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ORIGINAL ARTICLE

Fibrin-glycoprotein VI interaction increases platelet procoagulant activity and impacts clot structure

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Abstract

Background: The glycoprotein VI (GPVI) signaling pathway was previously reported to direct procoagulant platelet activity through collagen binding. However, the impact of GPVI-fibrin interaction on procoagulant platelet development and how it modulates the clot structure are unknown.

Objectives: To determine the effect of GPVI-fibrin interaction on the platelet phenotype and its impact on the clot structure.

Methods: Procoagulant platelets in platelet-rich plasma clots were determined by scanning electron microscopy (wild-type and GPVI-deficient murine samples) and confocal microscopy. Procoagulant platelet number, clot density, clot porosity, and clot retraction were determined in platelet-rich plasma or whole blood clots of healthy volunteers in the presence of tyrosine kinase inhibitors (PRT-060318, ibrutinib, and dasatinib) and eptifibatide.

Results: GPVI-deficient clots showed a higher nonprocoagulant vs procoagulant platelet ratio than wild-type clots. The fiber density and the procoagulant platelet number decreased in the presence of Affimer proteins, inhibiting GPVI-fibrin(ogen) interaction and the tyrosine kinase inhibitors. The effect of GPVI signaling inhibitors on the procoagulant platelet number was exacerbated by eptifibatide. The tyrosine kinase inhibitors led to an increase in clot porosity; however, no differences were observed in the final clot weight, following clot retraction with the tyrosine kinase inhibitors, except for ibrutinib. In the presence of eptifibatide, clot retraction was impaired.

Conclusion: Our findings showed that GPVI-fibrin interaction significantly contributes to the development of procoagulant platelets and that inhibition of GPVI signaling increases clot porosity. Clot contractibility was impaired by the integrin αIIbβ3 and Btk pathway inhibition. Thus, inhibition of GPVI-fibrin interactions can alleviate structural

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characteristics that contribute to a prothrombotic clot phenotype, having potential important implications for novel antithrombotic interventions.

KEYWORDS
clot structure, fibrin, GPVI, procoagulant platelets, tyrosine kinase inhibitors

1 | INTRODUCTION

Hemostasis is the primary response to vascular injury to avoid life-threatening blood loss. Platelets and fibrin are key players in this process, contributing to subsequent wound healing [1,2]. On the other hand, excessive platelet activity or fibrin formation can lead to severe thromboembolic disorders, which contribute significantly to global health burden and death [3–6]. However, all antithrombotic drugs that are currently used in clinical practice, which target molecular pathways pivotal for both normal hemostasis and thrombosis, are associated with an adverse risk of bleeding [7,8]. Observations that the absence of platelet receptor glycoprotein (GP)VI impairs experimental thrombosis in mouse models with no significant impact on hemostasis sparked interest in GPVI as an alternative antithrombotic target [9–11]. Additionally, GPVI-deficient individuals present with only mild bleeding diathesis, supporting the potential for this receptor to be a viable antithrombotic target, with reduced bleeding risk and minor impact on hemostasis [12–15]. The blockade of GPVI binding by humanized antigen-binding fragment glenzocimab (ACT-017) has been investigated in first-in-human trials [16] and progressed to phase 2 trials.

In addition to its role in platelet activation via collagen binding, the GPVI pathway has also been implicated in the formation of procoagulant platelets [17,18]. Procoagulant platelets, also known as "ballooning" platelets due to their round morphology, contribute to increased coagulation at the site of thrombus formation [19,20]. The negatively charged membrane of procoagulant platelets supports annexin V binding and thrombin generation, leading to lengthened activation relevant to thrombus stabilization [21].

A novel alternative ligand of GPVI, namely fibrin(ogen), has been identified in recent years, and studies have explored the potential binding sites for this interaction [22–24]. In the past year, we showed that the highest-affinity binding site for GPVI is located in the α C-region of the fibrinogen molecule [25]. Previous studies have identified the involvement of GPVI in platelet-mediated thrombin generation in response to both fibrin and collagen [24,26]. Specifically, fibrin-induced phosphatidylserine (PS) exposure was reduced in GPVI-deficient platelets [27]. Moreover, blockade of GPVI and Syk inhibition suppressed fibrin-induced platelet aggregation [28]. These mechanisms may have an impact on the clot structure, a risk factor for thrombosis, with an abnormal clot structure featuring in several thrombotic disorders [29–31]. The role of GPVI, specifically in the context of its interaction with fibrin, in procoagulant platelet formation and modulation of clot structure remains to be established.

In this study, we determined the impact of GPVI-fibrin interaction on procoagulant platelet development and characterized the effects of GPVI signaling inhibitors on the clot structure and function using a range of sensitive methods to analyze the procoagulant platelet number, clot density, clot porosity, and clot retraction. To compare with fibrin-integrin interactions, the impact of tyrosine kinase inhibitors on the clot structure and function was compared with effects observed following αIIbβ3 inhibition. Then, we found that fibrin-GPVI interaction contributes to the development of procoagulant platelets and that the clot structure and function are altered by the inhibition of fibrin-GPVI interaction and its subsequent tyrosine kinase-mediated signaling.

Essentials

- The effect of glycoprotein VI (GPVI)-fibrin interaction on procoagulant platelets and clot structure is not well known.
- The absence of GPVI and blockage of GPVI-fibrin interaction decreases procoagulant platelet number.
- Inhibition of GPVI signaling reduces procoagulant platelet number and alters clot structure.
- Inhibition of GPVI-fibrin interactions alleviates prothrombotic clot phenotype.

2 | MATERIALS AND METHODS

2.1 | Materials

Reagents were purchased from Thermo Fisher Scientific and stored at −20 °C, unless otherwise stated. The additional details of materials are included in the Supplementary material. The targets of tyrosine kinase inhibitors used in this study on the GPVI signaling cascade are shown in Supplementary Figure S1. The generation and characterization of GPVI-targeting Affimers, including functional parameters and aggregation data, will be described separately (manuscript in preparation). In short, Affimers were generated against a monomeric GPVI construct (D1 and D2 domains) and a dimeric GPVI-fragment crystallizable region fusion construct. Randomly selected clones were tested for binding to GPVI by phage enzyme-linked immunosorbent assay. GPVI-Affimer affinity measurements were performed by surface plasmon resonance. The competition between cross-linked collagen-related peptide/fibrinogen and Affimers binding to GPVI was characterized by MST and competitive ELISA. Recombinant wild-type

(WT) and α220 fibrinogen, lacking the αC-region including both the αC-terminal domain and the connector, were expressed in Chinese hamster cells and purified in house, as previously described [32].

2.2 | Murine samples

Comparisons were made between GPVI-deficient ($GPVI^{-/-}$; C57BL/6J background) and WT (WT, C57BL/6J) mice. The animals were maintained in individually ventilated cages at 21 °C with 50% to 70% humidity, light/dark cycle of 12/12 hours, and on standard diet ad libitum. Blood was collected from the inferior vena cava of anesthetized mice in 10% sodium citrate, in accordance with UK Home Office–approved procedures (nonrecovery) under the project license P144DD0D6. This whole blood was immediately centrifuged at 100 g for 5 minutes, without brakes, to obtain platelet-rich plasma (PRP). The platelet count in PRP samples was determined using a Beckman Coulter Z2 cell and particle counter. Murine PRP clots for scanning electron microscopy (SEM) were prepared as previously described [33,34], where clotting was initiated with thrombin (1 U/mL) and $CaCl_2$ (10 mM). Images were analyzed, and the platelet number was determined by counting the number of cells in a 6 × 8 grid at 20 000× magnification using ImageJ (v2.0, National Institutes of Health).

2.3 | Human blood collection

Blood samples were collected from the antecubital vein of 16 healthy volunteers in 0.109 M sodium citrate vacutainers. Informed written consent was obtained for blood donation according to the Declaration of Helsinki. Ethical approval for the study was granted by the University of Leeds Medicine and Health Faculty Research Ethics Committee (reference number: HSLTLM12045). Whole blood was used within 1 hour or immediately processed to obtain PRP or washed platelets. Then, it was centrifuged at 100 g for 20 minutes, without brakes, to obtain PRP. To obtain washed platelets, whole blood was diluted at a 2:1 ratio with warm modified Tyrode buffer and centrifuged at 230 g for 20 minutes, followed by the addition of apyrase (0.2 U/mL) and indomethacin (10 μM) and centrifugation at 980 g for 10 minutes. The platelet pellet was resuspended in warm modified Tyrode buffer and rested at 37 °C for 30 minutes prior to use.

