

# *The rate of platelet activation determines thrombus size and structure at arterial shear*

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## ORIGINAL ARTICLE

# The rate of platelet activation determines thrombus size and structure at arterial shear

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## Abstract

**Background:** The response of platelets to activating stimuli and pharmaceutical agents varies greatly within the normal population. Current platelet function tests are used to measure end-point levels of platelet activation without taking the speed at which platelets activate into account, potentially missing vital metrics to characterize platelet reactivity.

**Objectives:** To identify variability, to agonists and among individuals, in platelet activation kinetics and assess the impact of this on thrombus formation.

**Methods:** We have developed a bespoke real-time flow cytometry assay and analysis package to measure the rate of platelet activation over time using 2 parameters of platelet activation, fibrinogen binding and P-selectin exposure.

**Results:** The rate of platelet activation varied considerably within the normal population but did not correlate with maximal platelet activation, demonstrating that platelet activation rate is a separate and novel metric to describe platelet reactivity. The relative rate of platelet response between agonists was strongly correlated, suggesting that a central control mechanism regulates the rate of platelet response to all agonists.

**Conclusion:** For the first time, we have shown that platelet response rate corresponds to thrombus size and structure, wherein faster responders form larger, more densely packed thrombi at arterial, but crucially not venous, shear. We have demonstrated that the rate of platelet activation is an important metric in stratifying individual platelet responses and will provide a novel focus for the design and development of antiplatelet therapy, targeting high-shear thrombosis without exacerbating bleeding at low shear.

## KEYWORDS

flow cytometry, kinetics, platelet activation, platelet function tests, thrombosis

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## 1 | INTRODUCTION

Platelet responses to stimulating factors and pharmaceutical agents varies greatly within the population [1–5]. Antiplatelet therapy successfully reduces the recurrent rate of myocardial infarction and stroke; however, thrombotic disease remains a leading cause of morbidity and mortality [6–8]. This emphasizes the need to understand the interpopulation variability in platelet function and its implications for hemostasis and thrombosis. This will allow us to accurately stratify patients based on their individual measurements of platelet reactivity and propensity to form larger occlusive thrombi and enable bespoke pharmacotherapeutic preventative strategies.

Traditionally, platelet function is assessed using end point assays that measure fibrinogen binding, granule secretion, or aggregation in platelets stimulated for long enough to achieve maximal activation [9,10]. These tests do not consider the time taken for platelets to convert extracellular stimuli to intracellular signals and subsequently degranulate and bind to the growing thrombus. Blood flow in the arterial circulation exerts high shear [11,12], emphasizing the need for platelets to adapt to this by detecting, processing, and responding to stimuli rapidly to contribute to ongoing thrombus formation for effective hemostasis.

We have previously shown that the rate of platelet activation varies independently of the maximum extent of platelet activation. Integrin-linked kinase (ILK) is involved in  $\beta$ -integrin regulation in platelets [13,14]. We demonstrated that the rate, but not the maximal extent of platelet activation, was affected in the absence of ILK in mice. These mice also exhibited reduced thrombus formation [15], indicating that platelet activation kinetics could play a more important role in driving thrombus formation than the maximal extent of platelet activation. Differences between the rate of aggregation and maximum level of aggregation were also identified in mice deficient in CalDAG-GEFI [16,17].

These findings demonstrate that platelet activation rate and maximal activation can be independent and that measurements of maximal activation may not be descriptive of platelet behavior up to this point. Differences in platelet activation time may have a profound effect on their initial binding to a growing thrombus and impact propagation architecture and overall size of the forming thrombus.

The present study aimed to determine whether the rate of platelet activation varies within the normal population and whether platelet activation rate corresponds to thrombus size and density. Here, we assessed the rate of platelet activation in 143 healthy donors and 43 patients with established coronary artery disease (CAD) using an established real-time flow cytometry method and data analysis package [18] adapted for population testing. These kinetic measurements of platelet activation provide new insight into the behavior of platelets during the initial stages of thrombus formation, demonstrate that the rate of platelet activation is predictive of thrombus size and architecture, and provide evidence of the importance of assessing the dynamics of platelet activation when designing and developing antiplatelet therapy, particularly if we wish to target high-shear thrombosis without compromising hemostasis.

### Essentials

- Current platelet function tests often overlook the kinetics of platelet activation.
- Real-time flow cytometry assay and analysis package measures platelet activation kinetics.
- The rate of platelet activation varies considerably within the normal population.
- Platelet activation rate alters thrombus size and structure at arterial, but not venous, shear.

## 2 | MATERIALS AND METHODS

### 2.1 | Collection of blood and preparation of platelet-rich plasma

Peripheral blood from 143 fasting, healthy, aspirin-free donors aged 30 to 65 years was collected with their informed consent and with approval by the University of Reading Research Ethics Committee. A further blood sample was taken between 6 and 12 months later from 12 of these individuals to investigate *ex vivo* thrombus formation.

