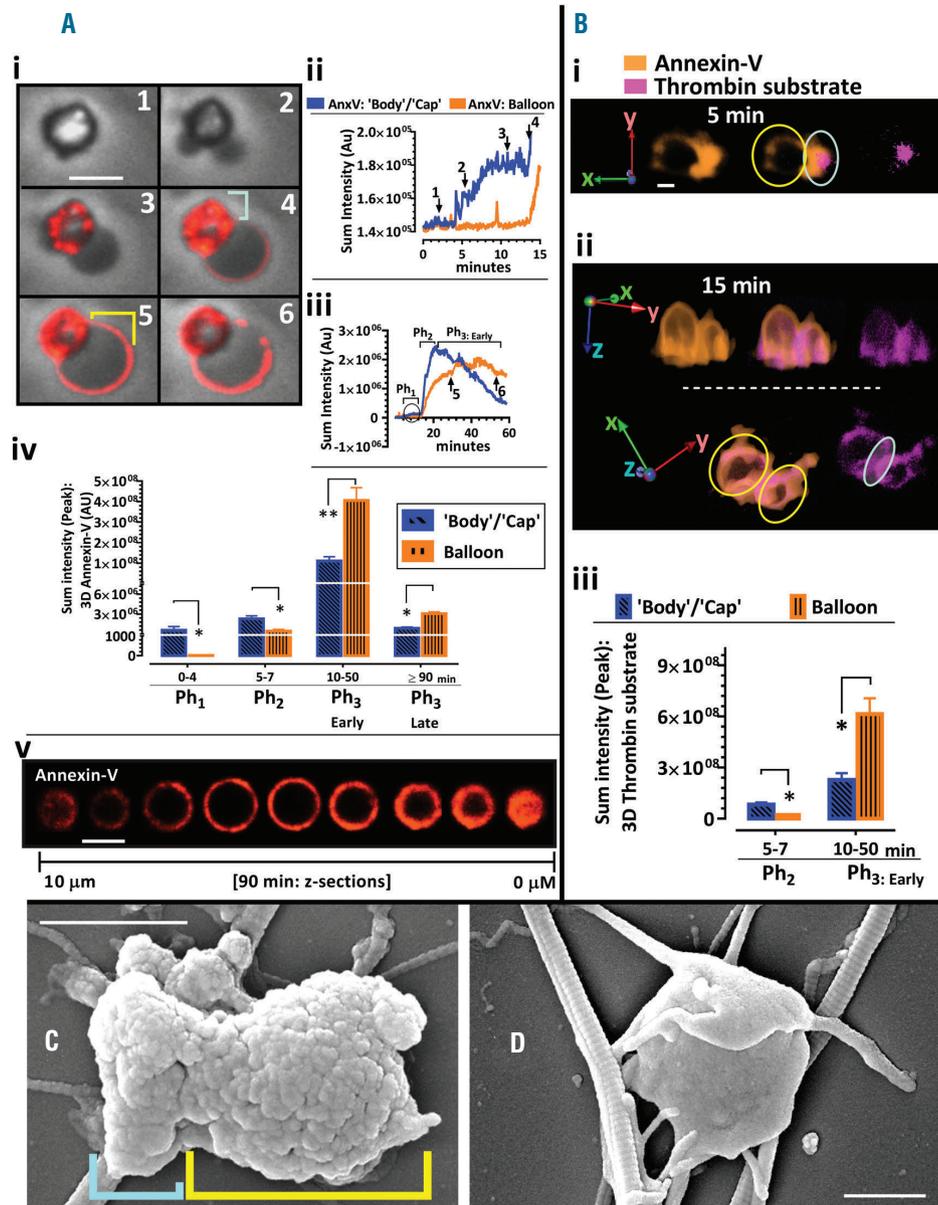


## Temporal contribution of the platelet body and balloon to thrombin generation

Ballooned platelets are part of the haemostatic plug and thrombus architecture; however, it is unclear whether the procoagulant nidus of the ballooned platelet is the platelet body or balloon. Here we show that both

the platelet body and balloon provide procoagulant surfaces but that these are temporally separated; procoagulant activity is primarily located on the platelet body at earlier stages of ballooning, whereas the ballooned membrane forms the predominant procoagulant surface at later time points.