2.4 | Scanning electron microscopy

Whole blood clots for SEM were prepared as previously described [33,34]. In short, GPVI signaling inhibitors were added to samples in varying concentrations in the absence or presence of eptifibatide. After 30 minutes, clotting was initiated with the activation mixture consisting of tissue factor (platelet-poor plasma-reagent, 1 pM) and $CaCl_2$ (10 mM). The samples were incubated at 37 °C in a humidity chamber for 2 hours and then immediately fixed in 2% glutaraldehyde (Sigma-Aldrich) overnight. Following washing with sodium cacodylate buffer (50 mM of $C_2H_{12}AsNaO_5$, pH 7.4, Sigma-Aldrich), the clots were dehydrated with increasing concentrations of acetone (30%-100%, Sigma-Aldrich) and further processed by critical point drying with CO_2. Prior to imaging on Hitachi SU8230 high-performance cold field emission (CFE) SEM (Chiyoda Corporation), the clots were sputter coated with 10 nm of iridium using a Cressington 208 HR (Cressington Scientific Instruments). ImageJ (v2.0, NIH) was used to add a magnified section to images.

2.5 | Laser scanning confocal microscopy

Sample preparation and imaging were performed as previously described [33]. Briefly, PRP was diluted at 1:6 with saline. Annexin V (1:8) was added to the samples and allowed to permeate for 30 minutes away from light. Affimers targeting GPVI-fibrin(ogen) interaction or GPVI signaling inhibitors (or dimethyl sulfide [DMSO] equivalent) were added to the samples in the absence or presence of eptifibatide. The samples were spiked with 50 μg/mL of Alexa Fluor 488-fibrinogen and clotting was triggered with the addition of thrombin (0.1 U/mL) and $CaCl_2$ (5 mM). The clotting of PRP was also initiated with tissue factor (1 pM, 24 μM of phospholipids). The same procedure using thrombin (0.1 U/mL) was followed for purified recombinant WT and α220 fibrinogen (0.5 mg/mL) clots with isolated platelets (2.3×10^8 platelets/mL) and 25-μg/mL–labeled fibrinogen. The clotting mixture was immediately transferred to Ibidi uncoated μ-Slide VI 0.4 mm (Ibidi GmbH) and placed in a dark humidity chamber for 2 hours prior to imaging. Imaging was performed using a Zeiss LSM700 inverted microscope (Carl Zeiss AG) with a 40× oil immersion lens. Optical z-stacks (45 × 0.7 μm) were combined, flattened, and projected using maximum intensity. The fiber density was determined by the average of fibers crossing an arbitrary straight line of fixed length (160 μm). Three fiber density measurements were obtained per clot. Image processing and measurements of fiber density and procoagulant platelet number were performed on ImageJ (version 2.0, NIH).

2.6 | Thrombin generation

Thrombin generation was measured using a calibrated automated thrombogram system based on the method of Hemker et al. [35]. Reagents were purchased from Stago. PRP in the absence and presence of GPVI signaling inhibitors, eptifibatide, and DMSO controls was loaded onto round-bottom 96-well plates in triplicates. Saline was used for volume adjustment. The thrombin calibrator (690 nM activity) was added to calibrator wells, and the PRP (1.2 pM of tissue factor) or platelet-poor plasma (6 pM of tissue factor, 24 μM of phospholipids) reagent for controls was added to the remaining wells. Thrombin generation was initiated by the addition of fluorogenic substrate Z-Gly-Gly-Arg-aminomethylcoumarin and 16.7 mM of $CaCl_2$. Fluorescence measurements were obtained every 20 seconds for 120 minutes using a Fluoroscan Ascent reader (Thermo Fisher Scientific). Data obtained were analyzed by the dedicated Hemker Thrombinoscope software (Stago). Peak thrombin was obtained from thrombin generation curves.

2.7 | Clot permeation

The clot permeability analysis was performed as previously described [36]. The PRP samples were diluted at a 1:6 ratio with saline. GPVI signaling inhibitors (or DMSO equivalent ones) were added to the samples in the absence or presence of eptifibatide. After 30 minutes, clotting was initiated by the addition of thrombin (0.5 U/mL) and $CaCl_2$ (10 mM). The clotting mixture was immediately transferred to Ibidi uncoated μ-Slide VI 0.4 mm (Ibidi GmbH) and placed in a humidity chamber for 1 hour. Constant pressure was applied to each clot by filling a plastic syringe, which was connected to the wells containing clots, to 4 cm. The volume (correlated to weight, assuming 1 g = 1 mL) of flow-through over time was plotted and fitted by linear regression ($R^2 \geq 0.99$) to determine the buffer flow rates through the clot. The permeation coefficient (Darcy constant [K_s]) was determined as previously described [37].

2.8 | Whole blood clot retraction

Whole blood was diluted at a 1:3 ratio with saline in a siliconized flat-bottom glass test tube (7.25 × 55 mm; Bio/Data) pretreated with Sigmacote (Sigma-Aldrich). GPVI signaling inhibitors were added to samples in the absence or presence of eptifibatide. After 30 minutes, clotting was initiated with tissue factor (1 pM, 24 μM of phospholipids) and $CaCl_2$ (10 mM). The test tubes were incubated at 37 °C for 2 hours. The supernatant was then removed, and clots were weighed. The percentage clot retraction over time was determined from images (every 15 minutes) using ImageJ (version 2.0, NIH).

Additional methods are included in the Supplementary material.

2.9 | Statistics

Data are presented as the mean of at least 3 replicates ± SEM. "n" refers to the number of independent individual repeats, in which technical repeats for each experiment were averaged and account for 1 repeat. The normal distribution of data was assessed with the Shapiro-Wilk test for normality. The differences between conditions were determined by 1-way analysis of variance or a Kruskal-Wallis test followed by a Tukey-Kramer or Dunn-Bonferroni post-hoc test to determine significance. Differences between WT and $GPVI^{-/-}$ groups were determined by a 2-tailed homoscedastic Student's *t*-test. *p* values of <.05 were considered statistically significant. Data analysis was performed using GraphPad Prism 9 (version 9.1.1).

3 | RESULTS

3.1 | Absence of GPVI in mice reduced the number of procoagulant platelets

SEM images of WT and GPVI-deficient ($GPVI^{-/-}$) murine PRP clots were obtained following clotting triggered with thrombin. Representative images of WT (Figure 1A–C) and $GPVI^{-/-}$ (Figure 1D–F) PRP clots showed the fibrin network, nonprocoagulant or aggregatory platelets (indicated by yellow arrows) displaying their distinctive filopodia, and (presumed) procoagulant platelets (indicated by light-blue arrows). The number of nonprocoagulant and procoagulant platelets in WT and $GPVI^{-/-}$ clots was determined from SEM images based on their distinctive morphology (Figure 1G). The percentage of procoagulant to nonprocoagulant platelets in WT clots was similar (49% ± 13% procoagulant platelets). In contrast, significantly less procoagulant platelets were present in the $GPVI^{-/-}$ clots compared with nonprocoagulant platelets (29% ± 6% procoagulant, $p < .05$; Figure 1G). There was no difference in total platelet count between $GPVI^{-/-}$ and WT murine PRP samples (Supplementary Figure S2A). To confirm these observations, differences in the procoagulant platelet number in these samples were further assessed by confocal microscopy, where procoagulant platelets were quantified using annexin V staining following clotting triggered by thrombin. Representative images of annexin V distribution in labeled platelets, showing PS exposure on the procoagulant platelet surface and accumulation in platelet "cap," are shown in Supplementary Figure S3. There were significantly fewer annexin V–positive platelets in the $GPVI^{-/-}$ murine PRP samples than that were in the WT samples ($p < .05$; Figure 1H, Ji, Ki, Supplementary Figure S4). Furthermore, the confocal microscopy analysis also allowed us to concurrently determine the impact of GPVI deficiency on the clot structure via the measurement of fiber density, visually and quantitatively. Albeit not significant, the average fiber number as analyzed by confocal microscopy showed a trend toward decrease in $GPVI^{-/-}$ murine PRP samples compared with WT (Figure 1I, Jii, Kii, Supplementary Figure S4). $GPVI^{-/-}$ mice showed slightly higher levels of fibrinogen than WT mice mice (Supplementary Figure S2B), and so, reduced fiber density observed in $GPVI^{-/-}$ clots were not attributable to a lower fibrinogen concentration. Clotting was induced in the absence of collagen, and therefore, changes observed in procoagulant platelet number in these samples were likely attributable to platelet interactions with fibrin.