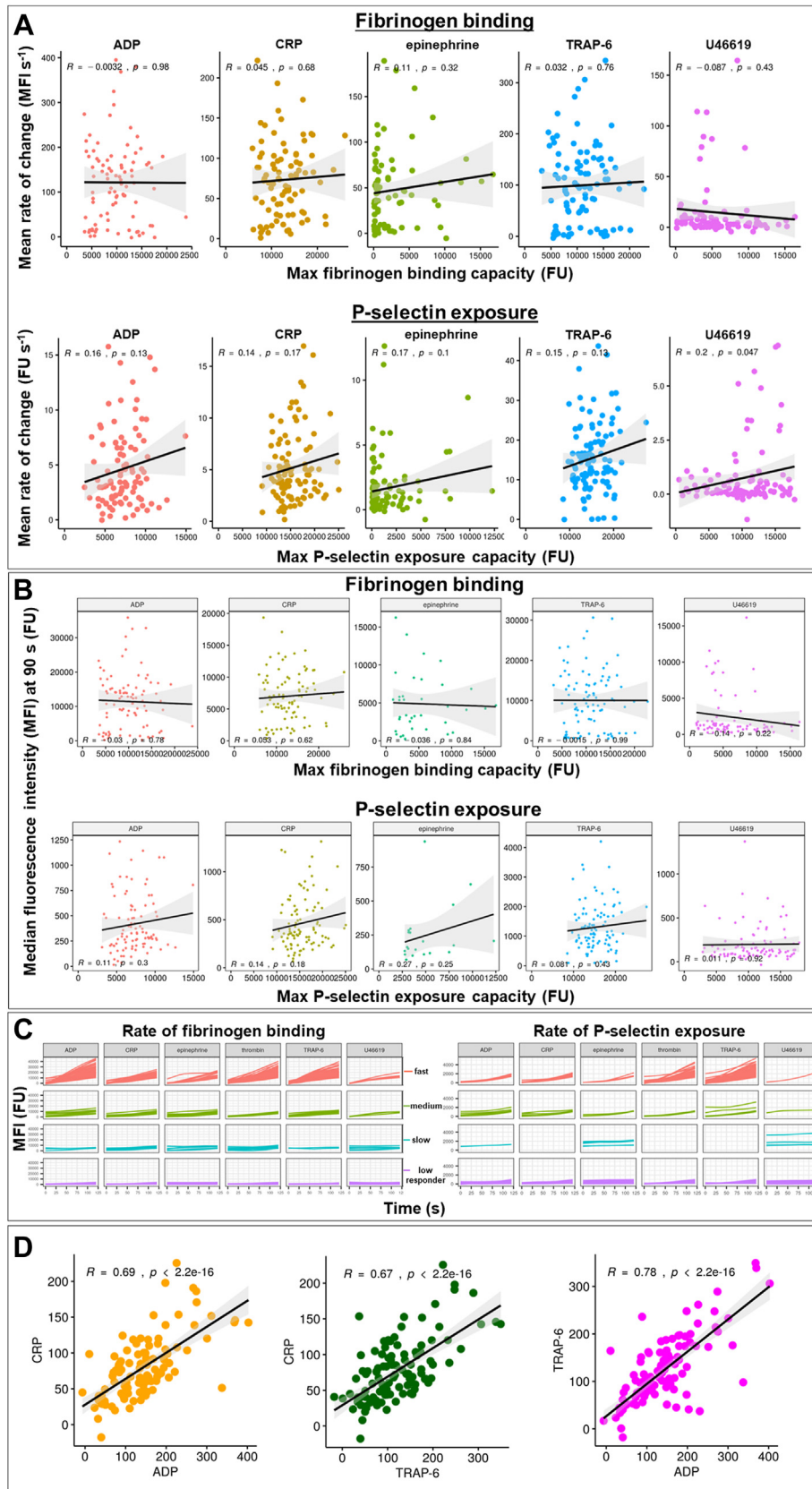
Patients were investigated for stable CAD as a part of their clinical workup, which includes coronary angiography at the Royal Berkshire Hospital in Reading, UK, and were classified into 3 different groups: those with no CAD following coronary angiography and no type 2 diabetes mellitus (T2DM) (control group;  $n = 8$ ); those with CAD and no T2DM ( $n = 25$ ); and those with CAD and T2DM ( $n = 10$ ). Exclusion criteria were as follows: acute coronary syndrome in the past 12 months, taking P2Y<sub>12</sub> inhibitors, anticoagulants, other metabolic dysfunctions, active or recent malignancy (<2 years), pregnancy, evidence of alcohol or drug misuse, and any underlying hematologic pathologies. All samples were collected with the patients' informed consent and with approval by the University of Reading Research Ethics Committee and NHS Health Research Authority (Ref: 19/LO/0572).

Blood was collected in vacutainers containing 3.2% sodium citrate (Greiner Bio-one LTD); the first 3 mL was discarded.

Platelet-rich plasma (PRP) was prepared by centrifugation at  $140 \times g$  for 20 minutes at 20 °C and incubated at 37 °C.

### 2.2 | Real-time flow cytometry

Real-time flow cytometry was carried out at 37 °C as described [18] using a Beckton Dickinson (BD) Accuri C6 flow cytometer with BD Csampl attachment and software (BD). Platelet activation was measured using antibodies to P-selectin and fibrinogen. A fibrinogen antibody was used rather than PAC-1 to limit competition for integrin binding by endogenous fibrinogen. The binding of fluorescein isothiocyanate (FITC)-labeled antifibrinogen was confirmed to be  $\alpha_{IIb} \beta_3$  mediated, which was abolished when platelets were preincubated



**FIGURE 1** There is no correlation between the rate of platelet activation and maximum platelet activation in response to any agonist, and the rate of platelet activation varies among agonists and is consistent across agonists in each donor. End point flow cytometry was used to measure maximal levels of fibrinogen binding and P-selectin exposure in platelets activated for 20 minutes with a range of agonist concentrations. The agonist concentration ranges were 0  $\mu$ M to 150  $\mu$ M adenosine diphosphate (ADP) (red), 0- to 15- $\mu$ g/mL cross-linked collagen-related peptide

(yellow), 0 to 150  $\mu$ M epinephrine (green), 0 to 75  $\mu$ M thrombin receptor activator peptide 6 (TRAP-6) (blue), or 0 to 500  $\mu$ M 9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin F2 $\alpha$  (magenta). Data were analyzed using PPAAnalysis. (A) Correlations between the mean rate of change (RoC), calculated over the first 3 minutes, and maximal levels of fibrinogen binding and P-selectin exposure after 20 minutes. (B) Correlations between median fluorescence intensity at 90 seconds and maximal levels of fibrinogen binding and P-selectin exposure after 20 minutes. R corresponds to Pearson correlation coefficient. There were no significant correlations. (C) Fibrinogen binding and P-selectin exposure in platelet-rich plasma (PRP) from 143 donors in response to stimulation with 1  $\mu$ M ADP, 0.5- $\mu$ g/mL cross-linked collagen-related peptide (CRP-XL) 20  $\mu$ M epinephrine, 1-U/mL thrombin, 5  $\mu$ M TRAP-6, or 1  $\mu$ M 9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin F2 $\alpha$ . The rate of platelet activation for fibrinogen binding or P-selectin exposure was characterized depending on the RoC, and curves were classified into 3 different categories, “fast” (fibrinogen binding; RoC > 80, P-selectin exposure; RoC > 10), “medium” (fibrinogen binding; RoC > 40, P-selectin exposure; RoC > 5), “slow” (fibrinogen binding; RoC  $\leq$  40, P-selectin exposure; RoC  $\leq$  5) and “low responders” (fibrinogen binding; fluorescence < 4000, P-selectin exposure, fluorescence < 1000). (D) Correlations between mean RoC in fibrinogen antibody binding over each agonists response where R corresponds to Pearson correlation coefficient. There was a significant positive correlation for the mean RoC between collagen-related peptide and ADP (left panel;  $p < .0001$ ), collagen-related peptide and TRAP-6 (middle panel;  $p < .0001$ ), and TRAP-6 and ADP (right panel;  $p < .0001$ ). ADP, adenosine diphosphate; CRP, collagen-related peptide; FU, fluorescence unit; MFI, mean fluorescence intensity; TRAP-6, thrombin receptor activator peptide 6; U46619, 9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin F2 $\alpha$ .

