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Procoagulant Platelets: -
Generation, Function and Therapeutic Targeting in Thrombosis

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Abstract
Current understanding of how platelets localise coagulation to wound sites has come mainly from studies of a subpopulation of activated platelets. In this review we summarise data from the last four decades which has described these platelets with a range of descriptive titles and attributes. We identify striking overlaps in the reported characteristics of these platelets which imply a single subpopulation of versatile platelets, and thus suggest that their commonality requires unification of their description. We therefore propose the term procoagulant platelet as the unifying terminology. We discuss the agonist requirements and molecular drivers for the dramatic morphological transformation platelets undergo when becoming procoagulant. Finally, we provide perspectives on the biomarker potential of procoagulant platelets for thrombotic events, as well as on the possible clinical benefits of inhibitors of carbonic anhydrase enzymes and the water channel Aquaporin-1 for targeting this subpopulation of platelets as antiprocoagulant antithrombotics.

Running title: Unifying review of procoagulant platelets

Keywords: ballooning, procoagulant platelets, mitochondrial permeability, procoagulant-spreading.

Acronyms/Abbreviations: BNS, BAPS, Fib-Cap, MPTP, ROS, PS, NADPH

ADP: Adenosine diphosphate
vWF: Von Willebrand factor
GSAO: 4-[N-(S-glutathionylacetyl) amino] phenylarsonous acid
SCIP: Sustained Calcium Induced Platelets
BAPS: Ballooned And Procoagulant-Spread
BP: Ballooned Platelets
BNS: Ballooned Non Spread
PS: Phosphatidylserine
FIB-CAP: Fibrinogen Capped Platelets
MPTP: Mitochondrial Permeability Transition Pore
HDBS: High Density Bubble Shaped
COATED: Collagen And Thrombin Activated
ROS: Reactive Oxygen Species
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
1. Introduction

The ability of platelets to multitask is critical to the arrest of bleeding after injury, and to the development of arterial thrombosis, which underlies stroke and coronary artery disease. Following the reflex vasoconstriction of an injured vessel, platelets are recruited to the wound site to which they adhere and aggregate to form a fragile plug in the initiation of haemostasis. Platelets then provide the requisite membrane for the assembly of the prothrombinase complex, thereby localising coagulation to the wound site. The local generation of thrombin catalyses the conversion of soluble fibrinogen to insoluble fibrin, which stabilises the plug into a firm clot that prevents bleeding and promotes healing. It is now apparent that different populations of platelets play different roles in the cascade of events in thrombus formation.

The concept of functionally different subpopulations of platelets or division of labour amongst platelets at wound sites is well reported. For example, Patel et al.,\(^1\) described differences between ‘vanguard platelets’, a subpopulation that is the first to adhere to collagen and a second population of ‘follower platelets’ that adhere over the top of these. Furthermore, it is now well accepted that even after strong activation, not all stimulated platelets become procoagulant. Accordingly, differential expression on the plasma membrane of phosphatidylserine (PS) or components of the prothrombinase complex have been reported after platelet activation with thrombin and/or collagen\(^2,3\) (see Fig. 1). Indeed, an increasing number of studies show heterogeneity in platelet responses to agonists. Two distinct phenotypes are discernible in the literature, a procoagulant phenotype which externalises phosphatidylserine, binds tenase and prothrombinase complexes and accelerates coagulation at the wound site and an aggregating and contractile phenotype characterised by active integrin $\alpha_{IIb}\beta_3$ which pulls fibrin over the platelet plug to tighten the clot into an impermeable cell mass\(^4-7\).

Over the last four decades, several subpopulations of platelets with different features have been identified, named and suggested as candidates for the procoagulant platelet. In this review we summarise these data, reveal attributes central to these platelet phenotypes and provide perspectives on the mechanisms and molecular targets in the procoagulant response of activated platelets. We suggest that the various phenotypes described actually form a spectrum of a single platelet phenotype, the procoagulant platelet. Finally, we examine the potential for targeting this platelet subpopulation as an antithrombotic approach. In this regard we discuss the potential of inhibitors of the water channel aquaporin-1 (AQP1) and the repurposing of carbonic anhydrase inhibitors as antiprocoagulant antithrombotics, which may selectively limit the platelet procoagulant responses while sparing platelet aggregation and secretion.