3.2 | Inhibition of GPVI-fibrin(ogen) binding by Affimers

The results from this section onward refer to human samples. We performed phage-display screening for Affimers [38] that specifically bind GPVI, the detailed characterization of which will be published separately (Xu et al., in preparation). Affimer reagents are small nonantibody-binding proteins with 2 variable regions for molecular recognition (Supplementary Figure S5). They have previously proved to be useful reagents for modulating proteins involved in fibrinolysis [39]. Affimer reagents positive for GPVI binding were next tested for the inhibition of fibrinogen binding to GPVI by competitive ELISA (Supplementary Figure S6). Three Affimer reagents (D16, M14, and M17) inhibiting GPVI-fibrin(ogen) interaction were next used in the investigations of procoagulant platelet and fibrin fiber number. The procoagulant platelet number was significantly decreased in the

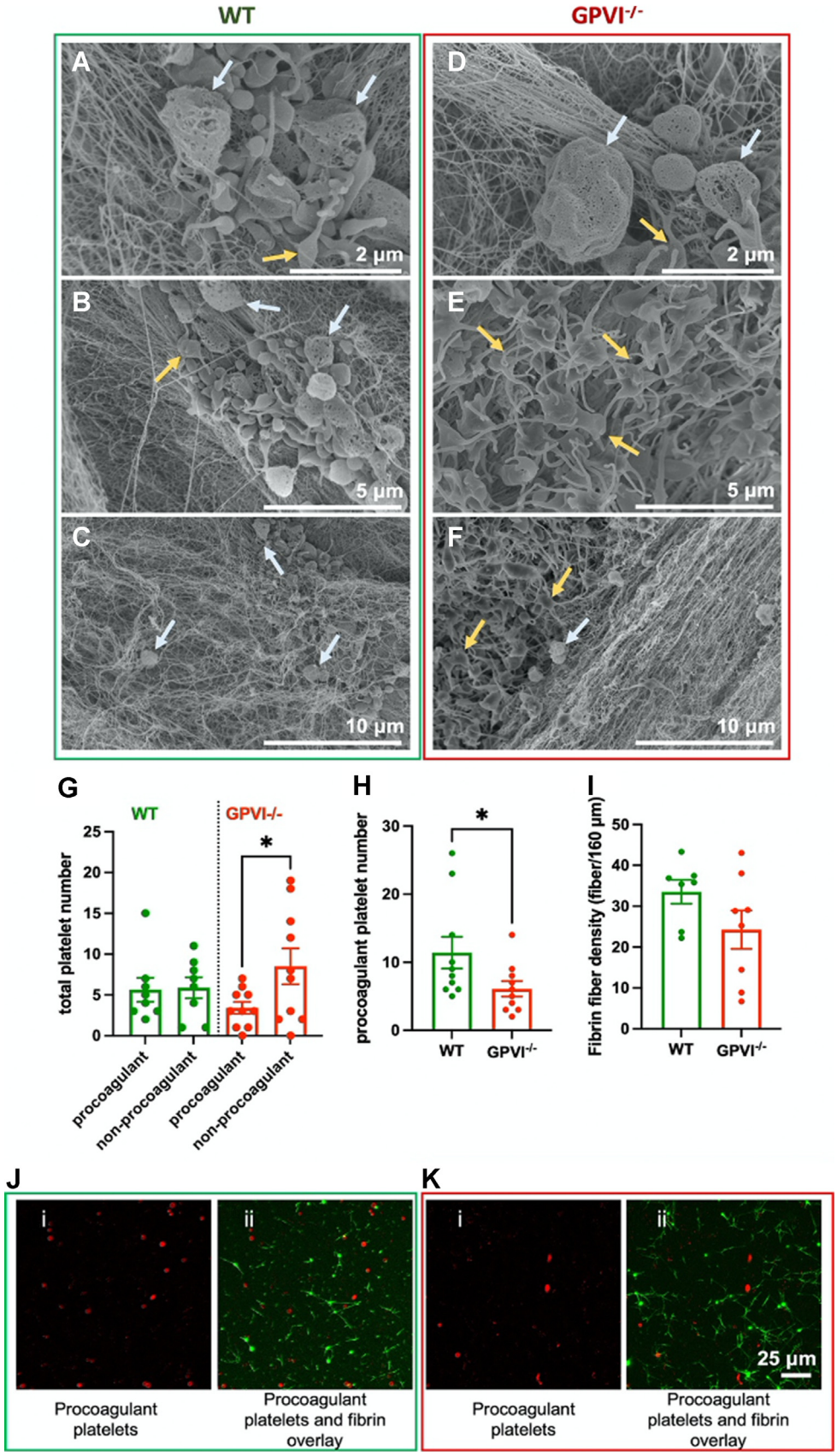

FIGURE 1 Platelet characterization and quantification in wild-type (WT) and glycoprotein VI (GPVI)-deficient ($GPVI^{-/-}$) murine clots. Representative SEM images of WT (A–C) and $GPVI^{-/-}$ (D–F) murine PRP clots under different magnifications. Examples of procoagulant

