 (ADAM10), however the precise mechanism by which fibrin induces GPVI shedding remains elusive. Plasma sGPVI levels are elevated in patients with coagulopathies, sepsis or inflammation and can predict onset of sepsis and sepsis-related mortality, therefore it is clinically important to understand the mechanisms of GPVI shedding under conditions of minimal collagen exposure. Our aim was to characterize mechanisms by which fibrin-GPVI interactions trigger GPVI shedding. Platelet aggregometry, sGPVI enzyme-linked immunosorbent assay (ELISA) and an ADAM10 fluorescence resonance energy transfer (FRET) assay were used to measure fibrin-mediated platelet responses. Fibrin induced α IIb β 3-independent washed platelet aggregate formation, GPVI shedding and increased ADAM10 activity, all of which were insensitive to pre-treatment with inhibitors of Src family kinases but were divalent cation- and metalloproteinase-dependent. In contrast, treatment of washed platelets with other GPVI ligands, collagen and collagen-related peptide (CRP) caused α IIb β 3-dependent platelet aggregation and GPVI release but did not increase constitutive ADAM10 activity. Thus, fibrin engages GPVI in a manner that differs from other GPVI ligands. Inclusion of polyanionic molecules disrupted fibrin-induced platelet aggregate formation and sGPVI release, suggesting that electrostatic charge may play a role in fibrin/GPVI engagement. It may be feasible to exploit this property and specifically disrupt GPVI/fibrin interactions whilst sparing GPVI/collagen engagement.

Essentials:

- Platelet exposure to fibrin increases ADAM10 activity and GPVI shedding, without requirement for activated signalling pathways
- Negatively-charged molecules and metalloproteinase inhibitors reduce fibrin-mediated release of GPVI
- Collagen and fibrin treatment of platelets induces ADAM10-mediated GPVI shedding via different mechanisms

Introduction

Glycoprotein (GP)VI is a platelet-specific receptor for collagen that has a critical role in maintaining vascular integrity,¹ and emerging roles in arterial thrombosis and thrombus stability, potentially through collagen-independent mechanisms.^{2, 3} GPVI was recently shown by several groups to bind fibrin^{2, 4-6} and this interaction is likely to be critical for thrombus stability under arterial shear rates, as thrombi formed in the carotid artery of GPVI-deficient mice are remarkably unstable. Collagen binds to a binding pocket within the first immunoglobulin-like domain of GPVI⁷ with strong preference for dimeric GPVI.^{7, 8} The site within GPVI mediating fibrin interactions is still under investigation.^{5, 6} Recent studies suggest immobilized fibrinogen can also interact with GPVI^{5, 9} although this remains controversial.¹⁰ Collagen and fibrin binding to GPVI triggers an intracellular signalling cascade initiated by phosphorylation of the immunoreceptor tyrosine-containing activation motif (ITAM) within the GPVI-linked Fc receptor (FcR) γ -chain by Src family kinases,^{2, 6, 11} leading to spleen tyrosine kinase (Syk) activation.¹² Notably, various charged and/or hydrophobic molecules including diesel exhaust particles, polysulfated sugars and histones can also indirectly induce GPVI-dependent ITAM signalling.¹³

Fibrin is formed by the action of the serine protease thrombin on fibrinogen and is critical for insoluble clot formation to control blood loss from damaged blood vessels. Thrombin cleaves fibrinopeptides A and B from the N-termini of A α and B β chains of fibrinogen, revealing a positively charged β N-terminal domain and allowing protofibril formation.¹⁴ Lateral aggregation of protofibrils and interactions with α C regions between protofibrils leads to fibrin polymerisation, and a fibrin network with pockets of positive charges. Fibrin is ultimately cross-linked by activated Factor XIII (FXIIIa),¹⁵ creating a stable latticed mesh that both encapsulates the platelet aggregate¹⁶ and forms at the base of a platelet aggregate¹⁷ to promote stable thrombus formation, sealing a damaged vessel wall and initiating wound healing processes. In a ferric chloride injury model, GPVI-deficient mice displayed normal onset of thrombosis but delays in reaching occlusion and increased embolization,¹⁸ implying that fibrin-GPVI binding might maintain thrombus stability in situations of minimal collagen exposure.

Platelet GPVI levels are stable under resting conditions but GPVI undergoes metalloproteolytic cleavage,¹⁹ releasing soluble GPVI (sGPVI) into the plasma, when platelets are activated by exposure to elevated shear stress,²⁰ active Factor X,²¹ or collagen, convulxin and collagen-related peptide (CRP).²² Proteolysis is mediated by A-Disintegrin And Metalloproteinase (ADAM) family

members, predominantly ADAM10, in human platelets. ADAM10 has a basal activity on resting platelets, which is increased following exposure of platelets to elevated shear stress, but not collagen or other classical GPVI ligands.²³ We have previously reported that polymerized fibrin, but not non-polymerized fibrin or fibrinogen, triggered GPVI shedding, which did not require active Src or Syk signalling.²⁴ This contrasts findings made with collagen,²² and suggests that fibrin and collagen engage GPVI via different mechanisms. We wanted to understand how fibrin induces GPVI shedding as this mechanism is likely to modulate thrombus size and stability with important implications for therapeutic intervention.

In this study, we demonstrate that fibrin exposure triggers $\alpha\text{IIb}\beta\text{3}$ -independent platelet aggregation, P-selectin upregulation and increased ADAM10 activity, leading to sGPVI release. This was a specific fibrin response as fibrinogen exposure or activation of protease-activated receptors (PARs) did not induce GPVI shedding or increase ADAM10 activity. Fibrin-induced ADAM10 activity and GPVI shedding also did not require ITAM signalling or cytoskeletal rearrangement. An anti-GPVI antibody Fab fragment which blocked collagen-induced aggregation, did not disrupt fibrin-mediated aggregation or increase ADAM10 activity indicating differences in GPVI engagement with fibrin and collagen. Finally, we examined whether electrostatic interactions controlled fibrin-mediated platelet events and demonstrated that fibrin-induced aggregation, ADAM10 activity and GPVI shedding were disrupted by inclusion of small polyanionic molecules. We conclude that several properties of GPVI/fibrin responses are unique and differ from collagen or CRP-mediated responses, thus providing an opportunity for specific therapeutic intervention.