1.1 How one platelet becomes procoagulant whilst another does not

A high and sustained calcium rise is required for PS externalisation, coagulation factor binding and calpain-mediated inactivation of $\alpha_{IIb}\beta_3$ integrin, and is a major difference between aggregatory and procoagulant platelets, possibly explaining their different derivations\(^8-11\). It is well known that the procoagulant response of strongly activated platelets is preceded by calcium mobilisation from intracellular stores\(^10,12,13\); this is associated with the activation of Ca\(^{2+}\) activated chloride channels, resulting in an initial salt entry which is then followed by the influx of water\(^8\). The chloride ion entry causes membrane hyperpolarisation and enhances the electrochemical drive for Ca\(^{2+}\) entry\(^14\) through both store-operated and store-independent pathways; together these ensure a high and sustained level of cytosolic calcium required to drive the procoagulant response\(^8,10,12-14\). The pattern of calcium signalling is different in adherent aggregating platelets, which are rather characterised by intermittent spikes in calcium levels or oscillatory calcium responses and the sustained engagement of $\alpha_{IIb}\beta_3$ integrins\(^8,10,15,16\). Furthermore, platelet age or level of oxidative stress may pre-commit platelets to aggregatory or procoagulant phenotypes as discussed later in this review.
2. Categorisation of procoagulant platelet types
Platelets reported in the literature to be procoagulant show several overlapping features, and may be classified as ballooning or MPTP phenotypes based on the broad descriptions provided in the corresponding papers here reviewed. We depict the features of these platelets, and explain their overlaps.

Figure 1: Procoagulant attributes and causative mechanisms of candidate procoagulant platelets. Figure shows platelet phenotypes by their published names or by their unique features as follows: 1) (BP/BNS)\(^8,10,17\): Ballooned Platelets/Ballooned Non-Spread Platelets, 2) (SCIP)\(^17\): Sustained Calcium-Induced Platelets, 3) (BAPS)\(^8,18\): Ballooned And Procoagulant-Spread platelets\(^6\); High Density Bubble Shaped (HDBS) platelets also show similar features\(^19,20\), 4) (FIB-CAP)\(^20,21\): Fibrinogen Capped platelets; characterised by PS exposure, the lack of active integrin \(\alpha IIb \beta 3\) and the retention of fibrinogen and thrombospondin as a single patch or cap on the procoagulant platelets. 5) (GSAO)\(^22,23\): 4-[N-(S-glutathionylacetyl) amino] phenylarsonic acid binding procoagulant platelets, 6) (MPTP)\(^24-28\): procoagulant platelets dependent on the formation/opening of Mitochondrial Permeability Transition Pore and 7) (COATED)\(^11,26-30\): Collagen And Thrombin-activated platelets. BP, SCIP, BAPS and FIB-CAP platelets are broadly referred to as ballooning/ballooned platelets; while GSAO, MPTP and COATED platelets are broadly classified as MPTP phenotype. The procoagulant attributes are qualitative features of procoagulant platelets which we scored 100 for ‘Yes’, 0 for ‘No’, and 50 for transient features or ‘Yes/No’. Features based on the ‘causative mechanisms’ are quantitative; for this we assigned values based on published fractional responses or the reported percentage procoagulant platelets formed. We then present these data as a colour map where the upper, middle and lower end of a 0 - 100 scale is represented by Red, Yellow and Blue, respectively. To determine the characteristic features of the procoagulant platelet based on pooled data, we used the mean value for each feature across candidates of the procoagulant platelet (BP, BNS, HDBS, FIB-CAP, SCIP, BAPS, GSAO, COATED & MPTP), as shown in the corresponding row of the ‘PROCOAG ID’ column. The colour map was generated using Prism 7 (GraphPad software). White spaces within map represent unknown parameters while the black column demarcates the individual scores from the mean score in the ‘PROCOAG ID’ column.
2.1 **Balloon shaped or ballooning phenotypes**