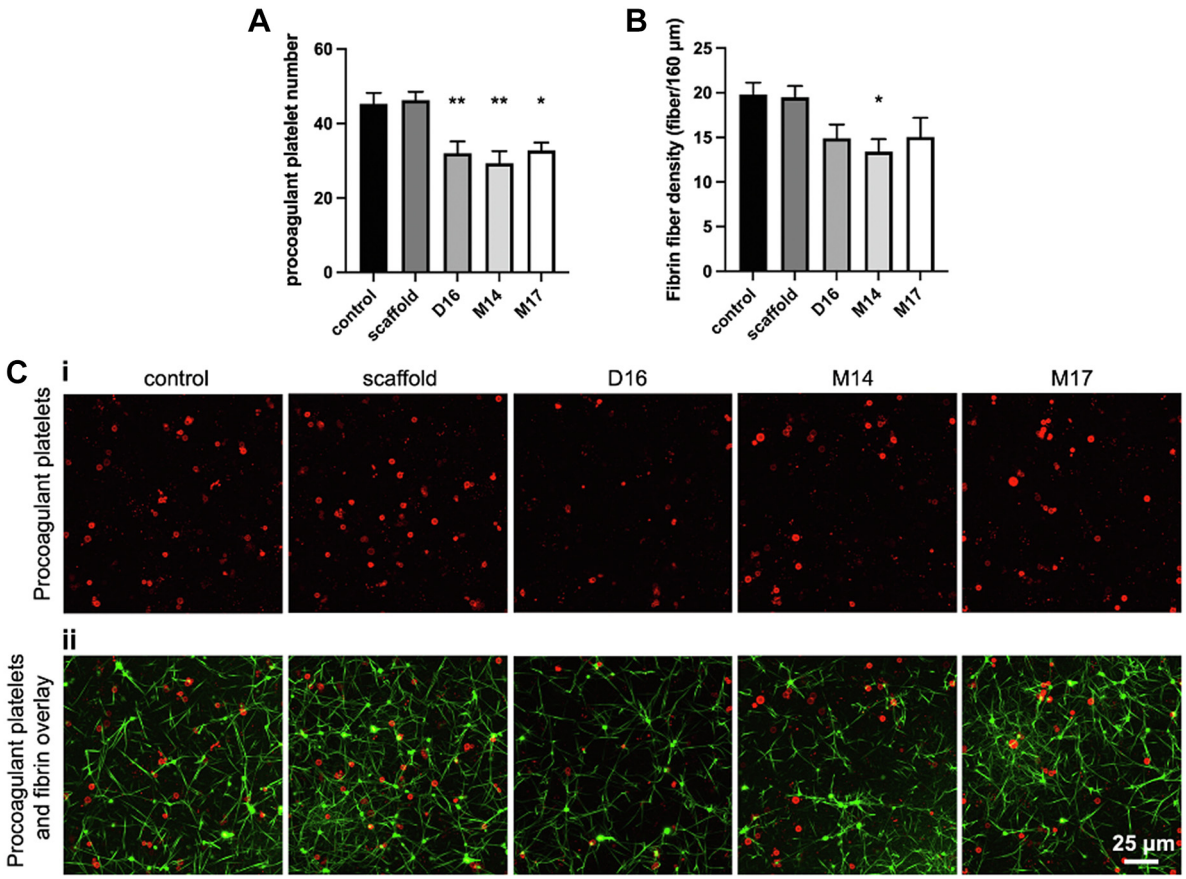

FIGURE 2 Procoagulant platelet quantification and fibrin fiber density in clots formed in the presence of Affimers. (A) Procoagulant platelet number and (B) fibrin fiber density of platelet-rich plasma (PRP) clots in the presence of 10 μM of Affimers targeting glycoprotein VI (GPVI)-fibrin(ogen) interaction. "Control" refers to clots formed in the absence of Affimers, and "scaffold" refers to a protein scaffold of similar size to Affimers but with no binding specificity, which is used as a control for the specific effects of Affimers on GPVI-fibrin interaction. Thrombin was used to initiate clotting. Results are shown as mean ± SEM, n = 4. *p < .05 and **p < .01 difference from control. (C) Representative confocal images of platelet-rich plasma (PRP) clots in the absence (control) and presence of scaffold, Affimer D16, Affimer M14, and Affimer M17. (i) Top images show procoagulant platelets only, and (ii) bottom images show the fibrin fiber overlay. Scale bar represents 25 μm.

presence of all Affimers ($p < .05$) compared with control (Figure 2A, Ci, Supplementary Table S1, Supplementary Figure S7) when clotting was triggered by thrombin. Moreover, there appeared to be a trend toward reduced average fiber number in the presence of all Affimers compared with control, with a significant change observed in the presence of Affimer M14 ($p < .05$) (Figure 2B, Cii, Supplementary Table S1, Supplementary Figure S7). No differences in procoagulant platelet number or fiber density were observed with Affimer scaffold–only protein control, which contains 3 alanines in the variable regions and showed no affinity toward GPVI.

3.3 | Inhibition of GPVI signaling

We next investigated the effects of GPVI signaling inhibitors on procoagulant platelet development and fiber number induced by fibrin-GPVI interaction–followed clotting triggered by thrombin. Compared with control, PRT-060318 and ibrutinib significantly reduced procoagulant platelets numbers at all 3 concentrations tested ($p < .05$) (Figure 3A, B, Gi; Supplementary Table S2; Supplementary Figure S8). Dasatinib also significantly decreased the number of procoagulant platelets relative to control at the highest concentration tested ($p < .0001$) when compared

platelets are indicated by light-blue arrows and those of nonprocoagulant platelets are indicated by yellow arrows. (G) Comparison of procoagulant and nonprocoagulant platelets in WT and GPVI$^{-/-}$ clots; GPVI$^{-/-}$ clots showed relatively fewer procoagulant and more nonprocoagulant platelets (29% ± 6% vs 71% ± 19%, respectively) than WT clots (49% ± 13% vs 51% ± 11%, respectively), which were measured from SEM images. (H) Procoagulant platelet number and (I) fibrin fiber density in WT and GPVI$^{-/-}$ clots measured by confocal microscopy; GPVI$^{-/-}$ clots showed relatively fewer procoagulant platelets and reduced fiber density (6 ± 1 and 26 ± 5, respectively) than WT clots (11 ± 2 and 33 ± 3, respectively). Thrombin was used to initiate clotting. Representative confocal images of (J) PRP WT and (K) GPVI$^{-/-}$ clots showing (i) procoagulant platelets only and (ii) fibrin fiber overlay. Scale bars represent 2 μm (A, D), 5 μm (B, E), 10 μm (C, F), and 25 μm (J, K). Results shown as mean ± SEM, n = 10 (G. H) or n = 8 (I). *$p < .05$. PRP, platelet-rich plasma; SEM, scanning electron microscopy.

with control (Figure 3C, Gi; Supplementary Table S2; Supplementary Figure S8). As the GPVI signaling inhibitors were solubilized in DMSO, the effect of different concentrations of this solvent was also investigated, with no differences in procoagulant platelet number observed (Supplementary Figure S9A). The procoagulant platelet number was not impacted by the addition of eptifibatide (Figure 3A–C, Gi, Hi; Supplementary Table S2; Supplementary Figure S8). Interestingly, the effects of all 3 GPVI signaling inhibitors at decreasing procoagulant platelet number were significantly exacerbated in the presence of eptifibatide ($p < .0001$) (Figure 3A–C, Hi; Supplementary Table S2; Supplementary Figure S8). A comparable decrease in procoagulant platelet number by PRT-060318, ibrutinib, and dasatinib was also observed when the tissue factor was used to initiate clotting (Supplementary Figure 10A, Ci, Di; Supplementary Table S3).

The average fiber number, corresponding to the clot fiber density, was significantly decreased in the presence of PRT-060318 ($p < .05$) relative to control, while in the presence of eptifibatide, PRT-060318 also significantly reduced fiber density ($p < .001$) compared with the eptifibatide-only sample (Figure 3D, Gii, Hii; Supplementary Table S2; Supplementary Figure S8). The same trend was observed for ibrutinib relative to control ($p < .01$) and eptifibatide-only sample ($p < .0001$; Figure 3E, Gii, Hii; Supplementary Table S2; Supplementary Figure S8). There were no changes in fiber density in the presence of dasatinib relative to control, and nevertheless, dasatinib in the presence of eptifibatide led to a reduction in fiber density ($p < .01$) compared with eptifibatide-only sample (Figure 3F, Gii, Hii; Supplementary Table S2; Supplementary Figure S8). No significant difference in fiber density was observed with DMSO, suggesting that the effects observed are attributable to the inhibitors not the solvent (Supplementary Figure S9B). Relative to control, fiber density was significantly increased ($p < .0001$) in the presence of eptifibatide (Figure 3D–F, Gii, Hii; Supplementary Table S2; Supplementary Figure S8). The impact of GPVI signaling inhibitors on decreasing fiber density was more pronounced in the absence of eptifibatide (Supplementary Table S2). A similar reduction in fibrin fiber density was observed in the presence of PRT-060318, ibrutinib, and dasatinib, when clotting was initiated with tissue factor (Supplementary Figure S10B, Cii, Dii; Supplementary Table S3).

The procoagulant platelet number was decreased in the presence of PRT-060318, ibrutinib, and dasatinib in clots made with recombinant WT fibrinogen clots and washed platelets following clotting triggered by thrombin (Figure 4A, B). However, these inhibitors had no effect on clots formed with α220 fibrinogen, a truncated form of fibrinogen lacking the αC-region, which is important for fibrin(ogen)-GPVI binding [25] (Figure 4A, C; Supplementary Table S4; Supplementary Figure S11). An abnormal fibrin fiber structure was observed for α220 fibrinogen clots (Supplementary Figure S12), as previously characterized [32]. The number of procoagulant platelets, adjusted for clot volume to account for the weakened clot structure, was decreased in α220 fibrinogen clots compared with WT clots.

Maximal procoagulant platelet response has been reported to be achieved by PAR1 and GPVI activation [8,17]. The procoagulant platelet number was not significantly altered by the addition of the PAR1 agonist TFLLR-NH_2 and antagonist vorapaxar when clotting was initiated with thrombin; a trend toward increased and reduced procoagulant platelet number was observed for TFLLR-NH_2 and vorapaxar, respectively (Supplementary Table 5, Figure 13). Addition of ibrutinib and PRT-060318 significantly reduced the procoagulant platelet number in the presence of TFLLR-NH_2 and vorapaxar, respectively, supporting a collaborative role of PAR1 and GPVI in maximal procoagulant platelet formation. Fiber density was unchanged by TFLLR-NH_2, whereas vorapaxar alone and in the presence of PRT-060318 and ibrutinib significantly decreased the fiber density (Supplementary Table 5; Figure 13).

