

## METHODS SECTION PAPER

# Dynamic analysis of platelet deposition to resolve platelet adhesion receptor activity in whole blood at arterial shear rate

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Platelet activation is traditionally quantified using turbidimetric aggregometry, which reflects integrin  $\alpha_{IIb}\beta_3$  activity, an important determinant of platelet function during pathophysiological thrombus formation. However, aggregometry does not recreate the shear conditions prevailing during thrombosis *in vivo*. Here we describe novel whole-frame analysis of real-time video microscopy to quantify platelet adhesion receptor activity under shear in parallel-plate flow chambers. We demonstrate that the rate of change of surface coverage (designated Platelet Population Mobility, *PM*) quantifies platelet mobility. On collagen, *PM* falls exponentially to a low level, corresponding to firm platelet adhesion, while on other substrates, *PM* remains high. Different receptor-specific thrombogenic surfaces reveal that the *PM* time constant reflects real-time changes in integrins  $\alpha_{IIb}\beta_3$  and  $\alpha_2\beta_1$  activity. This ensemble kinetic analysis has the potential to provide valuable diagnostic information about platelet thrombus formation with both academic and clinical applications.

**Keywords**

Activation, aggregation, platelets, shear

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**Introduction**

Since its development 50 years ago, turbidimetric (light transmission) platelet aggregometry has been the principal technique used to assess platelet activity in research and clinical settings [1, 2]. More recently, flow cytometry [3] has provided a measure of platelet activation by quantifying antibody binding to activated membrane receptors, while the PFA-100 [4] offers a shear-dependent assessment of platelet function. However, neither aggregometry nor flow cytometry recreates the physiological conditions experienced by circulating platelets *in vivo*, and although the PFA-100 operates at shear, it is unable to characterize receptor-specific platelet function. None of these techniques reports the full repertoire of hemostatic responses to the multiple activatory and adhesive receptors that regulate platelet behavior.

Imaging of thrombus deposition in perfusion chambers is increasingly employed to investigate platelet pathophysiology under hemodynamic blood flow [5]. *Surface coverage (SC)* is the parameter most frequently used to report platelet interaction with a thrombogenic surface. However, *SC* reveals little about platelet physiology as it takes no account of activation-dependent platelet-platelet interactions that are fundamental to thrombus formation. Where cooperativity exists between receptors, long recognized for the platelet collagen receptors [6], their specific roles cannot be resolved using *SC*. A similar argument can be made for

measurement of thrombus volume. Low volume could report an unactivated platelet monolayer or an activated thrombus with low adhesive potential. Improved methods of analysis are required to achieve a clear understanding.

Here, we introduce a novel image analysis technique that yields the parameter *platelet population mobility (PM)*, which allows us to quantify platelet recruitment to a thrombogenic surface under flow.

DIOC<sub>6</sub>-stained human blood was perfused over thrombogenic surfaces (collagen fibers or synthetic collagen peptides) in a parallel plate flow chamber mounted on a laser scanning confocal microscope. Blood was perfused at a shear rate of 1000 s<sup>-1</sup> for 5 minutes with images being acquired at the plane of the thrombogenic surface at 0.2 Hz.

Image series were thresholded empirically, and *SC* was calculated for each binary image. A duplicate image series was created, off-set by a single frame, from which the original series was subtracted on a frame-by-frame basis. The resultant *SC* for each frame was designated the *change in surface distribution (dSD/dT, %)*. Expressing *dSD/dT* relative to *SC* (of the corresponding, unprocessed frame) produces *dSD/dT/SC*, an ensemble measurement of the rate of change of platelet capture. We designated this parameter *PM (%)*. Data were fitted to an exponential decay model by non-linear regression. This simple mathematical procedure was performed rapidly using ImageJ (NIH) and Excel (Microsoft) (see Supplementary material for details).

*Platelet population mobility* was plotted against time (Figure 1A). Using both peptide VWF-III [6] and collagen fiber substrates, *PM* achieved a steady state after an initial period of high platelet mobility. On collagen, *PM* fell rapidly from its initial value of 58.7 ± 3.6%, reaching a *plateau* of 9.1 ± 0.2%