Materials and methods

Reagents

A complete reagents list is described in Supplementary Materials. 1G5 mouse monoclonal antibody against human GPVI extracellular domain was affinity-purified using protein A-Sepharose CL-4B (GE Healthcare, City, IL) as described.²⁵ 1G5 Fab fragment was generated and isolated using a Fab preparation kit (ThermoFisher Scientific; MA).²⁵ Small polyanions SPA.1 and SPA.2 are sulfated di- and trisaccharides respectively, which were generated using standard laboratory methods; their complete characterisation is the subject of a manuscript under review (Parish et al).

Platelet preparation

Human washed platelets were prepared as previously described.²³ Briefly, platelets were isolated from acid-citrate-dextrose (ACD; 97 mM trisodium citrate, 111 mM glucose, 78 mM citric acid, 6:1 blood:ACD ratio) anticoagulated blood drawn from healthy donors after provision of informed consent using a protocol approved by the Australian National University Human Research Ethics Committee (2016/317).

Light transmission aggregometry (LTA) and ADAM10 activity

LTA (Chrono-Log, Pennsylvania, USA) was used to measure fibrin-induced platelet aggregation. Washed platelets (5×10^8 /mL) in Tyrode's buffer containing 10 mM CaCl_2 and 5 μM ZnSO_4 were preincubated with 1 mM RGD peptide (GRGDSP) for 3 min at 37°C before agonist exposure. Other inhibitors or reagents were added 10 min before RGD addition. Washed platelets were placed in a cuvette and loaded into the aggregometer held at 37°C with constant stirring and 1 μM GPVI-Cy3 (ADAM10 substrate, see below) was added. After 1 min, 100 $\mu\text{g}/\text{mL}$ fibrinogen were added. After a further 3 min, 1 U/mL thrombin was added and $\alpha\text{IIb}\beta 3$ -independent aggregation was monitored for 20 min. Test agonists were also added 1 min after substrate addition and aggregation monitored for 20 min. 120 μL of each washed platelet suspension was mixed with 50 mM ethylenediaminetetraacetic acid (EDTA) before assaying ADAM10 activity and sGPVI levels.

Measurement of ADAM10 activity

ADAM10 activity was measured using an established fluorescence resonance energy transfer (FRET) assay²³ with a quenched GPVI-Cy3 peptide substrate comprising amino acid residues 236-248 of human GPVI that is known to be cleaved by ADAM10.^{23, 26} Fluorescence following cleavage of the peptide and quencher release at 37°C was detected by sample excitation at 540 nm and emission at 590 nm using a Tecan Infinite 200 Pro plate reader (endpoint or after 1 h in assays using recombinant human (rh) ADAM10). Increases in relative fluorescence units (RFU) represented increased ADAM10 activity.

Quantification of sGPVI levels

sGPVI levels were measured in supernatant samples generated by fibrin or other agonists exposure of washed platelets. Supernatants were obtained after centrifugation at 2500 g for 15 min and levels of sGPVI quantified using an established enzyme-linked immunosorbent assay (ELISA) as previously described.^{27, 28} Luminescence signal was detected with a Tecan Infinite 200 Pro plate reader.

Statistical analysis

Data are represented as mean \pm standard deviation (SD). GraphPad Prism version 8 (California, USA) was used for statistical analysis. Normal and log-normal distribution was tested using Anderson-Darling tests. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons tests were performed if data was normally distributed, or Kruskal-Wallis analysis with Dunn's post multiple comparisons test for non-parametric data, unless stated otherwise.

Results

Fibrin exposure triggers platelet aggregate formation and increases ADAM10 activity and sGPVI release

The interaction of fibrin with platelets has been shown by several groups to be mediated by engagement with GPVI,^{2, 5, 6, 28, 29} however, the nature of this engagement and consequences for platelet function have not been fully defined. Initially washed platelets were exposed to polymerized fibrin that had been generated by adding thrombin to platelet suspensions that contained fibrinogen and inhibitors of α IIb β 3 function, such as RGD.²⁴ Fibrin exposure alone produced an α IIb β 3-independent aggregation trace with 84% maximal change in light transmission (maximal aggregation) ($p < 0.001$, Figure 1A) or 80% aggregation ($p < 0.05$) in samples pre-treated with another α IIb β 3 inhibitor, GR144053 (Supplementary Figure 1D). Platelet exposure to fibrin in the presence of RGD also elevated P-selectin exposure over resting samples ($p < 0.01$, Figure 1B) to levels equivalent to those quantified in platelets treated with collagen or calcium ionophore (A23187), suggesting fibrin-exposed platelets triggered platelet activation and degranulation.

Metalloproteolytic shedding of human GPVI ectodomain is predominantly controlled by ADAM10 and is a consequence of platelet activation. Shedding can be triggered in the presence or absence of GPVI-ligand engagement and active signalling pathways.²³ We assessed the effect of fibrin exposure on platelet ADAM10 activity. Resting platelets have detectable ADAM10 activity that increases upon exposure to fluid shear stress or treatment of platelets with the thiol modifier *N*-ethylmaleimide (NEM), measured using an established ADAM10 FRET sensor tool.²³ Consistent with published data, Figure 1C (and Supplementary Figure 1B) shows resting platelets had detectable ADAM10 activity that was increased by treatment with NEM and reduced by inclusion of the broad spectrum metalloproteinase inhibitor GM6001, with a similar result observed with the ADAM10-specific inhibitor GI254023X (Supplementary Figure 2B).^{23, 30} Exposure of platelets to fibrin but not fibrinogen triggered increased ADAM10 activity (1.6-fold increase over resting, $p < 0.01$; Figure 1B and Supplementary 1A) that was reduced by inclusion of either GM6001 (64% reduction, $p < 0.001$) or GI254023X (39% reduction, $p < 0.01$) (Figure 1C and Supplementary Figure 2B). Fibrin-mediated platelet aggregation was unaffected by pre-treatment with the metalloproteinase inhibitors GM6001 or GI254023X (Figure 1A and Supplementary Figure 2A), suggesting that metalloproteinase inhibition did not alter fibrin/GPVI engagement.