3.4 | Thrombin generation and clot permeability

We also observed a significant decrease and increase in peak thrombin generation in the presence of the GPVI signaling inhibitors and eptifibatide, respectively (Figure 5A–E, Supplementary Table S6), indicating that the changes in fiber density observed are, at least in part, due to altered peak thrombin generation. Furthermore, the opposing effects on peak thrombin generation observed in the presence of the GPVI signaling inhibitors and eptifibatide elucidate the less pronounced effects of the GPVI signaling inhibitors on fiber density following the addition of eptifibatide. DMSO did not affect peak thrombin generation (Supplementary Figure 9C). These data complement our observations in $GPVI^{-/-}$ mice, suggesting a trend toward decreased fiber density in the absence of GPVI. Furthermore, they provide further evidence that procoagulant platelet development is at least in part mediated by GPVI-fibrin interaction and that these changes may have downstream effects on the clot structure. Thrombin generation on platelet-poor plasma was conducted as a control, showing no unanticipated direct effects of PRT-060318, ibrutinib, and dasatinib on the coagulation cascade (Supplementary Figure S14).

The clot pore size was determined by measuring the flow rate of buffer passing through the clot (thrombin used as clotting trigger) over time, from which the permeation coefficient (Ks) is calculated. The clot pore size (cm^2) is indicative of clot architecture and correlates with susceptibility to lysis, where clots with smaller pores (lower Ks value) and more densely packed fibrin fibers lyse at slower rates [36,40]. The K_s of PRP clots was significantly increased in the presence of PRT-060318 at 10 μM, ibrutinib at all concentrations tested, and dasatinib at 100-200 nM ($p < .05$), indicating larger pores relative to control (Figure 5F–H, Supplementary Table S2). In the presence of eptifibatide, only ibrutinib significantly increased the clot pore size relative to eptifibatide-only sample ($p < .05$) (Figure 5G, Supplementary Table S2). Clot pore size was not significantly affected by DMSO (Supplementary Figure S9D). The effects of the GPVI signaling inhibitors on clot pore size were more pronounced in the absence of eptifibatide (Supplementary Table S2).

3.5 | Clot retraction

We next analyzed whole blood clot development and retraction over time (120 minutes) following clotting triggered by tissue factor. Although differences in the percentage of whole blood clot retraction over time up to 105 minutes were observed for PRT-060318 and

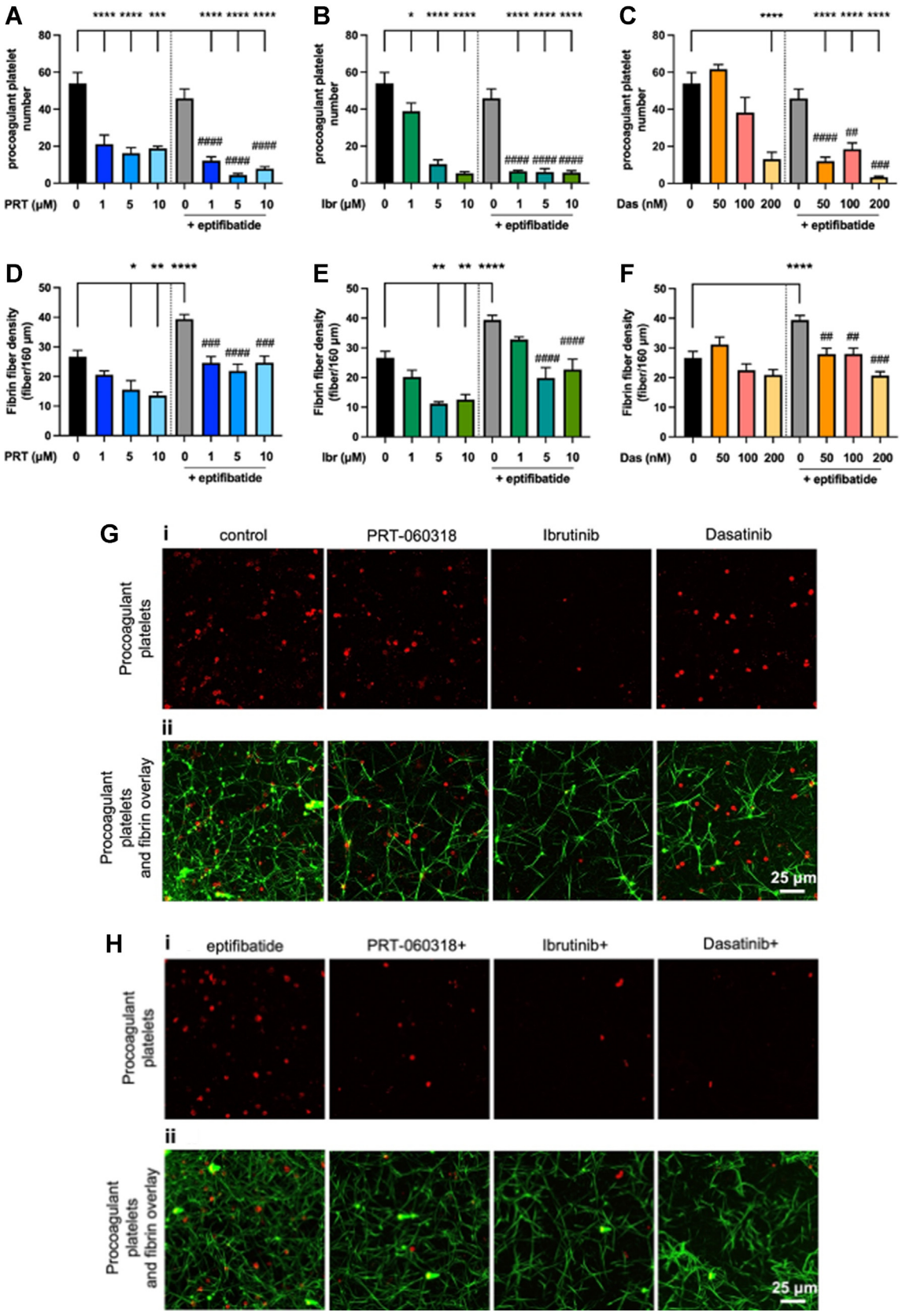

FIGURE 3 Procoagulant platelet quantification and fibrin fiber density in clots formed in the presence of glycoprotein VI (GPVI)-signaling inhibitors and eptifibatide. Procoagulant platelet count (top) and fibrin fiber density (bottom) of platelet-rich plasma (PRP) clots in the presence of varying concentrations of GPVI signaling inhibitor PRT-060318 (PRT; A, D), ibrutinib (Ibr; B, E), or dasatinib (Das; C, F) without and with (represented by '+') 25 µM of eptifibatide. Thrombin was used to initiate clotting. Results are shown as mean ± SEM, n = 4. *$p < .05$, **$p < .01$,