The earliest report of a ballooning platelet phenotype was in 1978 by Wester et al., who reported the ultrastructural changes platelets undergo to arrest bleeding after skin vessel transection. Changes in platelet morphology were investigated at high resolution by electron microscopy and a subset of platelets was observed to have assumed a ballooned shape, with fibrin deposited between platelet balloons. These ballooned platelets were an integral part of the platelet plug and stable clot. About two decades later, this phenotype was generated *in vitro* on a collagen matrix, and platelets shown to externalise PS after a sustained cytosolic calcium rise and in the presence of physiological levels of extracellular calcium. It is clear that ballooned platelets not only bind annexin-V as indication of PS exposure but provide an extended surface area for the assembly of the prothrombinase complex and contribute to the acceleration of coagulation at the wound site. Balooned phenotypes have been reported as either BP, SCiP, Fib-CAP, or BAPs (Fig. 1), and show attributes similar to other candidates of the ‘procoagulant platelet’ such as the MPTP and COATED platelets.

2.2 **Mitochondrial permeability transition pore (MPTP) phenotype**

In a recent study, Hua et al. showed that co-labelling of platelets with fluorescent conjugates of a tripeptide trivalent arsenical 4-[N-(S-glutathionylacetyl) amino] phenylarsonous acid (GSAO) and an α-granule marker identified a subpopulation of activated procoagulant platelets undergoing cyclophilin-D dependent necrosis. GSAO likely covalently binds to proteins containing cysteine thiols as it has been previously reported to covalently bind the molecular chaperone heat shock protein 90 (Hsp90). We have previously shown that the actin cytoskeleton undergoes remodelling and the cell membrane increases permeability during platelet transformation to the procoagulant phenotype, and this would allow GSAO to label intracellular proteins in necrotic platelets. Furthermore, GSAO platelets show striking similarities to the activated necrotic platelets described by Jobe et al. as ‘highly activated platelets’, characterized by high-level PS externalization, high-level fibrinogen retention, antigenic modulation of α\(_\text{IIbβ3}\) and marked membrane vesiculation. Like GSAO platelets, the formation of this subpopulation of platelets was dependent on cyclophilin-D induced mitochondrial permeability transition pore (MPTP) formation and opening. Cyclophilin-D is considered a modulatory component of MPTP which may regulate pore opening to allow for cytosolic molecule influx, leading to increased matrix volume, disruption of mitochondrial outer membrane and oxidative stress. Accordingly, Cyclophilin-D inhibition (by Cyclosporin A, coenzyme Q, or bongkrekic acid) markedly reduced the formation of both MPTP and GSAO platelets in independent experiments. Another platelet subpopulation exhibiting the MPTP phenotype is the COATED platelet. These were originally so named by Dale in 2005, as a subpopulation of platelets that were generated after ‘CO’llagen ‘A’nd ‘T’hrombin stimulation. Specific parameters characterize this PS exposing platelets, namely the surface expression of α-granule proteins such as FVa, strong binding of transglutaminase substrates, fibrinogen, von Willebrand factor, thrombospondin, fibronectin and α\(_2\)-antiplasmin.

2.3 **Commonalities of candidate procoagulant platelets**

Like the ballooning phenotype of procoagulant platelets (BP, SCiP, BAPs, FIB-CAP), COATED platelets bind α-granule protein, fibrinogen, externalise PS, promote microvesiculation and become membrane permeable after activation. Furthermore, convincing data also indicate COATED platelet formation is associated with rapid loss of loss of mitochondrial transmembrane potential (ΔΨ\(_m\)), a characteristic also described for MPTP and GSAO platelets. Other striking similarities in the formation and characteristics of the ‘procoagulant platelet’, as described in the literature, are shown in Fig. 1. The overlap in features of these platelets is therefore strongly suggestive of a single procoagulant versatile subpopulation of platelets. We therefore propose that platelets previously described by any of these range of descriptors, that show the common
characteristics indicated in the ‘PROCOAG-ID’ column of Fig. 1, be simply referred to as procoagulant platelets.

The features of the procoagulant platelet have differed depending on whether it was investigated in suspension (COATED, MPTP, BNS, FIB-CAP or GSAO) or adherent to solid agonist-coated matrices (SCIP, MPTP, BNS, BAPS, FIB-CAP). Unlike in suspension, a solid matrix provides procoagulant platelets with an adhesion platform upon which unique morphological transformations can occur. Features such as procoagulant-spreading, observed in SCIP and BAPS platelets, have been identified after adhesion to agonist-coated surfaces, but not in suspension. The characteristics of the procoagulant platelet, identified under either adhesion or suspension conditions, are summarised in Table 1.