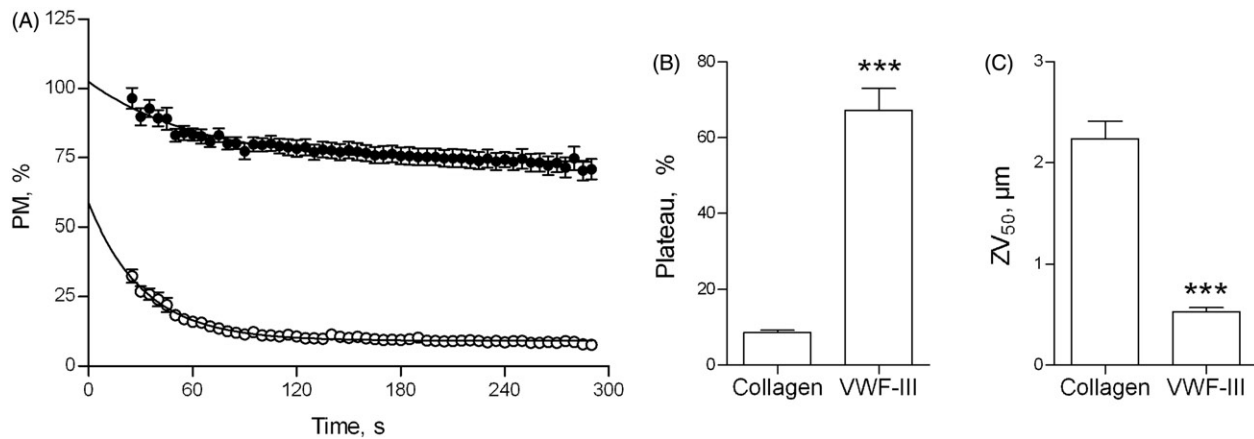


Figure 1. *PM* quantifies platelet adhesion under flow conditions. (A) Whole blood was perfused at  $1000\text{ s}^{-1}$  over type I collagen fibers (○) or VWF-III (●) and images were acquired at 0.2 Hz for 5 min. Image processing yielded *PM* measurements throughout the 5-min perfusion, which are plotted vs. time. *PM* curves were modeled as exponential decays, yielding the parameter of *Plateau* (B) (see Supplementary material for details). End-point image analysis yielded  $ZV_{50}$  (C).

(Figure 1B), progressing from its initial, transient interaction to stable platelet adhesion. On VWF-III, *PM* was initially higher ( $102.4 \pm 4.1\%$ ) and fell to a much higher steady state than on collagen (*Plateau*:  $73.3 \pm 1.0\%$ ), indicating that platelets interacted only transiently with this surface and did not achieve stable adhesion. This behavior indicates platelet rolling [7].

We recently described an end-point parameter of thrombus formation,  $ZV_{50}$ , (a volume measurement) which reports platelet–platelet interactions and thus directly quantifies  $\alpha_{\text{IIb}}\beta_3$  activity [6]. An inverse relationship is apparent between  $ZV_{50}$  and *PM*, the latter being low on surfaces where  $ZV_{50}$  is high. However, while  $ZV_{50}$  provides a *post hoc* estimate, *PM* reports platelet integrin functionality dynamically, in real-time. On type I collagen,  $ZV_{50}$  ( $2.2 \pm 0.2\ \mu\text{m}$ ) indicated that platelet activation and subsequent  $\alpha_{\text{IIb}}\beta_3$ -dependent platelet–platelet interactions had occurred by the end of the perfusion (Figure 1C). The corresponding low *PM* values are consistent with platelet activation. In contrast,  $ZV_{50}$  was lower on VWF-III ( $0.5 \pm 0.04\ \mu\text{m}$ ), reflecting the presence of a monolayer of platelets, where  $\alpha_{\text{IIb}}\beta_3$  is not activated and thrombus formation is absent [6]. These data demonstrate that *PM* describes and quantifies the transition from non-activated, rolling platelets to activated, stationary platelets.

Within this overall framework, *PM* profiles can be used to resolve the role of the collagen-binding integrin  $\alpha_2\beta_1$  in thrombus deposition. Experiments were conducted using surfaces coated with a series of GxOGER-containing peptides with differing affinity for  $\alpha_2\beta_1$ , in combination with VWF-III and the GpVI-specific collagen-related peptide (CRP) [6]. The different *PM* profiles reflected the known affinities of the peptides for  $\alpha_2\beta_1$  (Figure 2A and D; Supplementary Figure S1). Perfusion of whole blood over surfaces including GPP<sub>10</sub>, the inert control peptide, or GAOGER, the low-affinity integrin ligand, resulted in gradual reductions of *PM*. *PM* fell more rapidly using surfaces including the higher affinity peptides, GFOGER and GLOGER, indicating stable adhesion, while the mid-affinity GMOGER yielded an intermediate *PM* profile. *PM* curves from the different surfaces converged, showing *Plateau* (an end-point parameter) to be unsuitable to resolve the role of  $\alpha_2\beta_1$  (Figure 2D). The differences in *PM* are reflected in significantly higher decay constants with GFOGER and GLOGER than on the lower affinity peptides, GMOGER, GAOGER or GPP<sub>10</sub> (Figure 2E).