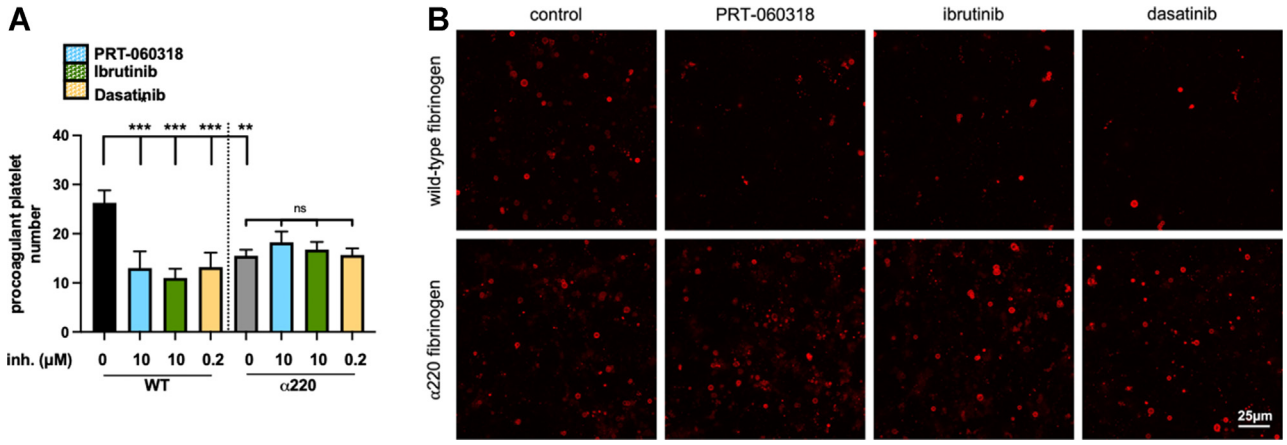

FIGURE 4 Procoagulant platelet quantification in clots made with recombinant purified wild-type (WT) and α220 fibrinogen in the presence of glycoprotein VI (GPVI)-signaling inhibitors. (A) Procoagulant platelet count of clots made with washed platelets (2.3×10^8 platelets/mL) and 0.5 mg/mL of recombinant purified WT and α220 fibrinogen (lacking the αC-region, C-terminal domains, and connectors) in the absence or presence of tyrosine kinase inhibitors (inh) PRT-060318 (10 μM), ibrutinib (10 μM), and dasatinib (200 nM). Procoagulant platelet number was adjusted by clot volume; WT fibrinogen clot volume = 788 480 μm^3 and α220 fibrinogen clot volume = 350 720 μm^3. Thrombin was used to initiate clotting. Results are shown as mean ± SEM, n = 2. $^{***}p < .001$ and $^{**}p < .01$ difference from WT control. Nonsignificant changes are represented by "ns." (B) Representative images of procoagulant platelets in clots made with WT and α220 fibrinogen. Scale bar represents 25

dasatinib (Supplementary Figure S15A, C), only ibrutinib impacted clot retraction beyond this time point (Supplementary Figure S15B) and significantly increased the clot weight at endpoint (120 minutes; $p < .05$) relative to the control (Figure 6A, B; Supplementary Table S7). Whole blood clots formed in the presence of eptifibatide showed impaired clot retraction over time (Supplementary Figure S15D) and increased clot weight at endpoint when compared with control ($p < .001$) (Figure 6A, B, Supplementary Table S7); however, the addition of GPVI signaling inhibitors did not further exacerbate this effect (Figure 6A, B; Supplementary Table S7). The SEM images of contracted whole blood clots showed that polyhedrocyte formation, a deformation of red blood cells driven by clot retraction, was reduced in the presence of eptifibatide (Figure 6C, D), whereas polyhedrocyte formation appeared unaffected in the presence of GPVI signaling inhibitors or DMSO (Figure 6E–H). Altogether, these data are consistent with a greater effect of eptifibatide and thereby αIIbβ3 than GPVI signaling inhibitors or GPVI on clot retraction.

Complementary to the results obtained for whole blood clot retraction, eptifibatide abolished washed platelet retraction entirely (Figure 6I, J). There were no significant changes in the percentage of washed platelet clot retraction observed in the presence of the GPVI signaling inhibitors (Figure 6J).

4 | DISCUSSION

Our study showed that the interaction of GPVI with fibrin contributes to the development of procoagulant platelets and that GPVI signaling inhibition has downstream effects on the fibrin clot structure/function. We showed fewer procoagulant platelets by 3 different mechanisms impeding GPVI-fibrin interaction: 1) absence of GPVI ($GPVI^{-/-}$ samples), 2) GPVI-ligand binding inhibition (Affimers), and 3) GPVI signaling inhibition (tyrosine kinase inhibitors). For the latter mechanism, integrin inhibition was used as a control, showing that blockage of integrin alone did not impact the procoagulant platelet number. Although previous reports have suggested that GPVI is involved in platelet procoagulant activity [24,27], currently, there are no reports exploring the potential impact of platelet procoagulant activity on the clot structure and function. The role of GPVI-collagen in thrombus initiation is well-established [1,41,42] and the impact of fibrin interaction with GPVI on thrombus consolidation and/or propagation has been previously observed [24,27,43]. Nevertheless, whether this specific interaction has potential as a target for novel therapeutics remains to be fully explored. GPVI blockage has previously been investigated in phase 2 trials with revacept, a dimeric fusion protein of the extracellular portion of GPVI, which did not lead to differences in myocardial injury in patients with stable ischemic heart disease undergoing percutaneous coronary intervention [44,45]. Currently, glenzocimab is at phase 2 stage following promising results from first-in-human trials [16]. Unlike previous studies using GPVI blockers, our study provides evidence for an important interplay between platelet GPVI and fibrin(ogen) specifically and highlights the importance of their synergistic contribution in the development of a prothrombotic phenotype.

Our data indicate a central new mechanism through which targeting GPVI may reduce thrombosis by impairing fibrin-GPVI

$^{***}p < .001$, and $^{****}p < .0001$ difference from control. Differences from eptifibatide only are represented by $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$, and $^{\#\#\#\#}p < .0001$. Representative confocal images of PRP clots in the (G) absence or (H) presence of GPVI signaling inhibitors without or with the addition of 25 μM of eptifibatide, indicated by "+"; PRT-060318 (at 10 μM); ibrutinib (at 10 μM); or dasatinib (at 200 nM). (i) Top images show procoagulant platelets only, and (ii) bottom images show the fibrin fiber overlay. Scale bar represents 25 μm.