In human platelets, ADAM10 cleaves GPVI releasing sGPVI into the plasma.^{27, 31} We measured sGPVI levels released in the supernatants of fibrin-exposed platelets, and showed that fibrin but not fibrinogen exposure induced sGPVI release ($p < 0.005$; Figure 1C and Supplementary Figure 1C), consistent with previous findings where fibrin exposure induced loss of intact GPVI and appearance of a 10-kDa remnant GPVI fragment, detected by western blotting.²⁸ Fibrin-mediated sGPVI release was blocked by GM6001 (Figure 1D) and reduced by treatment with GI254023X (Supplementary Figure 2C), although this reduction did not reach statistical significance, confirming release was mediated by metalloproteinases, with a contribution from ADAM10. To assess whether activation of PARs by thrombin during the fibrin generation step contributed to platelet aggregation, ADAM10 activity or GPVI release, RGD-treated platelets were mixed with either PAR-1 activating peptide (SFLLRN) or thrombin. Supplementary Figure 1 shows that neither treatment induced $\alpha\text{IIb}\beta 3$ -independent platelet aggregation, increased ADAM10 activity or triggered sGPVI release. This suggested that the contribution of thrombin to fibrin-mediated aggregation and shedding was limited to its fibrinogen-cleaving properties, consistent with previous findings.^{21, 22, 28}

Fibrin and collagen activate platelet aggregation and GPVI shedding via different mechanisms

Using the same experimental design, we directly compared collagen- and fibrin-mediated platelet responses. Addition of 30 $\mu\text{g/mL}$ collagen (an extremely high concentration) to RGD-treated platelets did not induce robust platelet aggregation ($< 40\%$ maximum $\alpha\text{IIb}\beta 3$ -independent platelet aggregation compared to $> 80\%$ maximum aggregation observed with fibrin exposure; Figure 2A). In line with previous studies using lower collagen concentrations,²³ 30 $\mu\text{g/mL}$ collagen treatment did not increase ADAM10 activity above resting levels and was significantly less than fibrin-mediated ADAM10 activity ($p < 0.05$, Figure 2B) but did result in significant sGPVI release (Figure 2C). High concentration collagen-induced sGPVI levels were 2-fold greater than fibrin-induced levels, and approached levels achieved with NEM treatment. Taken together, these data suggest mechanistic and regulatory differences between collagen- and fibrin-induced platelet functions.

GPVI utilizes ITAMs within the associated FcR γ chain to trigger platelet aggregation in response to collagen exposure^{3, 11, 32} and collagen-induced GPVI shedding also requires ITAM signalling.²² Fibrin activation of GPVI also triggers ITAM signalling, and inhibition of Src and Syk reduces

fibrin-mediated phosphorylation²⁹ and spreading on immobilized fibrin(ogen).⁹ Fibrin-mediated GPVI shedding however was shown to be Src and Syk independent.²⁸ We therefore wanted to determine whether fibrin-mediated aggregation, ADAM10 activity and sGPVI release was dependent on ITAM signalling. Figure 3 shows that inclusion of inhibitors of Syk (Bay-61-3606) or Src (Src-1) did not reduce fibrin-mediated platelet aggregation, ADAM10 activity or sGPVI release. These data indicate that fibrin interactions with GPVI to trigger GPVI shedding differ from collagen interactions with GPVI.

One explanation for the differences observed so far is that fibrin and collagen may interact with different sites within GPVI. To address this question, platelets were pre-treated with 10 µg/mL of a Fab fragment generated from the anti-human GPVI monoclonal antibody 1G5.²⁵ This concentration of 1G5 Fab was sufficient to inhibit collagen- and CRP-mediated PRP aggregation (data not shown). However, inclusion of up to 30 µg/mL 1G5 Fab did not affect fibrin-induced αIIbβ3-independent aggregation in washed platelets (Figure 4B) or fibrin-mediated ADAM10 activity (Figure 4C). These data suggest that fibrin engagement of GPVI utilizes a binding site that is distinct from that shared by collagen and 1G5 Fab.

Fibrin-mediated ADAM10 activity does not require intracellular Ca²⁺ flux.

Due to the importance of Ca²⁺ flux in GPVI activation,³³ we assessed roles for calcium and cation fluxes in fibrin-mediated ADAM10 activity and GPVI shedding. Washed platelets were treated with RGD and 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM), a membrane-permeable Ca²⁺-specific chelator that inhibits intracellular Ca²⁺ flux,³⁴ then exposed to fibrin. EDTA, an extracellular divalent cation chelator and broad metalloproteinase inhibitor, was included as a control. Inclusion of EDTA but not BAPTA-AM to platelet suspensions reduced fibrin-induced platelet aggregation (Figure 5A). Treatment with BAPTA-AM did not disrupt resting ADAM10 activity and only mildly reduced fibrin-induced ADAM10 activity (Figure 5B), and had no effect on fibrin-induced sGPVI release (Figure 5C). Because ADAM10 catalytic activity requires Zn²⁺, inclusion of EDTA inhibited both ADAM10 activity and sGPVI release induced by fibrin (Figure 5B and 5C) as expected. This effect of EDTA, and lack of major effect with BAPTA-AM was confirmed in an ADAM10 activity assay using 30 nM recombinant human ADAM10 (Supplementary Figure 4A). We conclude that fibrin-induced platelet ADAM10 activity and release of sGPVI did not require intracellular calcium flux.

Fibrin-mediated sGPVI release is reduced by negatively-charged molecules

Numerous electrostatically charged agents, including histones and diesel exhaust particles can activate platelets *via* GPVI and induce ITAM signalling, potentially through membrane perturbation and GPVI clustering.¹³ Fibrinogen, a negatively-charged molecule, is converted to fibrin by thrombin, creating a network of protein polymers with pockets of positive charges that can bind polyanions and negatively-charged polyphosphates, and disrupt fibrin binding properties.^{35, 36} As EDTA treatment disrupted fibrin-mediated platelet aggregation, we explored a role for electrostatic charge in contributing to fibrin-mediated responses. We assessed the ability of heparin, a negatively charged macromolecule of ~ 15-20-kDa and two small polyanions (SPA) termed SPA.1, a sulfated disaccharide and SPA.2, a sulfated trisaccharide, of approximately 0.9- to 1.5-kDa in size, respectively. The SPAs were evaluated at concentrations that were above levels shown to reduce histone toxicity in assays conducted *in vitro* and *in vivo* (manuscript under review). Whilst SPA.1 did not reduce fibrin-mediated platelet aggregation at any of the concentrations tested (Figure 6A), concentrations of 300-500 µg/mL of SPA.2 and 50 U/mL heparin reduced fibrin-mediated aggregation by 30-35% (Figure 6A).