<table>
<thead>
<tr>
<th>Adherent Platelets</th>
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<tr>
<td>• PS exposing</td>
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<tr>
<td>• FXa/ FVa binding</td>
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<td>• Thrombin generation</td>
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<td>• ↑_membrane permeability</td>
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<td>• Loss of Δψm</td>
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<tr>
<td>• Mitochondrial depolarisation</td>
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<td>• Cyclophilin-D dependence</td>
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<td>• Microvesiculation</td>
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<td>• Ballooning</td>
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<tr>
<td>• Procoagulant-Spreading</td>
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Table 1: Features of adherent and non-adherent procoagulant platelets. Procoagulant platelets show similar features, in the presence of physiological concentrations of extracellular calcium, whether studied adherent to a collagen matrix or stimulated with collagen in suspension. A distinguishing feature, unique to adherent platelets, is the formation of procoagulant-spread structures. There are currently no identified features that suspended procoagulant platelets show that are unique to this mode of activation.

3. Mechanisms of procoagulant platelet formation
The seven candidates for the procoagulant platelet shown in Fig. 1 share a common mechanism of formation which we will now discuss; a further indication that these comprise a spectrum of a single platelet phenotype.

3.1 Agonist requirement for procoagulant platelet formation
Collagen remains the most important single agonist for the formation of adherent procoagulant platelets in the presence of physiological concentrations of extracellular calcium; it accounts for 40-80% of the procoagulant platelets formed under this condition. In suspension however, co-stimulation with agonists of protease-activated receptors was often necessary to achieve a similar size of procoagulant subpopulation. With thrombin alone, the proportion of activated platelets that become procoagulant varied from 20-60% in suspended platelets to 1-30% in platelets adhered to inert surfaces (Fig. 1). These differences are likely to be due to variations in experimental conditions and to the different agonist stimulation pathways; however, in both adherent and suspended platelets, signalling via the glycoprotein VI (GPVI) receptor is a major pathway for the formation of procoagulant platelets. Platelet activation with adenosine diphosphate, von Willebrand factor, or a thromboxane A2 mimetic alone does not or only weakly generates procoagulant platelets. In addition, some studies report that platelet pre-treatment with aspirin has minimal effect on the generation of procoagulant platelets.
3.2 Calcium dependence of procoagulant platelets
Platelet activation via GPVI or Gq-coupled protease-activated receptors leads to a sustained increase in cytosolic calcium owing both to extracellular calcium influx and calcium mobilization from stores\textsuperscript{12,40}. The role of calcium signalling in the formation of procoagulant platelets is pivotal, as formation of these platelets is abrogated by the depletion of extracellular calcium or by BAPTA mediated chelation of cytosolic calcium\textsuperscript{8,10}, (Fig. 1); notably, there needs to be a sustained rise in cytosolic calcium to activate downstream signalling for a procoagulant response\textsuperscript{8,10,21,22,41} (Fig. 1).

3.3 The role of calpain
Sustained elevated cytosolic calcium required for the procoagulant response will in tandem breach the threshold of $[\text{Ca}^{2+}]_{\text{cyt}}$ required to activate the thiol protease, calpain\textsuperscript{17,42}. Major contractile cytoskeletal and membrane linker proteins such as actin, vinculin and myosin have been identified as calpain substrates\textsuperscript{43}. Once activated, calpain may induce the proteolysis of these proteins, and aid the remodelling associated with the dramatic transformation of the procoagulant platelet. The fragmentation of the procoagulant platelet membrane and eventual release of PS-positive microvesicles is consistent with calpain action\textsuperscript{44}. Indeed, we recorded a rise in levels of activated calpain during active membrane ballooning in collagen activated platelets (Fig. 2) consistent with previous reports of calpain activation in collagen-stimulated platelets\textsuperscript{45,46}.