Advances in video microscopy have enabled the detailed elucidation of the structural changes human



**Figure 1. Spatio-temporal dynamics of the procoagulant response of human platelets in contact with collagen.** Using spinning-disk confocal microscopy, human platelets were monitored while adhering to collagen fibres. Fluorescence intensity against time was recorded in platelets pre-incubated with Alexa568-Annexin-V. A: Chart and superimposed phase contrast and fluorescent annexin-V (red) images of the ballooning platelet captured the differential spatiotemporal accumulation of annexin-V on the platelet body (or cap) (indicated by cyan lines) and balloon (indicated by yellow lines). Sum intensity of annexin-V accumulated over time is plotted in A-ii & A-iii. The phases of membrane ballooning as previously described by Agbani *et al.*, 2015<sup>2</sup> is annotated on A-iii and A-iv. Number on images (A-i) corresponds to time points as indicated by the numbered arrows of chart (A-ii and A-iii). Sum annexin-V accumulated on the platelet body (or cap) and the balloon was evaluated for the various phases of membrane ballooning and plotted in A-iv. In A-v, Z-sections of a ballooned human platelet separated by 1  $\mu$ m is shown after 90 min. Platelet is loosely adherent to collagen matrix and stained with Alexa568-Annexin-V. B: By means of a fluorogenic thrombin substrate, Z-GGR-AMC, thrombin activity (magenta) was visualized in collagen adherent platelets of platelet-rich plasma showing annexin-V rich balloons (orange). Related to *Online Supplementary Movie S1*. B-i, B-ii show 3D images of these platelets at 5 and 15 min, respectively. The body (or cap) and balloons of these conjoined platelets are delineated by cyan and yellow circles, respectively. The sum intensity of the thrombin substrate was evaluated and shown in B-iii, for both the platelet body (or cap) and the balloon. C-D: show scanning electron microscopy (SEM) images of procoagulant (C) and non-procoagulant (D) phenotype of washed human platelets adherent to collagen. SEM images (C and D) and fluorescence live images (A-i) were derived from the same donor. Cyan and yellow brackets delineate the platelet body (or cap) and balloon, respectively in C. Data analysis was by Wilcoxon signed rank test,  $P < 0.05$  (\*) was considered significant. Scale bar represents 2  $\mu$ m (A-i, A-v, & C) or 1  $\mu$ m (B, D). Data are representative of platelets from 8 (A and B) and 4 (C and D) human donors. Details of the microscope and the software used for the image analysis are as previously reported by Agbani *et al.*, 2015.<sup>2</sup>

platelets undergo in the haemostatic process.<sup>1,2</sup> Recent studies are beginning to shed light on the physiological relevance of platelet membrane dynamics and its role in platelet-driven thrombosis.<sup>2-5</sup> These studies show that platelets undergo a dramatic transformation to generate balloon-like structures with surface-exposed phosphatidylserine (PS), after collagen stimulation.<sup>2-5</sup> However, the localisation of the procoagulant surface of the ballooned platelet, which amplifies coagulation, remains unclear. On the one hand, four-dimensional (4D) imaging showed that formation of membrane balloons (delineated by the yellow bracket in Figure 1Ai and C) coincided with the formation of procoagulant microvesicles and the amplification of thrombin generation.<sup>2</sup> Other studies however infer that a part of the ballooned platelet termed the 'cap' (delineated by the cyan bracket in Figure 1Ai and C), expresses a high density of surface PS, and is more important for the acceleration of coagulation and thrombus formation.<sup>4,5</sup> In this letter, we provide evidence that unifies these observations.

The procoagulant feature described as being the platelet cap<sup>3-5</sup> has recently been reported as the remnant platelet body in experiments observing membrane ballooning in real-time.<sup>2</sup> The platelet body (or cap) and the balloon are distinct parts of the activated platelet; strictly, the balloon is the inflated membrane of the platelet body (or cap) as shown in time-lapse images of Figure 1Ai. We suggest that both the platelet body and the ballooned membrane are likely to provide important procoagulant surfaces, but they are temporally separated so that the body is responsible for early PS exposure whereas the balloon, which has an extensive surface area, is responsible for a prolonged and substantial second wave of thrombin generation. Differences in interpretation of the derivation of the platelet body (or cap) are due largely to whether the dynamics of ballooning were followed in real-time over a prolonged period or observed at single time points.<sup>2,6</sup> Visualisation of platelet membrane ballooning in real-time by 4D imaging for 90 min reveals the time-dependent differential contribution of the remnant platelet body and the ballooned membrane to coagulation. We previously identified 3 distinct phases of platelet ballooning, which are critical to the correct interpretation of the role of the ballooned membrane in the localisation and acceleration of coagulation at wound sites.<sup>2</sup> We had termed these phases Ph1, Ph2 and Ph3; where Phase 1 (Ph1) was associated with blebbing and bleb retraction, phase 2 corresponded to the rapid membrane expansion or ballooning phase and in phase 3 (Ph3), the expansion plateaued.<sup>2</sup>