with integrilin and/or arginine-glycine-aspartate peptide. Briefly, FITC-labeled antifibrinogen antibody (FITC-FGN; dilution 1:37.5 [vol/vol]; Dako, Agilent Technologies) and allophycocyanin (APC)-labeled anti-P-selectin (APC-CD62P; dilution 1:37.5 [vol/vol]; BD) were added to HEPES buffered saline (HBS) containing 5mM glucose (HBS-G) (HBS; 10mM HEPES pH 7.4; 150mM sodium chloride, 5mM potassium chloride, and 1mM magnesium sulfate heptahydrate). Saturating amounts of antibodies were used to ensure detection of the earliest activation events and differentiate active and resting populations. Immediately before collecting events, PRP was added to the HBS-G-antibody mix at a dilution of 1:600 (vol/vol). For controls, 10 000 events were collected from samples containing unstimulated platelets with either FITC-FGN in the presence of EDTA to prevent fibrinogen binding or APC-labeled mouse antihuman immunoglobulin G1 isotype control. For the measurement of intracellular calcium studies, PRP at a dilution of 1:600 (vol/vol) was incubated at 37 °C for 30 minutes in a 1:1 mix of filtered Fluo-4-AM (Thermo-Fisher Scientific; 500  $\mu$ M) in HBS-G before collecting events. Agonists included thrombin (thr; 1 U/mL; Sigma-Aldrich) in the presence of Gly-Pro-Arg-Pro peptide (1.18 mM; Sigma-Aldrich) and a cross-linked collagen-related peptide (CRP-XL; 0.5  $\mu$ g/mL), adenosine diphosphate (ADP; 1  $\mu$ M; Sigma-Aldrich), epinephrine (epi; 20  $\mu$ M, Sigma-Aldrich), 9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin F2 $\alpha$  (U46619; 1  $\mu$ M, Sigma-Aldrich), and thrombin receptor activator peptide 6 (TRAP-6; 5  $\mu$ M, Bachem). Events in each well were recorded for 5 seconds before rapid addition of the activation mix. Events were recorded for 180 seconds. An in-depth description of the real-time flow cytometry method has been provided by Dunster et al. [18].

### 2.3 | Real-time flow cytometry data analysis

The results presented in this work used a bespoke open-source R package (Kinetx) developed using the original flow cytometry data and described in depth in the methods section in the article by Dunster et al. [18]. Briefly, responses of donors to each agonist were locally regressed to a curve that summarized fluorescence over time (Supplementary Figure S1). A moving average was generated using Locally Estimated Scatterplot Smoothing function. Low responders were determined as

those where fibrinogen-binding mean fluorescence intensity (MFI) was <4000 or P-selectin exposure MFI was <1000. The Locally Estimated Scatterplot Smoothing curve was also used to obtain the rate of change (RoC), defined as the average rise in the smoothed line >120 seconds. The RoC was then used to categorize fast, medium, and slow responders, who were defined as having an MFI of <40, between 40 and 80, and >80/s for fibrinogen binding, respectively, and <5, between 5 and 10, and >10/s for P-selectin exposure. The average RoC across all agonists was calculated for the Kinetx metric; however, because the general response to each agonist significantly differed and, hence, skewed the average toward results for certain agonists, the RoC per agonist for a given donor was first normalized against the maximum RoC for each agonist across all data sets. The Kinetx metric is, therefore, the average RoC across all agonists normalized for each agonist.

### 2.4 | Data and software availability

The latest version of Kinetx can be downloaded from <https://figshare.com/s/3a0a3b21cb9d27cbb522> and <http://github.com/cardiomaths/Kinetx>.

### 2.5 | In vitro thrombus formation under flow

Detailed methods for experimental procedures for and analysis of thrombus formation can be found in the supplementary methods. Briefly, thrombus formation under flow was performed on a selection of fast- and slow-responding individuals recalled from the original 143 volunteers. Cellix Vena8 Fluoro+ biochips were coated with collagen (100  $\mu$ g/mL) prior to thrombus formation studies. Images were taken every 2 to 4 seconds using a Nikon A1R fluorescence confocal microscope at 20 $\times$  magnification.

### 2.6 | Statistical analysis

Statistical analysis was performed using R and GraphPad Prism 7. Pearson correlations were performed to analyze relationships and



determine statistically significant correlations. Unpaired *t*-tests were used to analyze differences in thrombus formation parameters between the fast and slow responder groups, and 2-way analysis of variance (ANOVA) with Sidak multiple comparison posthoc test was used to analyze differences between dextran penetration in the thrombus core and shell.  $p < .05$  was considered significant.

### 3 | RESULTS

We designed a real-time flow cytometry assay and Kinetx analysis software [18] to measure platelet activation kinetics in a large number of subjects, understand the variability in the rate of platelet response within the population and between different signaling pathways, and understand how this impacts thrombus formation.

#### 3.1 | The rate of platelet activation varies widely in the normal population and does not correlate with maximum platelet activation

To determine the speed at which platelets become activated and how this varies among individuals, the RoC of platelet activation was measured based on the change in fibrinogen binding and P-selectin exposure over the first 3 minutes of platelet activation (Figure 1A).

The average RoC of platelet activation varied greatly among individuals (Figure 1A). The greatest differences in the rate of fibrinogen binding were observed in response to ADP (SD, 93 median fluorescence units/second [FU/s]), thrombin (75 FU/s), and TRAP-6 (93 FU/s). The rate of fibrinogen binding in response to CRP-XL (47 FU/s), epinephrine (36 FU/s), and U46619 (35 FU/s) varied among the donors but to a lesser extent. The rate of P-selectin exposure varied by a large amount among the donors in thrombin (6.1 FU/s) and TRAP-6 (7.7 FU/s)-stimulated platelets but less so in response to ADP (1.8 FU/s), CRP-XL (2.2 FU/s), epinephrine (1.5 FU/s), and U46619 (1.6 FU/s).

Maximal fibrinogen binding and P-selectin exposure were also measured in response to each agonist [19]. There was no correlation between the mean rate and final capacity of platelet activation for fibrinogen binding (ADP,  $p = .98$ ; CRP-XL,  $p = .68$ ; epinephrine  $p = .32$ ; TRAP-6,  $p = .76$ ; U46619,  $p = .43$ ) or P-selectin exposure (ADP,  $p = .13$ ; CRP-XL,  $p = .17$ ; epinephrine  $p = .1$ ; TRAP-6,  $p = .13$ ; U46619,  $p = .47$ ) (Figure 1A). Equally, there was no correlation between the MFI at 90 seconds and the maximal levels of fibrinogen binding and P-selectin exposure after 20 minutes (Figure 1B). This confirms that the rate and extent of activation are controlled separately.

#### 3.2 | The rate of platelet activation varies between agonists

Using the Kinetx software, the rate of platelet activation was categorized into fast, medium, or slow responses to each agonist for all the donors. The rate of platelet activation differed between agonists,

where ADP (61% fast, 9% medium, 30% slow), thrombin (42% fast, 21% medium, 37% slow), and TRAP-6 (58% fast, 13% medium, 29% slow) induced faster responses than CRP-XL (25% fast, 33% medium, 42% slow), epinephrine (Figure 1C; 5% fast, 24% medium, 71% slow), and U46619 (8% fast, 1% medium, 91% slow). These data show that the speed of platelet activation depends, to an extent, on the activating agonist.