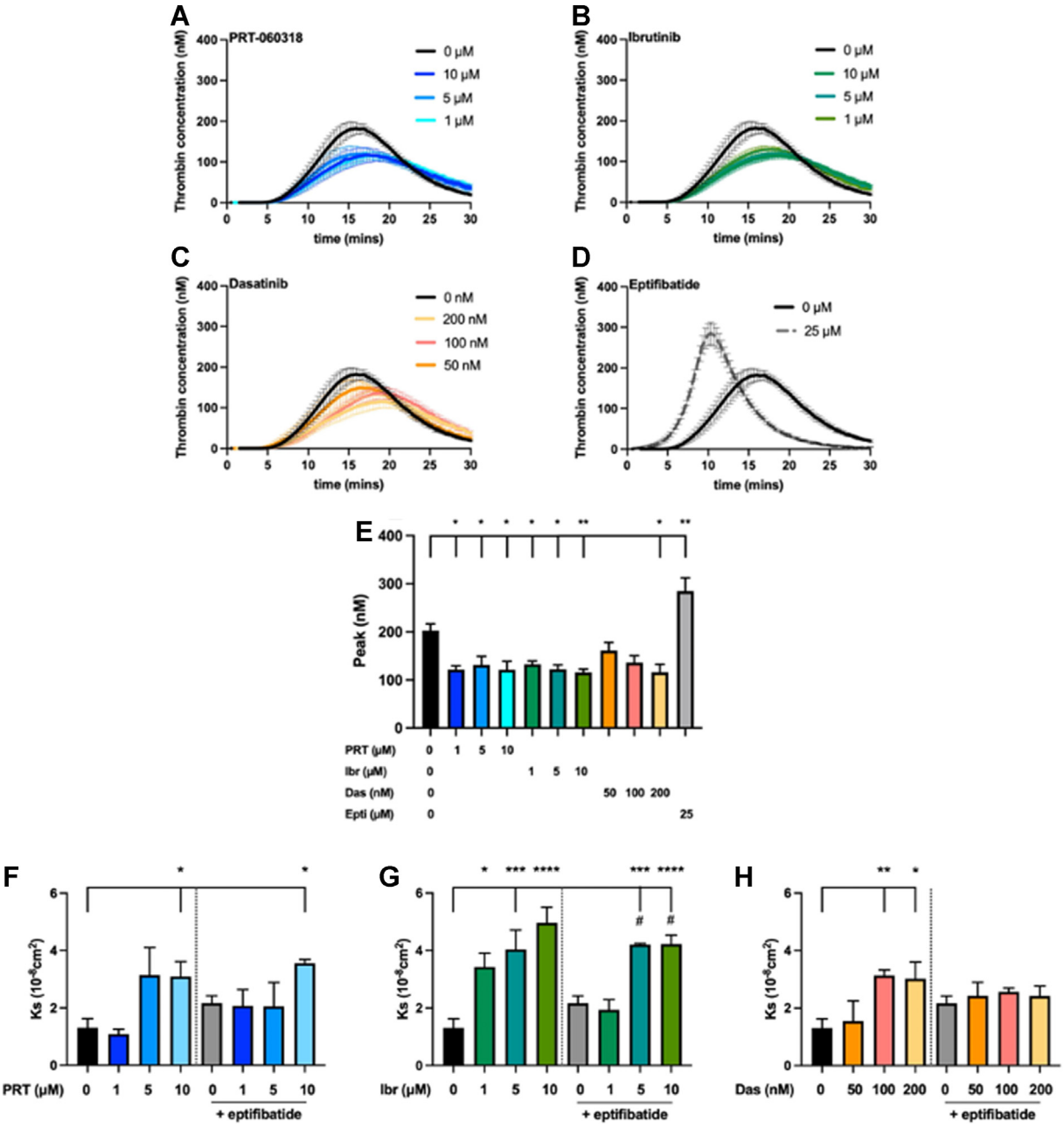

FIGURE 5 Thrombin generation and porosity of clots formed in the presence of glycoprotein VI (GPVI) signaling inhibitors and eptifibatide. Thrombin generation curves of platelet-rich plasma (PRP) clots in the presence of varying concentrations of the GPVI signaling inhibitor (A) PRT-060318, (B) ibrutinib, (C) dasatinib, or (D) eptifibatide at 25 μM. (E) From thrombin generation curves, the peak thrombin generation was determined. Porosity of PRP clots was determined by the permeation coefficient (K_s). Higher K_s values are indicative of a more porous fibrin fiber network, and lower K_s values are indicative of a less porous fibrin fiber network. Pore size of clots in the presence of varying concentrations of the GPVI signaling inhibitor (F) PRT-060318, (G) ibrutinib, and (H) dasatinib without and with (represented by "+") the addition of 25 μM of eptifibatide. Results are shown as mean ± SEM, n = 4. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, and $^{****}p < .0001$ difference from control. Differences from eptifibatide only in F–H are represented by $^{\#}p < .05$ and $^{\#\#}p < .01$.

interaction–mediated procoagulant platelet formation and subsequent improvements in clot structural characteristics. We observed a trend toward decreased fiber density in GPVI$^{-/-}$ samples and following GPVI-fibrin inhibition with Affimers, which is likely attributed to reduced procoagulant platelet numbers. Additionally, novel observations of the effect of GPVI signaling inhibition on the fibrin clot structure were determined using tyrosine kinase inhibitors. We observed significant reductions in procoagulant platelet and fiber number by PRT-060318 and ibrutinib, and dasatinib reduced procoagulant platelets at higher concentrations. It is important to note that tyrosine kinase inhibitors may interfere with other pathways distinct from those associated with GPVI-fibrin binding, and further

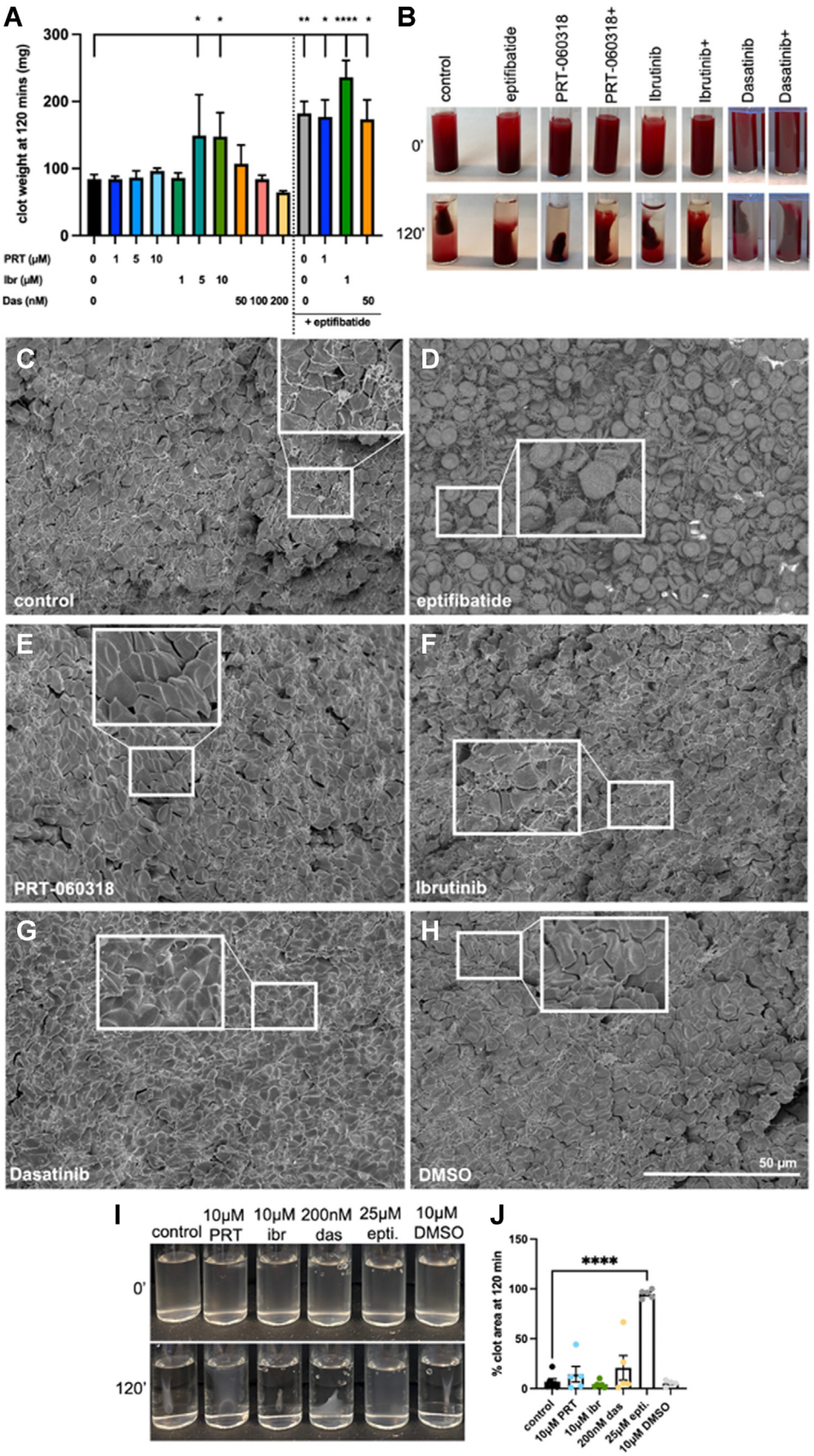

FIGURE 6 Whole blood clot and washed platelet retraction in the presence of glycoprotein VI (GPVI)-signaling inhibitors and eptifibatide. (A) Final clot weight (at 120 minutes) following clot retraction of clots formed in the presence of varying concentrations of the GPVI signaling inhibitor PRT-060318 (PRT), ibrutinib, or dasatinib without or with (represented by "+") 25 μM of eptifibatide. Clotting was initiated with tissue factor. Results shown mean ± SEM, n = 3. *$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$ difference from control. (B) Representative images of whole blood clots at time 0 and 120 minutes (end point) in the presence of GPVI signaling inhibitor eptifibatide (±25 μM). Representative SEM images of fully formed whole blood clots following clot retraction without (C) or (D) with 25 μM of eptifibatide or (E) GPVI signaling inhibitor PRT-060318 (10 μM), (F) 10 μM of ibrutinib, (G) 200 nM of dasatinib, or (H) 10 μM of DMSO equivalent. Scale bar represents 50 μm. Larger rectangles (inset) are a 2× magnified representation of smaller rectangles. (I) Representative images of washed platelet retraction at 0 and 120 minutes (end point) in the presence of the GPVI signaling inhibitor PRT-060318 (PRT), ibrutinib (Ibr) or dasatinib, 25 μM of eptifibatide (epti.) only sample, or DMSO equivalent. (J) Clot retraction at endpoint (120 minutes) was determined as a percentage clot area from time zero. Results shown as mean ± SEM, n = 3. ****$p < .0001$. DMSO, dimethyl sulfide; SEM, scanning electron microscopy.

research is required to characterize the specific mechanisms by which these inhibitors contribute to decreased procoagulant platelet formation and altered clot structure. Nevertheless, we showed that the procoagulant platelet number was decreased in recombinant purified WT clots with washed platelets, but not in the α220 fibrinogen clots, in the presence of GPVI signaling inhibitors. We recently showed that the αC-region, which is absent in α220 fibrinogen, is important for fibrin(ogen)-GPVI binding [25]. Therefore, this further supports our observations that GPVI-fibrin binding plays a role in the development of procoagulant platelets and that the fibrinogen αC-region is critical for this interaction. The strikingly abnormal structure of α220 fibrinogen clots has previously been shown to drastically impact fibrinolytic and mechanical resistance [32]. The procoagulant platelet number, adjusted for clot volume, was reduced in α220 fibrinogen compared with WT clots, further suggesting a role for the αC-region on GPVI-fibrin interaction. Furthermore, median fluorescence intensity per platelet was shown to be reduced in whole blood of fibrinogen-deficient mice supplemented with α220 fibrinogen [32], which may correspond to decreased GPVI binding sites. Further studies are required to identify the specific binding site of GPVI on the αC-region.