We could not assess fibrin-induced ADAM10 activity in samples containing any of these charged molecules, as concentrations of 500 µg/mL SPA.1 and SPA.2 or 50 U/mL heparin interfered directly with cleavage of the ADAM10 substrate by recombinant ADAM10 (Supplementary Figure 4A and 4B). However, the charged molecules did not interfere with NEM-induced sGPVI release (Supplementary Figure 4C), indicating that the sGPVI ELISA could be used to evaluate the effect of these molecules on fibrin-induced sGPVI release. Each of these charged molecules did not affect platelet spreading on immobilized fibrin (Supplementary Figure 5). Inclusion of SPA.1 or SPA.2 to fibrin-exposed platelet suspensions resulted in slight reduction (19% reduction SPA.1 and 20% reduction SPA.2, Figure 6C) in fibrin-induced release of sGPVI that was not statistically significant. However, inclusion of 10 or 50 U/mL heparin did significantly reduce fibrin-mediated sGPVI release. As heparin requires the presence of significant amounts of anti-thrombin III (unlikely to be present in our washed platelet preparations) to affect thrombin activity, we conclude that heparin interfered directly with the ability of fibrin to trigger shedding of GPVI, most likely via an electrostatic mechanism.

To further explore the effects of negatively charged molecules, we evaluated charged molecules, chondroitin sulfate (type A and C; 50-100-kDa) and dextran sulfate (45-50-kDa) to disrupt fibrin-

mediated platelet effects on aggregation and sGPVI release. ADAM10 activity measurements again could not be validated due to charge molecules interference with ADAM10 substrate cleavage by recombinant ADAM10 (data not shown). Comparable with data gained using 50 U/mL heparin, pre-treatment with 1000 µg/mL of chondroitin sulfate type A or C reduced fibrin-mediated sGPVI release ($p=0.037$ and $p=0.046$ respectively; Supplementary Table 1), although aggregation was unaltered. In contrast, 200 and 1000 µg/mL of a 50-kDa (but not a 45-kDa) preparation of dextran sulfate reduced platelet aggregation ($p<0.01$) but did not reduce sGPVI release (Supplementary Table 1). These data indicate that fibrin-mediated platelet responses were disrupted by inclusion of polyanions and the extent of inhibition was determined by specific properties of each polyanion.

Fibrin-mediated ADAM10 activity does not require cytoskeletal rearrangement but is inhibited by ATP hydrolysis

Platelet receptors including $\alpha\text{IIb}\beta 3$ and the GPIb-IX-V complex are linked to the cytoskeleton, through actin and filamin A,³⁷ which enable platelet shape change^{38, 39} and platelet signalling through interactions with Syk.⁴⁰ Disruption of actin function using cytochalasin D (cytoD) to block actin polymerization was shown to disrupt GPVI dimerization and clustering.⁴¹ To assess whether actin polymerization was required for fibrin-GPVI-mediated responses, we used 10 µM cytoD, a moderate concentration that reduced platelet spreading on collagen and immobilized fibrin (data not shown). Inclusion of cytoD to RGD-treated fibrin-exposed platelets did not disrupt $\alpha\text{IIb}\beta 3$ -independent aggregation (Figure 7A). Inclusion of cytoD also did not disrupt fibrin-mediated ADAM10 activity or sGPVI release (Figure 7B and 7C). This suggested that while fibrin can trigger platelet cytoplasmic events, fibrin mediates platelet aggregation and GPVI shedding via external effects on platelet receptors and metalloproteinase tertiary structure.

We consistently observed a two-step aggregation trace following fibrin exposure. However, one donor had significantly ablated fibrin-induced platelet aggregation (Figure 1A), together with a reduction of adenosine diphosphate (ADP)-induced aggregation in PRP (not shown). We therefore examined whether release of the secondary mediator ADP, that is important for integrin $\alpha 2\beta 1$ -modulated collagen-GPVI activation,⁴² contributed to fibrin-mediated ADAM10 activity or sGPVI release. Inclusion of the ADP scavenger apyrase did not disrupt fibrin-induced aggregation (Figure 7A), but reduced fibrin-mediated ADAM10 activity by 30% ($p<0.01$, Figure 7B) and slightly (but

not statistically significantly) reduced fibrin-induced sGPVI release (18% reduction; Figure 7C). Therefore, although apyrase interfered with fibrin-mediated ADAM10 activity, this was not sufficient for prevention of fibrin-mediated GPVI shedding.

Discussion

Hemostasis requires conversion of fibrinogen to fibrin to generate a distinctive fibrous network that mediates interactions with blood cells and initiation of tissue repair processes. Fibrin is present at all stages of thrombus formation.⁴³ Recently, fibrin and fibrinogen have been identified as ligands for GPVI and shown to induce signalling in support of thrombus formation and stabilization.^{2, 4, 5, 9} In addition, platelet exposure to fibrin was shown to induce metalloproteolytic loss of intact GPVI by western blot.²⁸ In this present study, we now show key differences between collagen and fibrin engagement of GPVI (Table 1). First, 1G5 Fab blocks collagen- but not fibrin-mediated aggregation of platelets in suspension, indicating that under these experimental conditions, fibrin engages at least one site within GPVI that is distinct from collagen. Second, whilst fibrin engagement of platelets in suspension is able to trigger ITAM signalling responses, fibrin-mediated platelet aggregation did not require ITAM signalling, actin polymerization, intracellular calcium flux or active integrin $\alpha\text{IIb}\beta 3$. Third, whilst both collagen and fibrin trigger shedding of GPVI, collagen does not achieve this via increased activation of ADAM10 activity whereas fibrin-treated platelets did elevate ADAM10 activity and induced sGPVI release. Blockade of fibrin-induced platelet aggregation by inhibitors of Syk and Src has been previously reported,²⁹ but integrin-independent aggregation by fibrin has not. Furthermore, we identified that the fibrin-mediated platelet aggregation and release of sGPVI involved an electrostatic charge component, as both of these outcomes could be disrupted by inclusion of small or large molecular weight polyanions.