#### 3.3 | The rate of platelet activation is consistent across agonists in each donor

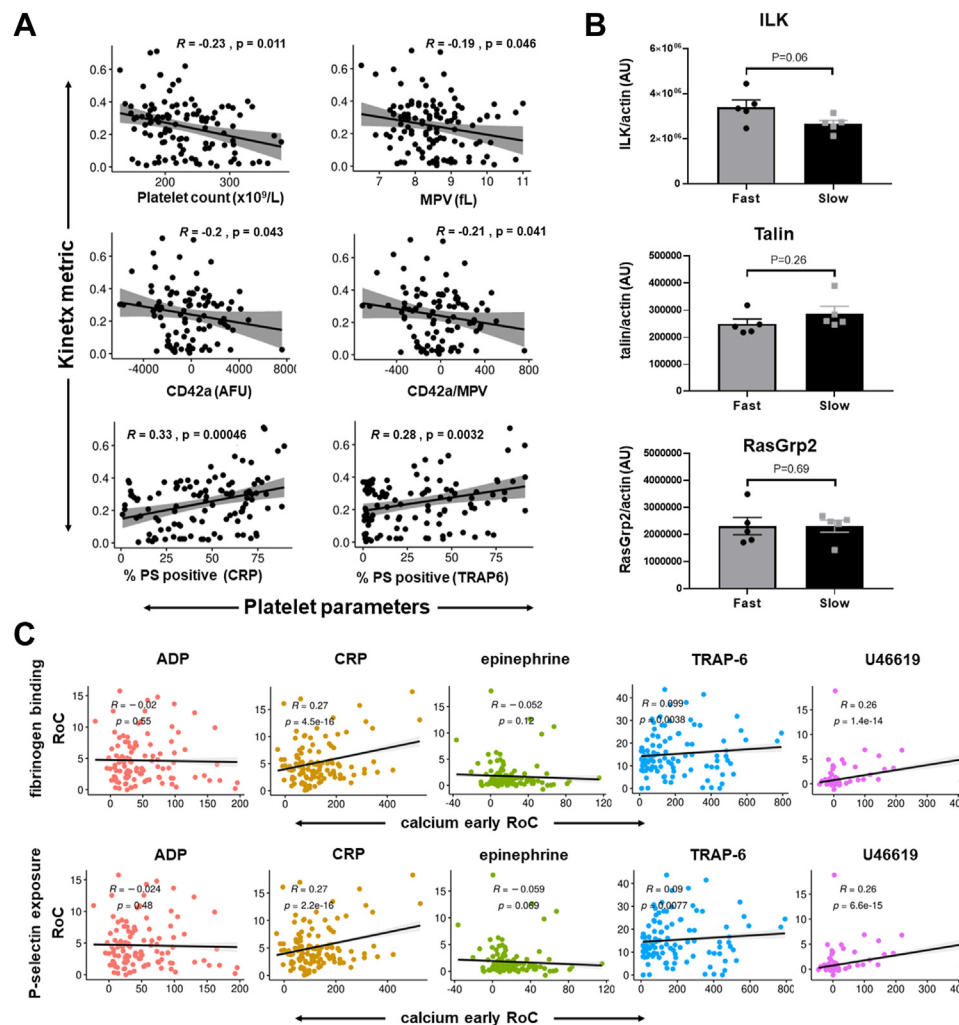
Regardless of differences in the rate of platelet activation between agonists, when the relative rate of response in fibrinogen antibody binding was compared between agonists across different individuals, there was a strong positive correlation between rate of response (CRP-XL vs ADP,  $p < .0001$ ; CRP-XL vs TRAP-6,  $p < .0001$ ; TRAP-6 vs ADP,  $p < .0001$ ) (Figure 1D). Similar positive correlations were observed with P-selectin antibody binding and other agonists (Supplementary Figures S2 and S3). This suggests that common mechanisms control the speed of platelet responses to every agonist.

#### 3.4 | The rate of platelet activation correlates with platelet count, mean platelet volume, surface expression of CD42a, phosphatidylserine exposure, and calcium flux

We investigated various potential mechanisms regulating the rate of platelet response. There were significant negative correlations associated with platelet count ( $p = .011$ ) and mean platelet volume (MPV;  $p = .046$ ), where a faster rate was observed in individuals with lower platelet count and smaller MPV but not other blood count parameters (Figure 2A and Supplementary Figure S4).

We assessed the impact of platelet receptor expression on the rate of platelet activation. Of 12 receptors measured, (Supplementary Figure S5), only CD42a (GPIX) showed a negative correlation with platelet count ( $p = .043$ ), which was unaltered by adjusting for MPV ( $p = .041$ ) to account for variation in receptor density (Figure 2A). Significant positive correlations were also identified between increasing platelet rate and greater levels of phosphatidylserine-positive, procoagulant platelets in response to CRP ( $p = .00081$ ) and TRAP-6 ( $p = .0021$ ) stimulation (Figure 2A).

Given these few and relatively weak correlations, it seems that platelet surface receptor density is unlikely to regulate platelet activation rate; we, therefore, investigated the impact of platelet signaling molecule expression in fast and slow responders. Western blotting was performed to measure levels of ILK, Talin, and RASGrp2, 3 molecules previously linked to changes in the rate of platelet response. Talin and RasGrp2 levels in platelets from fast and slow responders were similar. Levels of ILK were generally lower in slow responders than in fast responders; however, this did not reach statistical significance (Figure 2B).



**FIGURE 2** Fast responders have more intracellular integrin-linked kinase than slow responders, and the rate of platelet activation is correlated with metabolic and platelet parameters. (A) Pearson correlations were performed between Kinetx metric (average rate of change [RoC] across all agonists normalized for each agonist) and platelet parameters, including platelet count, mean platelet volume, CD42a, and CD42a/mean platelet volume (MPV) percentage of phosphatidylserine-(PS) positive platelets, including collagen-related peptide- (CRP) stimulated and thrombin receptor activator peptide 6-(TRAP6) stimulated platelets. (B) Western blotting for intracellular proteins integrin-linked kinase, (ILK), Talin, and RasGrp2 was performed on platelet lysates from fast and slow responders, and protein was quantified using densitometry in ImageJ and normalized to actin loading control.  $n = 5$  fast responders;  $n = 5$  slow responders. (C) Pearson correlations were performed between RoC to each agonist for fibrinogen binding and P-selectin exposure and the early RoC for calcium flux in response to each agonist. Pearson correlation coefficient ( $R$ ) and  $p$  values are stated on each graph. ADP, adenosine diphosphate; AFU, arbitrary fluorescent units; CRP, collagen-related peptide; ILK, integrin-linked kinase; MPV, mean platelet volume; PS, phosphatidylserine; RasGrp2, RAS guanyl-releasing protein 2; RoC, rate of change; TRAP-6, thrombin receptor activator peptide 6.