Figure 2: Spatio-temporal pattern of calpain activation, PS exposure and reactive oxygen species generation in the ballooning platelet. Extended focus images obtained at 20 min after platelet adherence to fibrillar collagen show the spatial location of exposed PS indicated in red (as monitored by membrane annexin-V accumulation), calpain indicated in cyan and reactive oxygen species (ROS) indicated in green. Calpain activity was monitored by the 7-Amino-4-chloromethylcoumarin (CMAC) based substrate, fluorogenic t-BOC-Leu-Met-CMAC substrate which yield fluorescent peptidase products with improved retention in live platelets. The generation of ROS during membrane ballooning was followed in real-time by means of MitoSox\textsuperscript{TM} (ThermoFisher Scientific), which is rapidly oxidized by superoxide to produce highly fluorescent products. Chart shows the temporal profile of calpain activation, PS exposure and ROS generation in a ballooning human platelet. Written informed consent was obtained in accordance with the Declaration of Helsinki. Human blood was obtained from healthy, drug-free volunteers under the University of Bristol, United Kingdom, Research Ethics approval (E5736). Live cell imaging was performed at 25 °C using a spinning-disk confocal system as previously described\textsuperscript{8,18}. Scale bar represents 3 µm. Data are representative of 4 independent experiments.

3.4 Integrin activation
The structure and function of the platelet integrin $\alpha_{\text{Iib}}\beta_3$ is extensively reviewed elsewhere\textsuperscript{47,48}. This integrin is the best characterised of the platelet integrins, yet reports of its role in the formation of procoagulant platelets are conflicting\textsuperscript{8,21,25}. For example after collagen stimulation, the BAPS phenotype of platelets showed high fibrinogen binding and $\alpha_{\text{Iib}}\beta_3$ activation, however BP, COATED, SCIP, MPTP and FIB-CAP platelets vary in this respect\textsuperscript{9} (Fig. 1). Some of the discrepancies may result from differences in kinetics of integrin activation. Platelets stimulated with convulxin/thrombin showed high fibrin and fibrinogen binding, which was associated with decreased PAC-1 binding after an initial peak\textsuperscript{11}; this supports previous reports of secondary inactivation of integrin $\alpha_{\text{Iib}}\beta_3$\textsuperscript{9,17} and a conclusion that its activity during the formation of procoagulant platelets is transient. Moreover, it is possible that fibrinogen and fibrin bind to distinct sites on integrin $\alpha_{\text{Iib}}\beta_3$\textsuperscript{15,49-51}, and binding to each may be modulated differently by the mechanisms of integrin inactivation.
3.5 Chloride ion (Cl\textsuperscript{-}) entry and a role for TMEM16F

Our pooled data indicate an association between the platelet procoagulant response and Cl\textsuperscript{-} entry (Fig. 1); strong stimulation of platelets with collagen and or thrombin induce Cl\textsuperscript{-} entry, which drives membrane hyperpolarisation and PS exposure\textsuperscript{14}. There is also good evidence that TMEM16F or Ano6 is a key channel for Cl\textsuperscript{-} entry in this response\textsuperscript{52,53}. Indeed, ablation of TMEM16F\textsuperscript{54} or the blockade of calcium-activated Cl\textsuperscript{-} channels with small molecule inhibitors markedly diminished the platelet procoagulant response\textsuperscript{8}. Accordingly, membrane ballooning was ablated in the Scott patient’s platelets, thus indicating an important role for TMEM16F in the platelet procoagulant response\textsuperscript{8,9,53-57}.

3.6 The procoagulant platelet is under oxidative stress

Independent of granular release, the oxidizing agent H\textsubscript{2}O\textsubscript{2} alone can induce a cyclophilin-D dependent loss of mitochondrial transmembrane potential (ΔΨ\textsubscript{m}) due to MPTP formation and opening\textsuperscript{24}; this effect is calcium-independent and is mediated by thiol oxidation\textsuperscript{58,59}. The pooled evidence (Fig. 1) indicates that thrombin alone is a weak agonist for inducing the procoagulant response in activated platelets; however once under oxidative stress, platelets are 6 times more likely to become procoagulant with thrombin stimulation alone\textsuperscript{24}. This suggests a role for intracellular oxidative stress and reveals an ancillary pathway independent of strong stimulation for the formation of the ‘procoagulant platelet’. Interestingly, oxidative stress alone has been shown to have no effect on fibrinogen binding, PS externalization, or granule release, raising the possibility that intravascular oxidative stress may play a role only in the priming of platelets for procoagulant response. This fits the observation that platelet aging, which has been reported to be associated with glutathione peroxidase-1 or NADPH oxidase dysfunction and oxidative stress\textsuperscript{60,61} may also prime and commit platelets to the procoagulant pathway. Equally, drugs or oxidizing agents that potentiated platelet oxidative stress or MPTP formation/opening have been shown to promote the formation of procoagulant platelets\textsuperscript{24,26}. Consistent with these and related findings previously reviewed\textsuperscript{60}, we observed that the procoagulant response of human platelets adhering to collagen was associated with increased superoxide generation (Fig. 2).