From our data using the collagen-blocking anti-GPVI antibody 1G5, and also the work of others,^{2, 4, 5, 9} it is apparent that fibrin engages GPVI in a manner that differs from collagen, and is likely to involve charge-charge interactions. Despite an overall net negative charge, regions of fibrin such as the central E region has a net positive charge, predominantly conferred through three positively charged clusters of residues within the βN -domain. This domain, which is shielded within fibrinogen by fibrinopeptide B and exposed upon thrombin-mediated cleavage of fibrinopeptide B, is solvent-accessible in fibrin, and mediates interactions with a number of cell receptor ectodomains. GPVI is a sialomucin with a negatively-charged membrane-proximal stalk region

and it is conceivable that fibrin is able to electrostatically engage and cluster GPVI via this domain. Fibrin D-dimer contains fragment E when formed by the action of plasmin on fibrin *in vivo*. Recently a D-dimer preparation was reported to disrupt GPVI-fibrin interactions *in vitro*,²⁹ however it is not clear whether fragment E was present in the commercial preparation used in that study. We were able to ablate fibrin effects by including high and low molecular weight polyanionic reagents. Consistent with our findings that heparin disrupts fibrin-GPVI effects, the β N-domain of fibrin also contains a heparin-binding site.^{44, 45}

Fibrin treatment also increased ADAM10 activity indicating that fibrin-platelet interactions were able to illicit changes to platelet ADAM10 that mimicked changes induced by exposure to elevated shear stress.²³ ADAM10 is active on the surface of resting platelets and collagen exposure does not increase ADAM10 activity, but is likely to increase GPVI shedding by altering the access of the catalytic domain of ADAM10 for the cleavage site within GPVI. How fibrin exposure increases the activity of platelet ADAM10 is not yet known, however it does not require platelet cytoskeletal rearrangement or intracellular signalling events. Interestingly, the β N-domain of fibrin has been shown to interact with a cysteine-rich domain within the very low density lipoprotein (VLDL) receptor on endothelial cells.⁴⁶ Cysteine-rich domains are present in both α IIb β 3 and ADAM10 and it is intriguing to speculate that fibrin elicits at least a portion of its platelet activity by interacting with these domains on the platelet surface. In other vascular systems the positively-charged amino terminus of the β chain of fibrin (but not fibrinogen) interacts with VE-cadherin^{47, 48} to orchestrate the transmigration of leukocytes across endothelium. Taken together, our data and the work of others suggests that fibrin has roles in the vasculature beyond maintenance of structural integrity of a thrombus.

Numerous reports using a variety of experimental conditions have concluded that thrombin treatment of platelets does not mediate direct release of GPVI.^{21, 22, 28, 49} We confirmed no role for thrombin activation of PARs in ADAM10 activity increase and sGPVI release, as PAR-1 peptide or thrombin exposure alone did not increase ADAM10 activity or increased sGPVI release. Likewise, although platelets have been shown to spread on immobilized fibrinogen and invoke single platelet Ca^{2+} signals through ITAM signalling,⁹ inclusion of either the actin polymerization inhibitor cytochalasin D, or BAPTA-AM an inhibitor of intracellular Ca^{2+} flux did not interfere with fibrin-mediated platelet aggregation. It remains unclear why BAPTA-AM treatment reduced fibrin-mediated ADAM10 activity but GPVI shedding was not affected. It is possible that platelet

Ca²⁺ flux facilitates an increase in ADAM10 activity as resting platelets become activated, but that fibrin engagement of GPVI also improves access of the ADAM10 catalytic site to the cleavage site within GPVI, resulting in no change to ADAM10 activity but increased GPVI shedding.

Finally, whether fibrin binds to monomeric²⁹ or dimeric GPVI⁵ or mediates its effects on platelets via interaction with membrane or other platelet surface proteins remains to be resolved¹⁰. We provide evidence here that fibrin-GPVI engagement can be disrupted by negatively charged molecules, including heparin or small polyanionic molecules. GPVI pathways have previously been reported to be activated by several electrostatically charged molecules including diesel exhaust particles and histone proteins that trigger ITAM-dependent platelet signalling, and may work to trigger GPVI clustering,¹³ either directly or via involvement with a co-associated receptor such as GPIb-IX-V,⁵⁰ or an adjacent molecule such as members of the tetraspanin family.⁵¹

As expected and consistent with published data, both collagen and CRP treatment of washed platelets induced GPVI shedding, and did not induce α IIB β 3-independent aggregation, or any increase in ADAM10 activity beyond resting levels. These findings strongly support the notion of molecular differences between collagen and fibrin engagement of GPVI. Fibrin-mediated effects were disrupted by inclusion of heparin, or small polyanionic and higher concentrations of certain types of chondroitin sulfate and dextran sulfate molecules. Properties including charge density and stoichiometry of each of these molecules are likely to be important determinants of inhibitory capacity. Our data suggests that it will be possible in the future to design and synthesis low molecular weight polyanions with appropriate conformation and chemical attributes for the targeted disruption of fibrin-GPVI interactions.

In conclusion, understanding mechanisms enabling fibrin-GPVI engagement and resultant effects on platelet activation and function, is of fundamental importance to our understanding of how a thrombus is formed and stabilized. Specifically targeting fibrin-GPVI engagement may be a therapeutic option that targets thrombus formation under conditions where there is minimal collagen exposure (sepsis, DIC and other coagulopathy settings) or in situations where modulation of thrombus stability is important such as in atherothrombosis and stroke.

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Authorship:

Contributions: S.J.M designed, performed and interpreted research and wrote the manuscript. S.M.H. and C.S-M.L. interpreted data and processed samples. L.A.C and C.R.P supplied crucial reagents and interpreted data. R.K.A and W.M.L designed and interpreted research. E.E.G designed and interpreted research and wrote the manuscript. All authors reviewed the manuscript.

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Table 1 – Summary of fibrin- *versus* collagen-induced platelet responses

<i>Event</i>	<i>Fibrin</i>	<i>Collagen</i>
Binds GPVI and activates ITAM signalling pathways	Y	Y
Platelet αIIbβ3-independent aggregation	Y	N
Increased ADAM10 activity	Y	N
• Reduced with 1G5 Fab fragment	N	Y
Induces GPVI shedding	Y	Y
• Metalloproteinase-dependent	Y	Y
• Src- and Syk-dependent	N	Y
Increases P-selectin surface levels	Y	Y
<i>GPVI monomer/dimer binding</i>	ND Charge- dependent	Dimeric GPVI

Responses described in the current study or reported in other studies. Y = yes, N= no, ND = not determined

Figure legends

Figure 1 – Fibrin exposure triggers α IIb β 3-independent platelet aggregation, increased P-selectin upregulation, increased ADAM10 activity and sGPVI release. Washed platelets (5×10^8 /mL) were pre-treated with 1 mM RGD for 3 min before agonist exposure. Fibrin was generated when 1 U/mL thrombin (T; arrowed) was added 3 min after fibrinogen (Fgn; 100 μ g/mL) under stirring conditions. In some cases, platelets were pre-treated with 250 μ M GM6001 (GM), a general metalloproteinase inhibitor, 10 min before agonist exposure. (A) Platelet aggregation was monitored for 20 min. (Left = representative aggregation trace, right = quantitation. B) P-selectin upregulation was measured by flow cytometry following washed platelet exposure to vehicle (resting), fibrinogen (Fgn) or fibrin (as above). 30 μ g/mL collagen and 10 μ M A23187 (calcium ionophore) stimulation for 1 h (static) were used as GPVI-ligand control and maximum degranulation control respectively. CD62P-PE fluorescence measured as geomean. C) ADAM10 activity in fibrin-exposed washed platelets was measured as relative fluorescence units (RFU; endpoint value) using an established ADAM10 FRET assay (see methods). D) sGPVI levels in supernatants obtain following agonist exposure using a sGPVI ELISA. 5 mM NEM was used as a positive control for ADAM10 activity and sGPVI release. Aggregations = Kruskal-Wallis test with Dunn's multiple comparisons test performed. All other experiments= One-way ANOVA with Tukey's multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. Individual dots = individual donors (n=3-29). Bars and error bars are mean \pm SD.