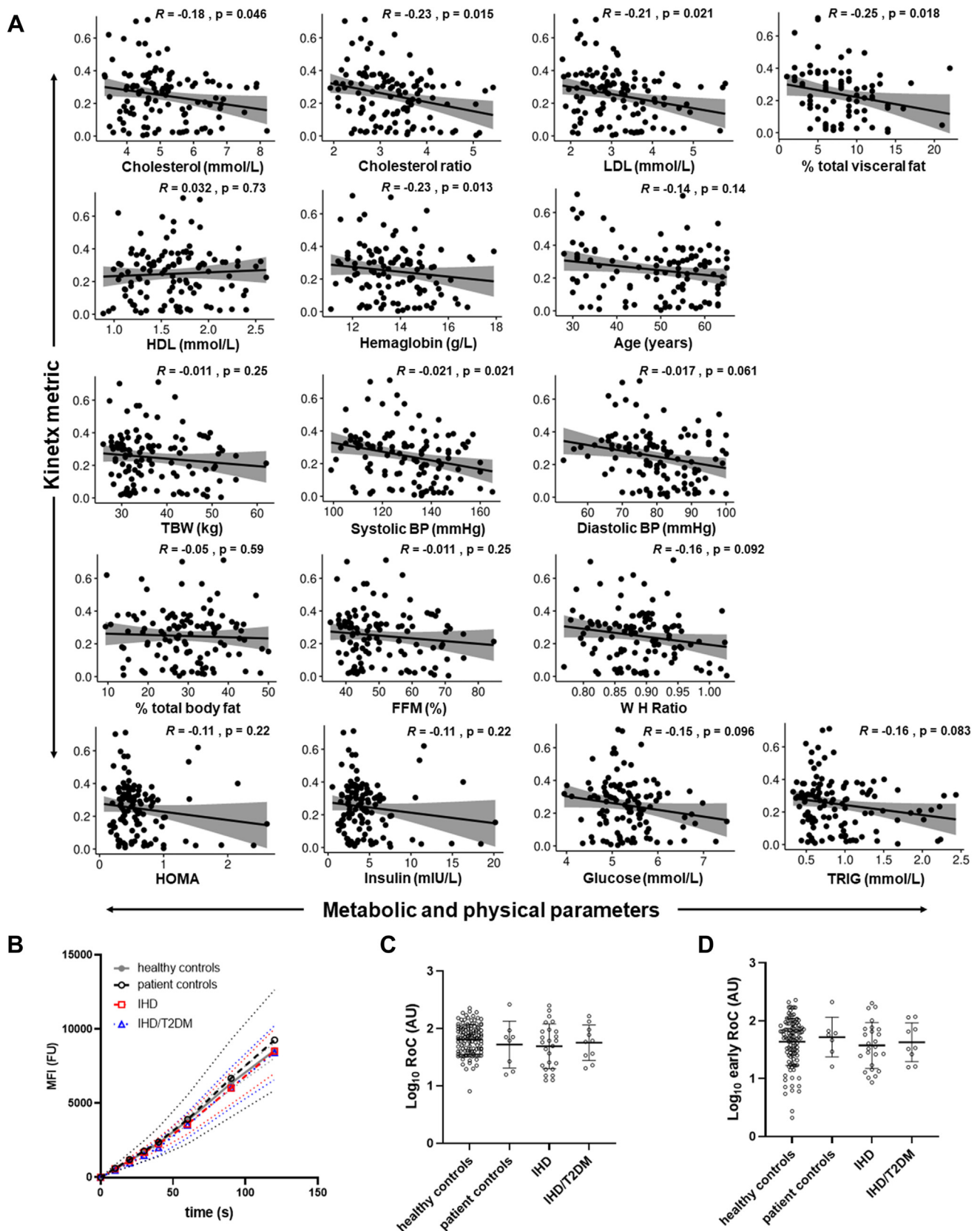
We then investigated intracellular calcium release as a possible central mechanism controlling the rate of platelet response. The rate and levels of intracellular calcium flux did vary among individuals and agonists. Correlations were performed between RoC for fibrinogen binding and P-selectin exposure and early RoC for calcium flux, which was derived from the first 20 seconds of calcium flux at a point where the rate was increasing. Significant positive correlations were observed for fibrinogen binding in response to CRP-XL ( $p < .0001$ ), TRAP-6 ( $p < .001$ ), and U46619 ( $p < .0001$ ) but not ADP or epinephrine. For P-selectin exposure, significant positive correlations were observed in response to CRP-XL ( $p < .0001$ ), TRAP ( $p < .01$ ), and U46619 ( $p < .0001$ ) but, again, not ADP or epinephrine. The incidence

of both correlation and lack of correlation between rate of fibrinogen binding or P-selectin exposure and calcium suggests that the central mechanism controlling the rate of platelet activation is not dependent on the rate and levels of intracellular calcium influx (Figure 2C).

### 3.5 | The rate of platelet activation is not related to established risk factors for cardiovascular disease

To assess the impact of established cardiovascular disease risk factors we measured common physical and metabolic parameters to investigate if they influence the rate of platelet response. There were weak





**FIGURE 3** The rate of platelet activation is not related to metabolic and physical parameters related to cardiovascular disease. (A) Pearson correlations were performed between Kinetx metric (average rate of change [RoC] across all agonists) and physical and metabolic parameters. The parameters included cholesterol, cholesterol ratio, low-density lipoprotein, visceral fat, high-density lipoprotein, hemoglobin, age, waist-to-hip ratio, fat-free mass diastolic blood pressure, high-density lipoprotein, total body water, triglycerides, fat percentage (fat), glucose,

homeostatic model assessment, and insulin. Pearson correlation coefficient (R) and  $p$  values are stated on each graph. (B) Platelet activation rate was measured in response to cross-linked collagen-related peptide (CRP-XL) stimulation (0.5  $\mu\text{g}/\text{mL}$ ) in 3 patient cohorts diagnosed with either IHD and no type 2 diabetes mellitus (T2DM) ( $n = 25$ ), IHD and T2DM ( $n = 10$ ), or without IHD or T2DM (control patients) ( $n = 8$ ) and compared with that of healthy controls ( $n = 121$ ). Neither (C) maximum RoC nor (D) early RoC (mean  $\pm$  SD) was significantly different between the patient and healthy groups. AU, arbitrary units; BP, blood pressure; FFM, fat-free mass; HDL, high-density lipoprotein; HOMA, homeostatic model assessment; IHD, ischemic heart disease; LDL, low-density lipoprotein; MFI, mean fluorescence intensity; RoC, rate of change; T2DM, type 2 diabetes mellitus; TBW, total body water; W H, waist-to-hip.

negative correlations between the levels of cholesterol, cholesterol ratio, low-density lipoprotein, and percentage of visceral fat and the rate of platelet activation as well as between hemoglobin and systolic blood pressure and the rate of platelet activation (Figure 3A). It is worth noting that changes in the rate of platelet activation did not alter with changes in subjects' age or markers of metabolic disease, such as increased insulin levels, that have previously been shown to affect other markers of platelet function such as aggregation (Figure 3A) [20–24].