3.7 A phenotype that gains function by dying

Although it is clear that procoagulant platelets are undergoing a death process, the literature is ambivalent on whether this is by apoptosis\textsuperscript{26,62} or necrosis\textsuperscript{8,22}, indicating that the process may be strictly neither. Instead, it follows a pathway analogous to necrosis, but differentiated by a gain of procoagulant function. This pathway is induced by external ligand and characterised by the following features: (1) calcium dependence\textsuperscript{8,10,12,18} (2) cyclophilin-D and but not Bax/Bak ablation dependence\textsuperscript{9,24} (3) formation/opening of mitochondrial permeability transition pore\textsuperscript{24,58}, (4) early and rapid loss of ΔΨ\textsubscript{m}\textsuperscript{24}, (5) intracellular oxidative stress\textsuperscript{8,22,26}, (6) membrane ballooning due to a tightly regulated fluid entry system\textsuperscript{8,10}, (7) an early increase in membrane permeability associated with microtubule unwinding and (8) calpain mediated remodelling of the actin-cytoskeleton\textsuperscript{44,63}. Characteristically, these events are caspase-independent (Fig. 1), yet they drive the amplification of thrombin generation through the combined increase in membrane PS exposure and membrane surface area and microvesiculation\textsuperscript{8,18,24}. It is interesting to note, that while cell death is classically associated with a loss of function, for the procoagulant platelet the death process is clearly mechanistically important.

3.8 The procoagulant response is regulated by fluid entry

A key event during procoagulant platelet formation is the irreversible membrane swelling or ballooning which results from the physical disruption of the membrane-cytoskeleton interaction, and an increase in internal hydrostatic pressure provided by a coordinated fluid entry system\textsuperscript{8}. Balloon formation is reported in adherent platelets\textsuperscript{8,18} as well as in stimulated platelet suspensions\textsuperscript{9,64}; here in Fig. 3, we illustrate its formation mechanism. The fluid entry requirement for ballooning distinguishes this process from blebbing, which is the formation of retractable
membrane protrusions. Blebbing is often used interchangeably with ‘ballooning’\textsuperscript{10,65}, whereas both events illustrated in Fig. 3 are distinct and driven by separate mechanisms\textsuperscript{8}. For example, (i) balloons, but not transient membrane blebs, are procoagulant; (ii) ballooning but not bleb formation requires disruption to the platelet microtubule cytoskeleton and increased membrane permeability; (iii) unlike blebs, procoagulant ballooning is irreversible and consequent upon Na\textsuperscript{+}, Cl\textsuperscript{−} and water entry, and (iv) whereas the hydrostatic pressure required for bleb formation is fluid entry-independent, the rapid membrane expansion associated with ballooning requires fluid entry driven by the osmotic pressure of salt entry\textsuperscript{8} (Fig. 3). Precision is therefore required when discussing these events in platelets. 

![Figure 3: Membrane blebbing and ballooning in human platelets.](image)

4.0: Clinical relevance and translational potential for procoagulant platelets

Globally, cardiovascular disorders (CVD) remain the largest single contributor to mortality and morbidity\textsuperscript{68,69}. The role of the procoagulant platelet in platelet-driven thrombosis may underlie this statistic. For example, on the one hand low levels of procoagulant platelets have been shown to correlate with increased frequency of acute ischemic stroke complications, especially after thrombolytic therapy and increased spontaneous intracranial haemorrhage\textsuperscript{70-72}; and on the other, high levels of these platelets correlate with transient ischemic attack and stroke\textsuperscript{73,74} and with milder haemorrhagic phenotype in severe hemophilia\textsuperscript{75}. It may therefore be possible to exploit procoagulant platelets either through targeting them pharmacologically or through identifying them as biomarkers, in the management of thrombotic cardiovascular disease.