Figure 2 – Fibrin-mediated GPVI shedding is different from collagen-induced GPVI shedding. RGD-treated washed platelets (5×10^8 /mL) were treated with fibrin (fibrinogen: Fgn 100 μ g/mL + thrombin: T 1 U/mL) or collagen (Coll; 30 μ g/mL). A) Representative platelet aggregations after agonist exposure measured by LTA for 20 min. B) ADAM10 activity or C) sGPVI levels released from untreated platelets or platelets treated with 30 μ g/mL collagen, or fibrin or 5 mM NEM. One-way ANOVA with Tukey's multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. Individual dots = individual donors, n=8-9. Bars and error bars are mean \pm SD.

Figure 3 – Fibrin-mediated increase in ADAM10 activity and GPVI release do not require ITAM signalling. Washed platelets were pre-treated with 10 μ M Bay61-3606 (Syk inhibitor) or 10 μ M Src-1 (Src inhibitor) before exposure to fibrin and A) platelet aggregation; B) ADAM10 activity or C) sGPVI release were evaluated. One-way ANOVA with Tukey's multiple

comparisons (for aggregations and ADAM10 activity), Friedman test with Dunn's multiple comparisons (for sGPVI release) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Individual dots = individual donors, $n = 6-7$. Bars and error bars are mean \pm SD.

Figure 4 – Anti-GPVI antibody 1G5 Fab reduces CRP- and collagen-mediated ADAM10 activity but not fibrin-mediated ADAM10 activity. A) RGD-treated washed platelets were preincubated with 10 $\mu\text{g/mL}$ 1G5 Fab for 10 min before exposure to 25 $\mu\text{g/mL}$ CRP. B) RGD treated washed platelets were preincubated with 10 or 30 $\mu\text{g/mL}$ 1G5 Fab 10 min before exposure to fibrin- (Fgn 100 $\mu\text{g/mL}$ + thrombin: T 1 U/mL). Aggregation measured for 20 min C) ADAM10 activity measured after aggregation. One-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Individual dots = individual donors, $n = 3-4$, Bars and error bars are mean \pm SD.

Figure 5 – BAPTA-AM and EDTA reduces fibrin-mediated ADAM10 activity. Washed platelets were pretreated with vehicle or 50 μM BAPTA-AM or 50 mM EDTA then exposed to Fibrin (Fgn + T) and monitored for A) platelet aggregation (Left = representative aggregation trace, right = quantification); B) ADAM10 activity or C) sGPVI release. Aggregations = Kruskal-Wallis with Dunn's multiple comparisons test. ADAM10 activity and sGPVI release = One-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. Individual dots = individual donors. Bars and error bars are mean \pm SD.

Figure 6 – Fibrin-mediated platelet aggregation and sGPVI release is reduced by negatively-charged molecules. RGD-treated washed platelets ($5 \times 10^8/\text{mL}$) were preincubated with 100 – 500 $\mu\text{g/mL}$ SPA.1, 50 – 500 $\mu\text{g/mL}$ SPA.2 or 10 or 50 U/mL heparin (corresponding to 67 and 333 $\mu\text{g/mL}$ of heparin) for 10 min before exposure to fibrin. A) Platelet aggregations quantified from $n = 3-8$ donors; R = resting platelets, F = Fibrin-exposed platelets. B) sGPVI release measured after fibrin-mediated aggregations. One-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. Individual dots = individual donors, $n = 3-8$. Bars and error bars are mean \pm SD.

Figure 7 – Fibrin-mediated ADAM10 activity does not require actin polymerization but is reduced with ADP scavenging. RGD-treated washed platelets were pre-treated with 2 U/mL apyrase (Apy) or 10 μM cytochalasin D (CytoD) or 250 μM GM6001 10 min before exposure to fibrin. A) Fibrin (Fgn + T) mediated platelet aggregation (left and middle panels are representative

aggregation traces; right = quantification. B) ADAM10 activity and (C) sGPVI levels. One-way ANOVA with Tukey's multiple comparison tests performed. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.005$ **** $p < 0.001$. Individual dots = individual donors. $n=3-4$. Bars and error bars are mean \pm SD.