The difference in the rate of platelet activation in response to CRP-XL was also compared between normal healthy individuals and the following 3 patient cohorts: 1) individuals without CAD or T2DM; 2) those with CAD and no T2DM; and 3) those with CAD and T2DM. No significant differences were observed among groups in any measure of platelet response kinetics (Figure 3B–D). Taken together, these data suggest that the rate of platelet activation is relatively stable and not altered by physiologic changes or comorbidities.

### 3.6 | The rate of platelet activation predicts thrombus size and density *in vitro*

We assessed the physiological relevance of the rate of platelet activation during thrombus formation *in vitro*. Blood from fast responders formed larger platelet thrombi than blood from slow responders who formed smaller thrombi under conditions of arterial shear (Figure 4A). Platelet thrombus formation was compared in fast and slow responders across shear rates ranging from 100 to 1500/s. At higher arterial shear rates of 1000 and 1500/s, a clear distribution can be identified between fast and slow responders (Figure 4B, C), wherein fast responders form larger thrombi (Figure 4C;  $p < .05$ ) at a faster rate (Figure 4D;  $p < .05$ ). At lower venous shear rates of 100 and 500/s, no difference in thrombus size (Figure 4B, C) or rate (Figure 4D) was observed between fast and slow responders. This demonstrates that the rate of platelet activation is predictive of thrombus size *in vitro* at arterial, but not venous, shear rates; furthermore, the rate of platelet activation corresponds to the rate of thrombus formation, where fast responders form thrombi at a faster rate than slow responders.

### 3.7 | The rate of platelet activation predicts thrombus density

Previous studies have revealed distinct layers of platelets within thrombi, specifically the “core” and “shell,” which are areas of varying porosity within thrombi [25]. We sought to determine whether the

rate of platelet activation had effects on thrombus density. Thrombus density was analyzed by flowing labeled dextrans over thrombi and measuring their ability to penetrate the thrombus core and shell (Figure 5A). There was no significant difference in the ability of 3-kDa dextran to penetrate the shell or core of thrombi, showing that the pores of both areas are large enough to allow this smaller molecule to pass through. However, a significant difference in porosity between the core and shell was measured based on the ability of 70-kDa dextran to penetrate the thrombus shell more readily in all donors, indicating that the shell is more loosely packed than the core (Figure 5B;  $p < .01$ ).

There were no differences between fast and slow responders in the diffusion of 3-kDa dextran throughout the thrombi and no difference in the penetrance of 70-kDa dextran between the shell of fast responders and that of slow responders (Figure 5B;  $p > .05$ ). This indicates that the shell has pores large enough for both dextrans to pass through and that the shell pore size is not affected by the rate of platelet response. The core of thrombi formed by fast responders were, however, denser than those formed by slow responders, indicated by the penetrance of 70-kDa dextran, which was more pronounced in the core of slow compared to fast responders (Figure 5B;  $p < .05$ ). This shows that the pore size is larger in the core of thrombi formed from slow responders than the pore size in the core of thrombi formed from fast responders, demonstrating that fast responders form more densely packed thrombi.

P-selectin exposure, a measure of platelet activation and degranulation, was also measured and compared between thrombi formed from blood from fast responders with those formed from slow responders (Figure 5C); however, no differences were observed (Figure 5D).

## 4 | DISCUSSION

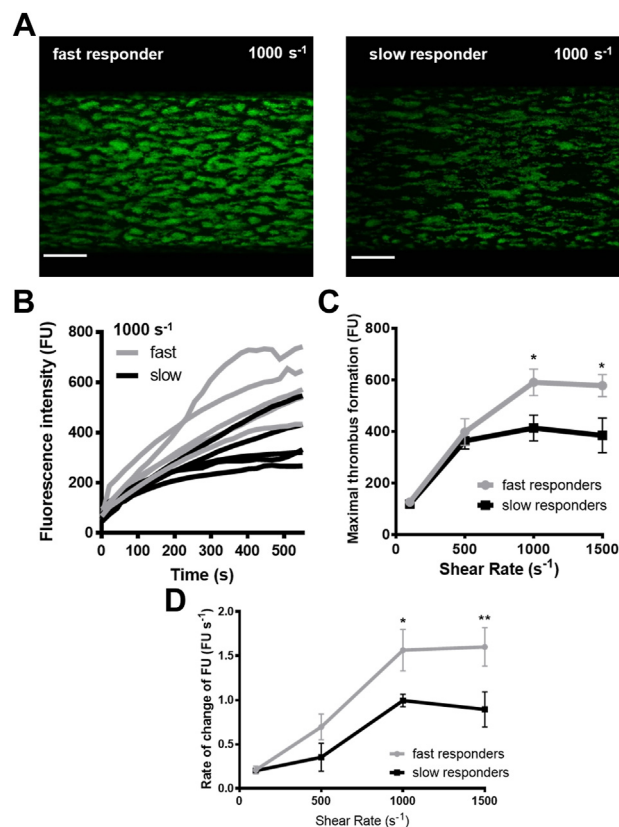
We used an innovative flow cytometry assay coupled with bespoke open-source analysis software to measure real-time platelet activation in 143 healthy subjects and 43 patients. This study shows that the rate of platelet activation varies considerably between individuals and different platelet agonists; however, the relative rate of platelet response between agonists is strongly correlated and not altered by conventional risk factors for cardiovascular disease. These findings suggest that a central control mechanism regulates the rate of platelet response to agonists. We have shown that the rate of platelet activation alters thrombus formation and architecture, and crucially, this occurs in high-shear conditions but not at low shear. This suggests that the rate of platelet response may be an important independent

risk factor for thrombosis and that, if therapeutically targeted, has the potential to limit arterial thrombus formation without impacting bleeding or enable targeting of specific patients who would benefit from more aggressive therapies in the setting of acute presentation.

Previous reports have shown that platelet reactivity is highly variable among normal individuals [26,27]. We have now demonstrated that the rate of platelet activation also varies between individuals but is independent of maximum platelet response, suggesting differential regulation of these parameters and the value of assessment of both if we are to accurately assess platelet function. The rate was not related to surface receptor density or calcium release. The rate of response varied weakly with physiologic and metabolic parameters; however, the subjects in whom metabolic parameters were measured in this study were all healthy volunteers; therefore, these findings should not be directly related to patient populations. We also compared our population of normal healthy volunteers with 3 cohorts of patients with established CAD. Interestingly, the data demonstrates no difference in the rate of platelet activation between the normal and patient populations despite differences in age and comorbidities, suggesting that platelet reactivity and activation speed are remarkably stable in the presence of physiologic changes that alter other measures of platelet activity.