4.1 Procoagulant platelets as clinical biomarkers

There is a need for universally accepted markers and/or identity criteria for the clinical assessment of thrombotic or bleeding tendency. It is possible that clinical markers could be designed based on the shared attributes of procoagulant platelets, and their identity criteria could be based on their commonalities shown in Fig. 1. For example, evaluation of blood from healthy donors for procoagulant platelets, based on the combined expression of activated coagulation proteins and PS externalisation, showed that about 31.67 ± 13.2% (mean± SD), of platelets can assume this phenotype after dual stimulation with collagen and thrombin as previously reported\textsuperscript{27,73}. This figure varied widely between donors but appeared conserved within donors over time\textsuperscript{29}, a clinical feature that may be exploited to individualise and monitor bleeding or thrombotic tendency in anticoagulation therapy or surgical units. Furthermore, classical features of the procoagulant platelet, such as increased membrane permeability\textsuperscript{8}, will enable their in vitro identification by quick tests utilising low molecular weight cell impermeant dyes such as propidium iodide which label DNA and RNA\textsuperscript{76}. Also, the report of GSAO earlier described as a marker of procoagulant platelets demonstrates the feasibility of this approach\textsuperscript{22}. Other features such as a strong fibrinogen or coagulation factors (FVa, FXa) binding (Fig. 1), can also be used to identify and quantify the preponderance of procoagulant platelets in clinical settings, using image based or flow cytomteric analysis\textsuperscript{3}. Such assessment, when done before major surgical intervention, may provide reliable
data to enable the prediction of whether a patient has a tendency to bleed or will be at risk of thrombosis. In addition, these data provide a lead for the development of new bedside medical devices, for measuring platelet function and with predictive capabilities for bleeding disorders. For example, the *in vitro* formation of BP, BAPS, FIB-CAP, GSAO or COATED platelets was a strong correlate of *in vivo* arterial thrombus formation (Fig. 1).

### 4.2 Molecular drivers of procoagulant membrane dynamics as new antithrombotic targets

Newer data provide insight into the molecular mediators and drivers of the platelet procoagulant response.

These molecular mechanisms reveal alternative targets for the regulation of thrombosis, which may spare essential secretion and other platelet functions. Since we had recently shown that blockade of P2Y1 and P2Y12 does not inhibit platelet procoagulant balloon formation or microvesiculation, there is an opportunity for development of a new class of antithrombotics that target platelet procoagulation. For example, carbonic anhydrases 1, 2 and 13 have been recently identified as new potential anti-thrombotic targets, for which there are already inhibitors in clinical use such as the mild diuretic acetazolamide. We have recently shown that acetazolamide is a potent antithrombotic *in vivo,* and has the potential to be a genuinely novel approach to the management of thrombotic disease. The signalling pathway involved in the antithrombotic actions of acetazolamide might include the regulation of reactive oxygen species by carbonic anhydrase enzymes, but at the moment there is no evidence in the literature. However, several reports indicate that carbonic anhydrase inhibitors are also capable of blocking water entry via the water channel AQP1, and this may also be an important mechanism of action. Consistent with this, we have recently shown AQP1 to potently regulate thrombus formation *in vivo,* implicating it as a target for development of novel antithrombotic drugs.

![Figure 4: Acetazolamide suppresses thrombus formation *in vivo.*](image)

Mice were administered acetazolamide (7 mg/kg) or vehicle by single bolus intravenous injection, followed immediately by DyLight 488–conjugated anti-GPIbβ antibody to label platelets. Carotid artery damage was achieved by treatment with FeCl3 as previously described. Fluorescently labelled platelets adhering at the site of injury could then be imaged continuously by intravitral fluorescence microscopy. Images at frames indicated in A correspond to time points indicated in Bi, which shows median fluorescence intensity, quantified by using ImageJ. Analysis of the area under the curve for media fluorescence is shown in Bi as interleaved box plots with whiskers showing minimum to maximum values, median, and interquartile range. Data analysis was by Wilcoxon signed rank test, *P*<0.05 (*) was considered significant. Scale bar= 500 μm). Data are from 8 pairs of mice. (Reproduced with permission from Circulation. 2015; 132:1414-1424).