Consistent with this, we show in Figure 1A that annexin-V accumulates on the membrane of the platelet body alone from 0-7 min after adhesion to collagen, corresponding to phases Ph1 and Ph2 of ballooning (Figure 1Ai,ii), followed by binding subsequently to the ballooned membrane in early phase Ph3 (Figure 1Ai,iii). The earlier time points correspond more closely to those used in other studies<sup>4,5</sup> which had made similar observations of a higher density of annexin-V on the platelet body (or cap) compared to the ballooned membrane.<sup>2,4,5</sup> However, visualizing the formation of the balloon as well as the spatial distribution of annexin-V for over 45 min, revealed that the PS localisation switches from the remnant platelet body to the ballooned membrane in time (Figure 1Ai-iv), possibly mediated through a wave of Ca<sup>2+</sup> along the membrane.<sup>2</sup> Consistent with this, we show in Figure 1Aiv, that at early phase 3 (10-50

min) and late phase 3 ( $\geq 90$  min) adhesion to collagen, the ballooned membrane plays the predominant role in PS exposure. We also present data to indicate that the platelet body (or cap) is gradually lost, such that at later time points the only remaining structure is the platelet balloon. Figure 1Av shows an example of this, where the platelet body (or cap) has completely disappeared. At present we do not know whether the platelet body is incorporated into the balloon, internalised or shed into the external environment. However, the presence of PS +ve microvesicles nearby<sup>2</sup> may suggest that it can potentially contribute to PS +ve membrane shedding into the external environment. Throughout their lifetime ballooned platelet membranes remain able to support not only annexin-V binding, but also active thrombin generation as demonstrated by use of the fluorogenic thrombin substrate peptide (Figure 1B; *Online Supplementary Movie S1*). Likewise, consistent with annexin-V binding data (Figure 1Aiv), thrombin generation was significantly higher at the platelet body when compared to the ballooned membrane at early time points (Phase 2, Figure 1Bi, iii), but this reversed to higher thrombin generation on the ballooned membrane at later time points (early Phase 3, Figure 1Bii, iii; *Online Supplementary Movie S1*).

Our 4D imaging data also clarify the nature of the platelet body (or cap) during ballooning,<sup>3-5,7,8</sup> clearly showing that what has previously been termed the platelet cap, is the remnant platelet body. Capping was originally described by Taylor *et al.* in lymphocytes<sup>9</sup> as a characteristic of certain motile cells, and is a readily reversible process induced by antibodies directed against lymphocyte surface immunoglobulins, causing them to cluster at one region of the cell surface.<sup>9</sup> Data on platelet capping are limited, but it is unlikely that human platelets actually undergo this process. The reported features of platelets described as capped<sup>5</sup> do not match the description by Taylor *et al.*<sup>9</sup> For example, unlike classical capping, the process for capped platelet formation is irreversible, and this phenotype shows striking similarities with previously characterised ballooned platelets,<sup>2,10</sup> the formation of which was driven by salt and water entry. Also, while cap formation in lymphocytes was attenuated by drugs inhibiting actin filament network formation such as cytochalasin-B or D, these compounds promoted the formation of ballooned platelets after collagen stimulation.<sup>2</sup> Capping in lymphocytes involves clustering of surface immunoglobulins at the cap region, resulting in distinct changes in surface morphology. To test whether the membrane characteristics of the platelet body differed from that of the ballooned membrane, we used high resolution scanning electron microscopy (SEM), simultaneously with imaging of platelets (of the same donor) fluorescently labelled with annexin-V after adhesion to collagen surface; we then identified the platelet phenotype by their morphology as previously described.<sup>2</sup> While the results did not indicate that ballooned platelets are exclusively procoagulant, it showed that procoagulant human platelet membrane surfaces displayed a 'cauliflower-like' transformation, when compared to non-procoagulant platelets (compare Figure 1C with 1D). Also, membrane undulations were similar at the region of the platelet body to that of the balloon (Figure 1C) and we therefore failed to identify a distinct surface morphology of the platelet body that would suggest receptor clustering or 'capping' in this region.

Although, the physiological significance of thrombin generation starting at the platelet body and then moving

to the balloon is presently unknown, it may lie with the spatiotemporal importance of PS exposure in the developing thrombus. PS exposure on the platelet body begins almost immediately after platelet adhesion to collagen,<sup>2</sup> and is likely to ensure rapid initiation of thrombin generation at wound sites. This may then become a seed procoagulant agonist, for the stimulation and recruitment of more platelets as well as fibrin deposition at the wound site itself. The increased procoagulant surface provided by the balloon at later times will amplify thrombin generation, microvesiculation and hence coagulation. This differential contribution of the platelet body and balloon could be a way to propagate the thrombin gradient throughout a growing thrombus, and therefore temporally coordinate coagulation and microvesicle formation.<sup>11</sup> From a physiological perspective we therefore suggest that the differential time lines of procoagulant activation enable platelets to generate thrombin on both a rapid timescale (from the cell body) and a more prolonged timescale (from the balloon). This may allow rapid development of platelet activation within the thrombus core whilst also allowing for more prolonged activation that will be required for consolidation and stabilisation of the thrombus at the site of injury.<sup>12</sup>

In conclusion, the 4D imaging approach adopted here shows that what has previously been referred to as a cap is actually the remnant platelet body. We conclude that PS is predominantly expressed on the platelet body at earlier stages of ballooning, whereas the balloon surface itself forms the predominant procoagulant surface at later time points. The localisation and density of PS exposure at different time points may contribute to propagation of the thrombin gradient, and therefore coagulation, throughout a growing thrombus.

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