DM-BAPTA-AM treatment abolished stable adhesion on GMOGER, whereas adhesion was merely attenuated on

GFOGER and GLOGER (Figure 2B and S2). Therefore, an activation-dependent rise in intracellular calcium is an absolute requirement for platelet adhesion to the medium-affinity ligand, and mediates observable increases in integrin activity even to high-affinity peptides. *PM* thus reveals the rate at which  $\alpha_2\beta_1$  is activated in platelets adhering to a thrombogenic, activatory surface. This study provides quantitative evidence that the role of  $\alpha_2\beta_1$  under shear conditions is to accelerate recruitment of platelets to collagen surfaces.

Inhibition of  $\alpha_{\text{IIb}}\beta_3$  with  $5\ \mu\text{M}$  GR144053 reduced  $ZV_{50}$  to  $\sim 0.5\ \mu\text{m}$  on all surfaces, consistent with the presence of a platelet monolayer (Figure 2F). On a surface containing CRP, VWF-III and GPP<sub>10</sub>,  $\alpha_{\text{IIb}}\beta_3$  blockade caused the *plateau* of *PM* to increase from  $21.5 \pm 5.4\%$  to  $69.1 \pm 11.4\%$ , consistent with platelet rolling (Figure 2C and D). *Plateau* on surfaces containing the higher-affinity peptides, GFOGER and GLOGER, was insensitive to GR144053 treatment, while GMOGER supported intermediate behavior.

Hence, a high-affinity  $\alpha_2\beta_1$  ligand is necessary for stable platelet adhesion in the absence of  $\alpha_{\text{IIb}}\beta_3$  activity, and as a corollary,  $\alpha_{\text{IIb}}\beta_3$  contributes to stable adhesion to a surface that cannot bind  $\alpha_2\beta_1$ . It follows that the *PM* profile on such a surface quantifies  $\alpha_{\text{IIb}}\beta_3$  activity, further confirmed by the rise in *plateau* following DM-BAPTA-AM treatment (Figure 2D). Thus, on such surfaces, the *PM* profile is functionally similar to an aggregometry trace, in that it quantifies changes in the  $\alpha_{\text{IIb}}\beta_3$  activation state in real time.

In conclusion, we demonstrate a novel imaging technique that offers parametric kinetic analysis of thrombus deposition. By manipulating platelet receptor function and the composition of the thrombogenic surface, we demonstrate that *PM* measurement can be used to report changes in the activity of integrins  $\alpha_{\text{IIb}}\beta_3$  and  $\alpha_2\beta_1$ . Although similar in some respects to aggregometry, *PM* profiling has the advantage of using whole blood under flow conditions that occur during pathogenic thrombus formation. Real-time, perfusion-based measurements are therefore less reductive and more direct. Additionally, the analysis is mathematically simple and quick and easy to perform, making our technique an alternative to aggregometry, but crucially under physiologically relevant flow conditions. *PM* profiling could be developed to analyze platelet function in a clinical setting using simpler, widefield fluorescence microscopy.