A major clinical side effect associated with current antithrombotic regimens in the management of CVD is the significant bleeding resulting from the use of dual anti-platelet therapy, for example, aspirin and P2Y12 blockers usually targeting the inhibition of platelet secretion. This status quo therefore demonstrates a need for alternative targets for the control of thrombotic events associated with CVD. With the procoagulant response of activated platelets critically reliant on morphological transformations, molecular mediators critical to the fluid entry mechanism which drive this event provide new approaches or target genes for the control of bleeding and thrombotic disorders. Furthermore, this may reveal new molecular mechanisms in diseases associated with disorders of haemostasis. It is well established that platelets secrete a plethora of releasates essential for the maintenance of vascular integrity and cardiovascular haemostasis.

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Consequently, a new antithrombotic approach that spares essential platelet secretion will potentially limit or eliminate the well-known side effects of antiplatelet therapies.

It is rather surprising that 25% or more of patients on antiplatelet drugs go on to suffer an ischaemic event\(^8^7\). Controlled trials of aspirin for the long-term secondary prevention of ischaemic events reports only a 13% relative reduction in risk of recurrent stroke\(^8^8,8^9\). Similar low percent reductions were reported in studies evaluating the effects of aspirin in the 4-week risk period of recurrent stroke or intracerebral haemorrhage after short-term treatment for stroke. Together, these indicate that the present ‘secretion-driven’ approach to controlling platelet function in thrombosis is not optimal. Also, newer data show that platelets pre-treated with aspirin or from patients administered aspirin still showed full procoagulant response upon stimulation\(^7^2,9^0\) (Fig. 1). The heterogeneity of activated platelets comes with unique challenges for drug therapy; aspirin for instance, will likely affect only aggregating platelets, which may present as conventional spread, non-ballooning platelets (CSNB) on collagen matrix\(^8\). Therefore, co-administration of agents like acetazolamide that suppress platelet procoagulant responses and thrombus formation by distinct mechanisms, may prove effective to limit the procoagulant response of platelets in patients already on aspirin. The existing literature suggests a need for such clinically effective antiplatelet-antiprocoagulant regimen\(^8^7\).

4.3 Potential Challenges of Procoagulant Platelet Inhibition
Platelet procoagulant activity has been linked to stroke and coronary artery disease\(^7^1,9^1,9^2\), however this activity is also vital for haemostasis after vessel injury\(^7^1\); thus its blockade as an antithrombotic approach may precipitate a bleeding diathesis if not fine-tuned. For example, the bleeding defect of Scott patients may be attributable to an aberrant procoagulant activity\(^8,9^3,9^4\). Also, prolonged bleeding times have been reported in TMEM16F null mice which showed a deficiency in Ca\(^2^+\)-dependent PS exposure and procoagulant activity in platelets\(^5^3,5^5\). Fine tuning of therapy may therefore be required. Targeting platelet aquaporins however may achieve this fine tuning, since we have recently shown that mice lacking AQP1 show normal haemostasis whereas thrombus formation was significantly attenuated\(^7^7\). In addition, it may be possible to envisage the use of lower dose acetazolamide in combination with lower dose aspirin, thereby achieving antithrombotic activity at doses lower than currently recognised to cause their clinical adverse effects.

Conclusion
The major platelet phenotypes identified over the last 40 years, to support coagulation are essentially a versatile subpopulation of activated platelets, which we now suggest be referred to as procoagulant platelets. Procoagulant platelets undergo morphological transformations which amplify the surface area required for phosphatidylserine exposure and the acceleration of coagulation. Molecular drivers of procoagulant membrane dynamics provide distinct targets for the regulation of procoagulant platelets’ role in thrombosis. Targeting the water channel AQP1 or carbonic anhydrase enzymes may therefore represent novel approaches in the management of thrombotic disease, both arterial and venous, with potential to suppress the function of procoagulant platelets and selectively limit thrombosis with minimal effect on other platelet functions.

Authorship
Agbani E.O. and Poole A.W. wrote the review

Conflict of Interest Disclosure
None
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