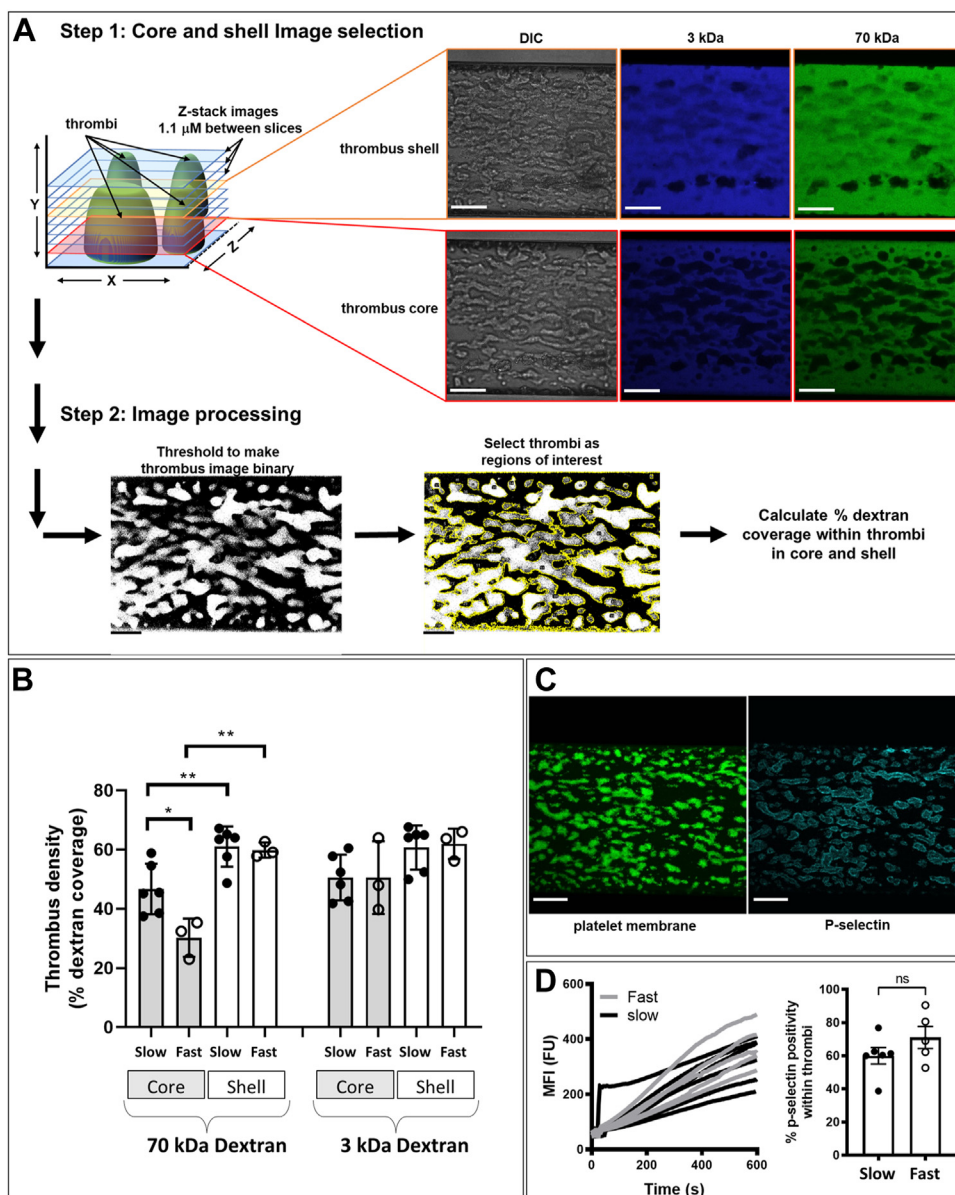
The rate of platelet response differed among agonists, which is consistent with differential potency in the stimulation of platelet activation. A much larger percentage of “fast” responses was observed in response to ADP, thr, and TRAP-6. This is likely to result from differences in the complexity of signaling cascades they induce as well as the strength of signal and involvement of secondary mediators. All receptor activation pathways in platelets converge at the point where the activation of a phospholipase C isoform converts phosphatidylinositol (4,5)-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol(1,4,5)-trisphosphate, which are considered as second messengers of platelet activation. These, in turn, activate protein kinase C and facilitate  $Ca^{2+}$  mobilization into the platelet cytosol [26–28].

Despite differences between individual signaling pathways, the relative rate of response between agonists shows a high degree of correlation. This strongly suggests that common mechanisms control the rate of platelet response that are independent of differences between receptor-initiated signaling pathways. We showed that there was no correlation between platelet activation rate and levels of receptors that platelets were stimulated through, indicating that the rate is governed by an internal mechanism. We also demonstrated that platelet calcium flux, although variable among individuals and agonists, does not correlate consistently in response to each agonist with respect to platelet activation rate, suggesting that the central mechanism controlling rate is independent of the rate of intracellular calcium influx. It is worth noting that calcium influx is highly transient and the fast on-off rates of calcium flux make it difficult to capture into a single metric for correlations, which could also account for the lack of correlation with platelet activation rate. Second messengers are generated in response to all platelet agonists, and central control of platelet activation rate is likely to comprise signaling elements downstream of this common point in the activation pathway, of which there are several potential



**FIGURE 4** The rate of platelet activation predicts thrombus size *in vitro*. Thrombus formation was performed under flow with whole blood from recalled slow and fast responders for 10 minutes at shear rates of 100, 500, 1000, and 1500/s in the presence of 3,3'-dihexyloxycarbocyanine iodide to label platelet membranes. Images were recorded using Nikon A1R, and data were analyzed by measuring the fluorescence intensity of 3,3'-dihexyloxycarbocyanine iodide over time. (A) Representative image of maximal thrombus formation after 10 minutes in a fast responder and slow responder. Scale bar represents 100  $\mu$ m. (B) Thrombus formation over time as represented by an increase in fluorescence units (FU) in all individual fast (gray line) and slow (black line) responders at a shear rate of 1000/s. (C) Average maximal thrombus formation in FUs was compared between fast (gray line) and slow (black line) responders over increasing shear rates from 100 to 1500/s. (D) Average rate of change of thrombus formation in FUs was compared between fast (gray line) and slow (black line) responders over increasing shear rates from 100 to 1500/s. \* $p < .05$ , \*\* $p < .01$  ( $n = 5$  fast responders,  $n = 6$  slow responders). FU, fluorescence unit.