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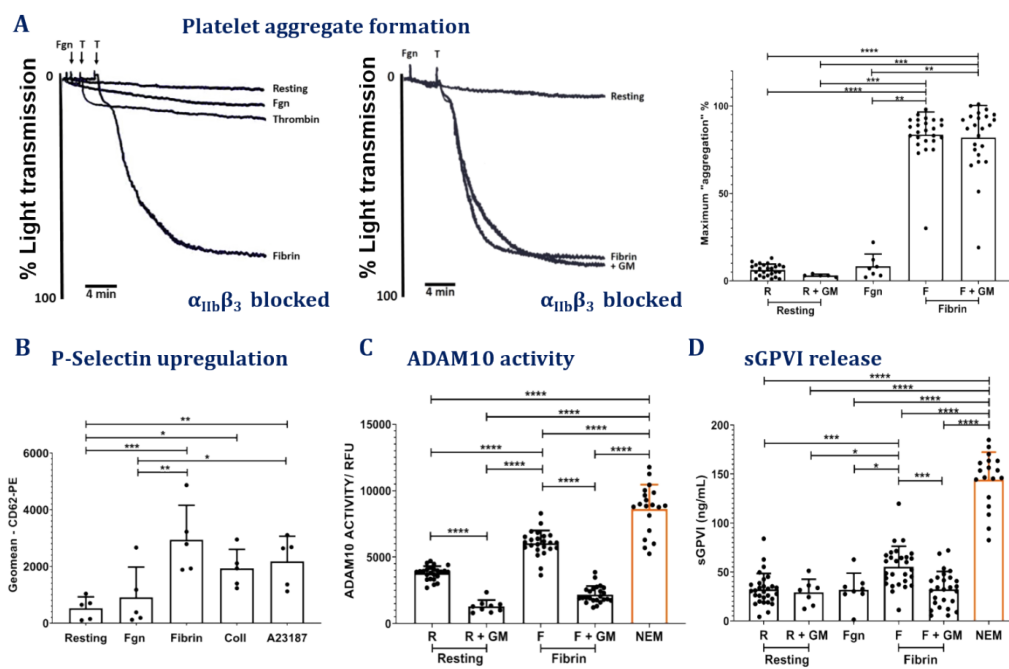
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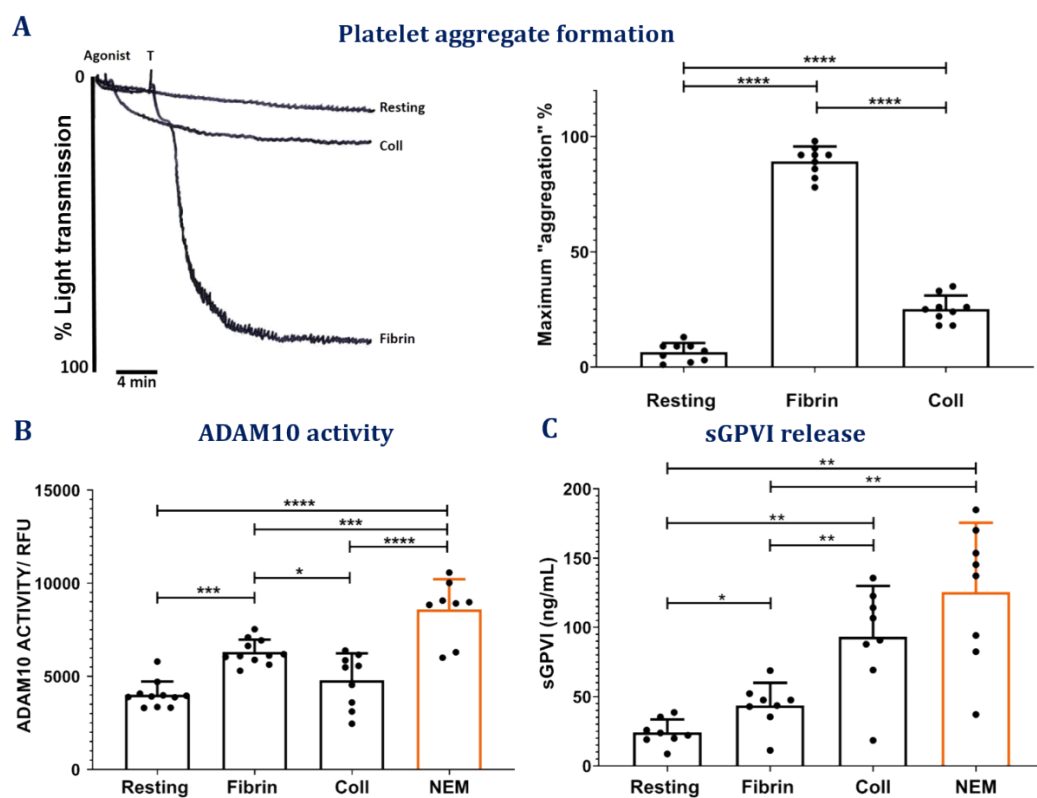
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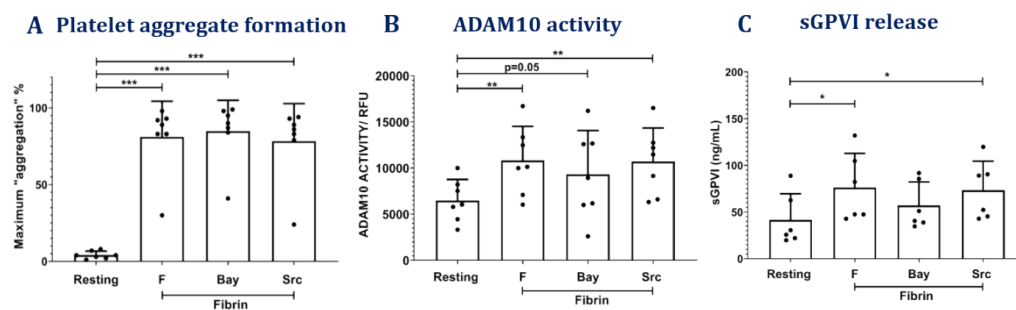
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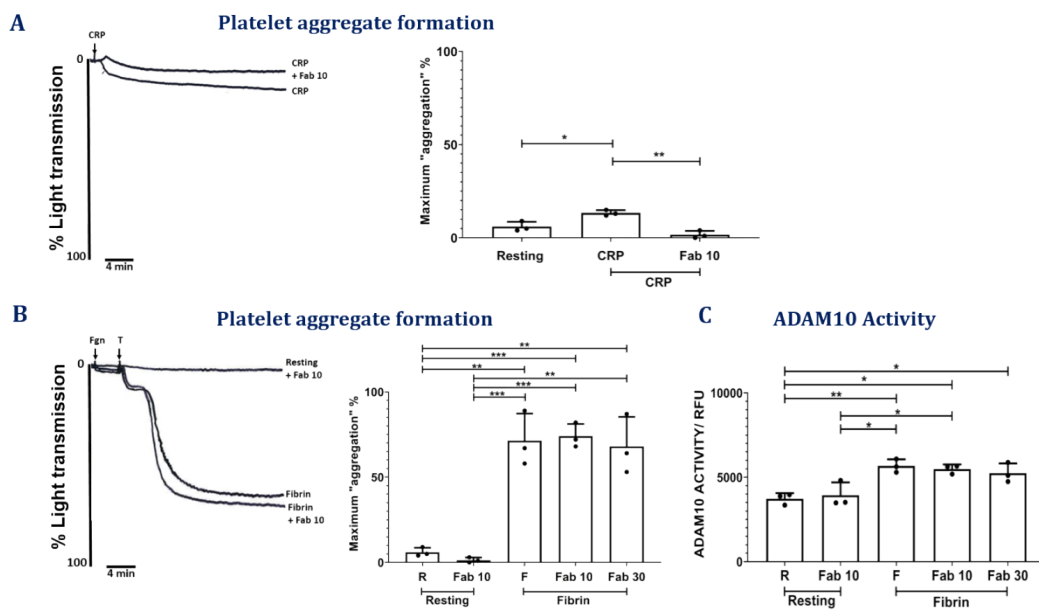
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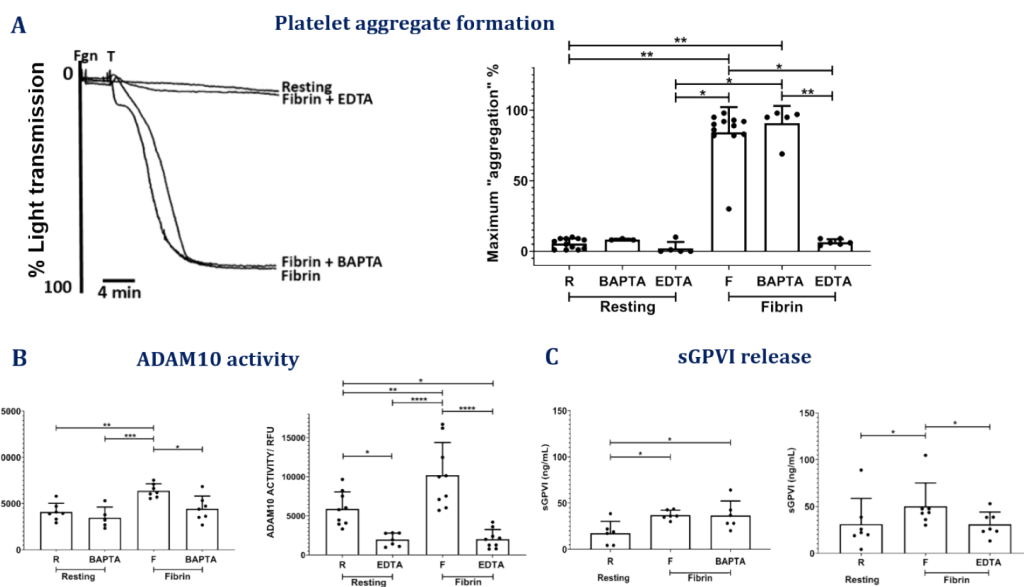
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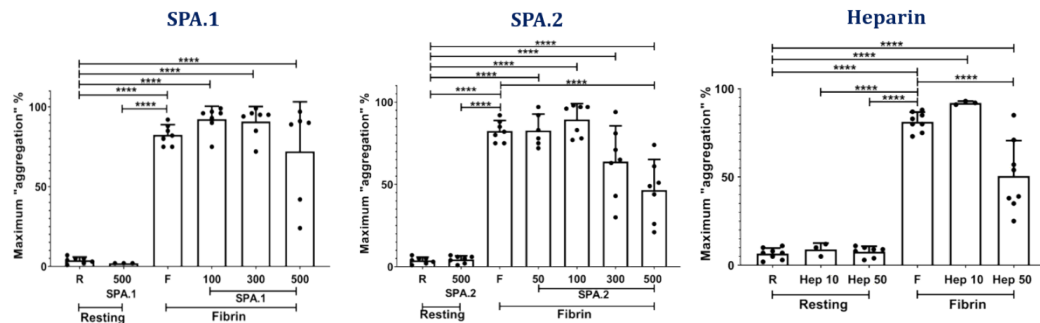