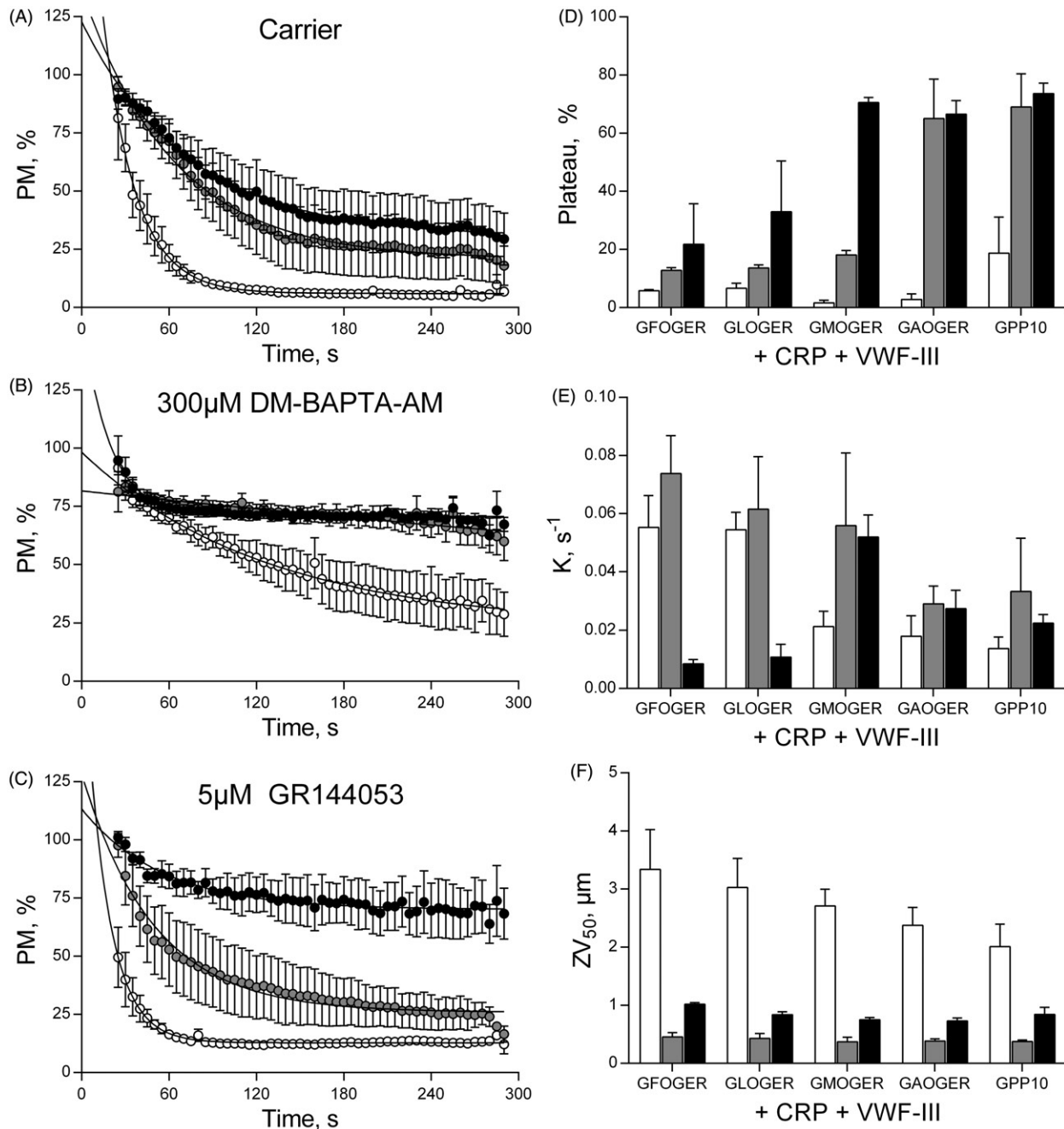


Figure 2. *PM* quantifies integrin activation under flow conditions. Whole blood was pre-treated with carrier (A), 300 μM DM-BAPTA-AM (B) or 5 μM GR144053 (C), before perfusion at 1000 s<sup>-1</sup> over coverslips coated with combinations of CRP, VWF-III and one of a panel of integrin α<sub>2</sub>β<sub>1</sub>-adhesive peptides of varying affinities. *PM* was calculated as before. For clarity, only GFOGER, (○), GMOGER (●) and GPP<sub>10</sub> (●) are shown. Other data sets (GLOGER and GAOGER) are shown in Supplementary Figure S2. End-point measurements were calculated as described in the text, Plateau (D), Decay constant (E) and ZV<sub>50</sub> (F). Blood was pre-treated with: Carrier (□), 5 μM GR144053 (■), 300 μM DM-BAPTA-AM (■).

### Contributions

N.P. and R.W.F. conceived this project. N.P. designed and conducted experiments, analyzed and interpreted data and wrote the manuscript. D.B. generated reagents. R.W.F. and D.J.P. analyzed and interpreted data and wrote the manuscript.

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### Declaration of interest

The authors declare no competing financial interests. This work was supported by the British Heart Foundation (RG/09/003/27122), the Medical Research Council (G0500707) and The Newton Trust.

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### Supplementary material available online

Figure S1. Illustration of the subtraction of subsequent frames to yield a dynamic measure of platelet deposition on a thrombogenic surface.  
Figure S2. *PM* quantifies the activation state of  $\alpha_2\beta_1$  and  $\alpha_{IIb}\beta_3$  under flow conditions.

Supplemental material can be viewed and downloaded at <http://informahealthcare.com/plt>