Our findings are supported by previous reports with comparable concentrations of dasatinib (100 nM; equivalent to therapeutic concentration) showing diminished thrombin generation and PS exposure in PRP clots induced with convulxin [46]. Furthermore, previous studies have observed decreased thrombin generation in PRP from GPVI-deficient patients [24,47] and in PRP from healthy volunteers, where GPVI was blocked by Fab of monoclonal antibody 9012 [24]. Interestingly, the addition of Fab 9012 to fibrinogen-deficient samples did not decrease thrombin generation, further supporting a role of GPVI-fibrin interaction in this process [24]. All 3 inhibitors investigated in our study significantly increased the clot pore size (consistent with reduced thrombin generation), with ibrutinib demonstrating the most potent effect on clot permeability. PRT-060318 and ibrutinib have previously been shown to impair thrombus formation of whole blood perfused over collagen-coated surfaces [48,49]. PRT-060318 has also been reported to prevent fibrin-induced platelet aggregation, and studies have demonstrated impaired thrombosis in vivo in the presence of this Syk inhibitor [28,50].

The effects of GPVI on the clot structure and function were compared with those of αIIbβ3 blockade (by eptifibatide), as a control for fibrin-integrin interactions, with novel observations on the impact of combined inhibition of integrin and GPVI signaling on the clot architecture and functionality. We observed a small reduction (albeit not significant) in procoagulant platelets in the presence of eptifibatide, which is consistent with previous reports that integrin αIIbβ3 antagonists decrease PS exposure [51,52]. Intriguingly, substantial reduction in procoagulant platelet number occurred following combined integrin and GPVI signaling inhibition, suggesting that both αIIbβ3 and GPVI are involved in the development of procoagulant platelets mediated by their interaction with fibrin. Moreover, unlike GPVI signaling inhibition, blockage of integrin αIIbβ3 significantly increased fiber density. Interestingly, in combination with eptifibatide, the reduction in fiber density by tyrosine kinase inhibitors was less pronounced. There are conflicting reports on the impact of integrin inhibition on thrombin generation, with reports observing no impact [53,54] or a reduction in thrombin generation by αIIbβ3 blockers [24,55]. In our hands, eptifibatide increased peak thrombin generation, which supports our findings of increased fibrin fiber density in the presence of this integrin blocker. Furthermore, we observed no significant change in the clot pore size following αIIbβ3 inhibition, and the combined effects of tyrosine kinase inhibitors in the presence of eptifibatide were less pronounced than their individual effects on increasing clot pore size. This suggests that GPVI plays a central role in clot structure and indicates that inhibition of GPVI signaling may contributes to more porous clots.

We next examined the effect of GPVI signaling inhibition on clot retraction to compare the effects of each inhibitor on normal whole blood clot functionality and contractibility. We observed a significant reduction in clot retraction after 120 minutes following treatment with ibrutinib, but not with PRT-060318 or dasatinib. However, it is worth noting that some changes in percentage clot retraction over time were observed for all tyrosine kinase inhibitors up to 60 minutes, which was the end point in a previous study of convulxin-induced PRP clot retraction reporting attenuated clot retraction following treatment with dasatinib [46]. Our study distinguishes ibrutinib as the single inhibitor impacting the whole blood final clot weight at later time points, which is supported by previous reports of ibrutinib inducing αIIbβ3 shedding and impairing PRP clot-retraction kinetics [56,57]. We observed no significant changes in washed platelet retraction in samples containing GPVI signaling inhibitors, suggesting that lack of contact with other cells or fibrin reduces the impact of ibrutinib on platelet retractability.

Clot retraction in the presence of eptifibatide was also performed to control fibrin-integrin interaction. The inhibition of αIIbβ3 with eptifibatide impaired whole blood and washed platelet retraction much more clearly, and the addition of tyrosine kinase inhibitors did not further exacerbate the effect of αIIbβ3 inhibition, suggesting that although the integrin is essential for optimal clot retraction, GPVI is not critical in this process. The importance of αIIbβ3 for optimal clot retraction is highlighted by the absence of clot retraction in patients with Glanzmann thrombasthenia, a bleeding disorder caused by the lack or impairment of αIIbβ3 [58–60]. Furthermore, previous reports have identified outside-in signaling processes elicited by integrin-ligand [ie, fibrin(ogen)] binding to play a crucial role in clot retraction [61–63]. We have also observed a reduction in polyhedrocyte formation in the presence of eptifibatide but not GPVI signaling inhibitors, further supporting a key role of αIIbβ3 but not GPVI in clot retraction. Ibrutinib has been previously proposed to inhibit integrin αIIbβ3 outside-in signaling as well as collagen GPVI signaling [49,57]. More recently, it was identified that ibrutinib induces αIIbβ3 shedding from the platelet surface [56]. Therefore, it is possible that the effect of ibrutinib on whole blood clot retraction, at least in part, is due to integrin shedding by reducing αIIbβ3 on the platelet surface, which is crucial for fibrin interactions underpinning clot contraction [64]. Further investigations are required to determine the specific contributions of integrin shedding by ibrutinib on clot retraction.

Further studies are required to explore the impact of GPVI-fibrin inhibition on thrombosis and procoagulant platelet formation under flow and in vivo. In addition, studies are required to establish the role of GPVI signaling inhibition on the localization of coagulation and fibrinolytic factors previously shown in procoagulant platelet "cap" [20,65,66]. The effects of GPVI-fibrin and GPVI signaling inhibition on single fibrin fiber thickness and strength and how they affect fibrinolysis also remain to be characterized. Moreover, further studies are needed to characterize the role of fibrin-GPVI interaction in the clustering of GPVI. Previous research has suggested a joint role of PAR1 and GPVI in procoagulant platelet development and that calcium mobilization and mitochondria number may contribute to the development of different platelet populations (procoagulant vs aggregatory) [8,17]. Further research is also required to identify the specific mechanisms involved in the development of different platelet populations and the potential impact of GPVI-fibrin interaction in these processes.

In conclusion, our study shows that GPVI-fibrin interaction contributes to the development of procoagulant platelets and suggests that inhibition of GPVI-fibrin interaction and subsequent signaling alters the clot structure and function. Procoagulant platelets were decreased in GPVI$^{-/-}$ samples in the presence of GPVI-fibrin inhibiting Affimers and in the presence of GPVI signaling inhibitors, an effect exacerbated by integrin blockage. Ibrutinib, followed by PRT-060318, had the most pronounced effects on the clot structure through the reduction of fiber density and increased permeability. The Syk inhibitor ibrutinib also impacted clot contractibility. These findings suggest that inhibiting the GPVI signaling cascade can provide an avenue to ameliorate the features of a prothrombotic clot phenotype and, thereby, that targeting this pathway has potential implications for interventions aimed at reducing prothrombotic risks.

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AUTHOR CONTRIBUTIONS

J.S.G. performed the experimental work, planned and designed the research, analyzed data, and wrote the manuscript. C.D. obtained murine samples and designed and optimized the methodology. R.G.X. characterized Affimer inhibition of GPVI-fibrin(ogen) interaction. F.L.M. performed SEM sample analysis, imaging, and interpreted the data. H.R.M. purified recombinant full-length and truncated fibrinogen. D.T. and C.T. isolated Affimers targeting GPVI. S.P.W. conceived the study and provided advice on experimental work. R.A.S.A. conceived the study, provided advice on experimental design and interpretation, and edited the article. All authors critically reviewed and approved the final version of the article.

DECLARATION OF COMPETING INTEREST

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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