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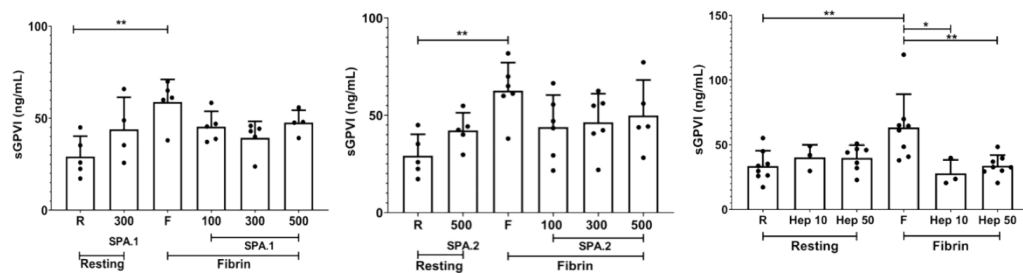


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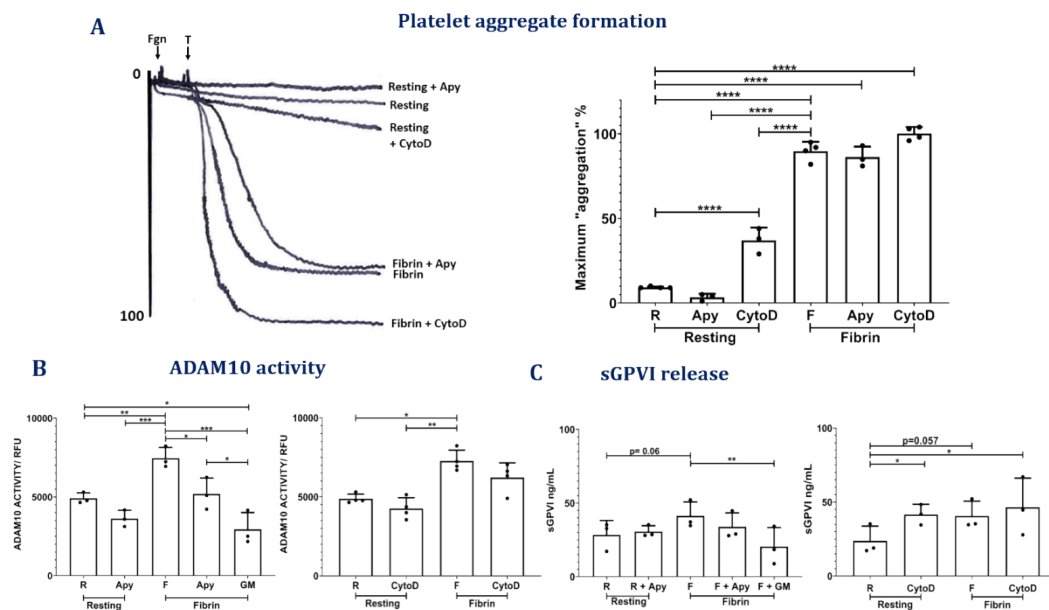
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B sGPVI release



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