candidates. Interestingly, varying PIP2 levels among donors have been reported in the literature, where differences in polyphosphoinositide content and higher turnover of PIP2 were positively correlated with increased aggregation to ADP [29]. This suggests that PIP2 turnover varies within the population and could be a limiting factor affecting the speed of platelet activation. The main pathway of PIP2 synthesis in platelets is through phosphorylation of phosphatidylinositol 4-phosphate by phosphatidylinositol 4-phosphate 5-kinase type I (PIP5KI) [30]. Rapid PIP2 synthesis during platelet activation is lost in PIP5KI $\beta$ -knockout mice, causing reduced platelet aggregation *ex vivo*



**FIGURE 5** The rate of platelet activation predicts thrombus density but not P-selectin exposure in thrombi. (A, B) Thrombi were formed for 10 minutes at 1000/s before flowing modified Tyrode buffer containing Cascade Blue (CB)-labeled 3-kDa dextran (83  $\mu\text{g}/\text{mL}$ ) and fluorescein isothiocyanate-labeled 70-kDa dextran (83  $\mu\text{g}/\text{mL}$ ) over formed thrombi for 3 minutes to label the background and spaces between formed thrombi. Z-stack images with 1.1- $\mu\text{M}$  slices were taken from the base to the top of thrombi. (A) Step 1: the schematic on the left depicts how z-stack slice images, which most accurately resembled the thrombus core and shell, were identified. The core is located at the tightly packed, highly activated base of the thrombus, and the shell is at the less densely packed, weakly activated top section of the thrombus. Images on the right are representative of a z-stack image of the shell (top panel) and core (bottom panel) of the thrombus showing platelet thrombi using DIC (left), and background staining and thrombus penetration of 3-kDa dextran (middle) and 70-kDa dextran (right). The thrombus core and shell slices of these z-stacks were identified separately for each thrombus formation experiment. Step 2: image processing was performed in ImageJ. Images were thresholded and converted into a binary image (left panel). Regions of interest were selected using a combination of the “analyze particles” function, the “magic wand” tool, and freehand area selection to ensure all thrombi in each image were selected (right panel) before the percentage of dextran coverage within the areas identified as thrombi was calculated. (B) Thrombus density was calculated as the percent coverage of 70- and 3-kDa dextran within the thrombus core and shell in fast and slow responders. The density was compared between the thrombus core (gray bars) and shell (white bars) in both fast (open circles) and slow responders (closed circles).  $**p < .01$  ( $n = 3$  fast responders,  $n = 6$  slow responders). (C, D) Thrombi were formed for 10 minutes at 1000/s in the presence of DiOC6 to label platelet membranes and allophycocyanin-labeled anti-CD62P to label P-selectin. (C) Representative image of maximal thrombus formation and P-selectin exposure on thrombi after 10 minutes. Scale bar represents 100  $\mu\text{m}$ . (D) Left panel—P-selectin exposure within thrombi over time as represented by an increase in fluorescence units in all individual fast (gray line) and slow (black line) responders at a shear rate of 1000/s. Right panel—Percentage of P-selectin positivity within thrombi was determined by defining the pixels positive for both P-selectin and DiOC6 and calculating P-selectin-positive pixels as a percentage of total DiOC6-positive pixels. The percentage of P-selectin positivity within thrombi was compared between fast (gray bar) and slow (black bar) responders. ( $n = 5$  fast responders,  $n = 6$  slow responders). DIC, differential interference contrast; DiOC6, 3,3'-dihexyloxycarbocyanine iodide; MFI, mean fluorescence intensity; ns, not significant.



and impairment of thrombus formation *in vivo* [31]. This dramatic phenotype in the absence of PIP5K1 $\beta$  suggests that varying levels of this enzyme in platelets from different individuals is a rate-limiting factor, wherein lower levels could limit PIP2 turnover, resulting in a slow platelet rate and reduced thrombus formation.

Fibrinogen binding occurs downstream of CalDAG-GEFI (RasGRP2) activation by second messengers Ca<sup>2+</sup> and diacylglycerol [32,33]. The absence of RasGRP2 in mice delayed production of Rap1-GTP and reduced aggregation rates [16], suggesting that RasGRP2 levels influence platelet rate. This study, however, found no difference between RasGrp2 levels between fast and slow responders, although this does not rule out the potential involvement of regulators of their function.

We have previously demonstrated ILK as a regulator of platelet activation rate [15]. Toward the end of the inside-out signaling pathway, ILK acts as an adaptor protein and plays roles in  $\beta$ 3 clustering [34,35]. ILK-knockout mice had significantly reduced initial rates of platelet activation, which recovered over time to have only a modest effect on the maximal extent of activation. Thrombus formation in these mice was reduced, indicating that platelet activation rate influences thrombus formation and stability [15,36]. We showed that platelet ILK levels in fast responders trend toward being greater than those in slow responders; however, this difference is not significant, and future studies will be required to ascertain whether ILK is involved in controlling platelet activation rate.

We demonstrated for the first time that variability in the rate of platelet response is a determinant of thrombus development. Individuals whose platelets responded more rapidly to stimulation developed larger, denser thrombi *in vitro*. Critically, changes in thrombi only occurred at arterial, but not venous, shear. This, combined with our findings of the central control of platelet activation rate, suggests that therapeutic strategies targeting the rate of platelet activation may have the potential to limit arterial thrombus formation without causing bleeding. In addition to targeting platelet activation rate therapeutically, the rate of platelet activation in individuals may potentially be predictive of thrombosis *in vivo*. The propensity of fast responders to form larger thrombi at arterial shear suggests that individuals with faster platelet activation rates are more likely to form large, dense, occlusive thrombi, which may, in turn, lead to larger end-organ damage as well as increased mortality and morbidity. Measuring the rate of platelet response may, therefore, be a better assessment of an individual's risk of thrombosis than current platelet function assays. Further understanding of the dynamics of platelet activation will help unravel complex platelet responses *in vivo*. It will uncover new targets for assessing risk of thrombosis and developing novel antiplatelet therapeutics.

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#### AUTHOR CONTRIBUTIONS

J.L.M. designed the research, performed experiments, analyzed data, and wrote the manuscript. J.L.D. designed the analysis package, wrote

the analysis software, analyzed data, and wrote the manuscript. A.J.U. performed experiments, analyzed data, and edited the manuscript. N.K. performed experiments, analyzed data, and edited the manuscript. T.S. performed experiments and analyzed data. Y.M.M.M. performed experiments and analyzed data. I.D.S. performed experiments and analyzed data. A.P.B. performed experiments and analyzed data. K.A.T. performed experiments, analyzed data, and edited the manuscript. G.Ó. analyzed data and edited the manuscript. M.B., S.M., L.D.D., A.W., N.R., and C.M. designed the patient study, recruited patients, and collected patient samples. J.M.G. designed elements, analyzed data, and edited the manuscript. C.I.J. designed the research, designed the analysis package, analyzed data, and wrote the manuscript.

#### FUNDING INFORMATION

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#### DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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#### SUPPLEMENTARY MATERIAL

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