Consensus report on markers to distinguish procoagulant platelets from apoptotic platelets

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ISTH SSC COMMUNICATION

Consensus report on markers to distinguish procoagulant platelets from apoptotic platelets: communication from the Scientific and Standardization Committee of the ISTH

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Abstract

Background: Procoagulant platelets are a subpopulation of highly activated platelets that promote coagulation through surface-exposed, negatively charged phospholipids, especially phosphatidylserine. Procoagulant platelets are important for clot stabilization during hemostasis, and an increased number of these platelets is associated with thrombotic risk. There is a need for harmonization in this area since many of the markers and methods used to assess procoagulant platelets are not specific when used in isolation but are also associated with platelet apoptosis.

Objectives: We initiated this project to identify a minimum set of markers and/or methods that can detect and distinguish procoagulant platelets from apoptotic platelets.

Methods: The study design involved a primary panel with 27 international experts who participated in an online survey and moderated virtual focus group meetings. Primary and secondary panel members were then invited to provide input on themes and statements generated from the focus groups.

Results: This led to a recommendation to use flow cytometry and a combination of the following 3 surface markers to differentiate procoagulant platelets from apoptotic platelets: P-selectin (CD62P), phosphatidylserine (recognized by annexin V), and the platelet-specific receptor GPIX (CD42a) or α_{IIb} integrin (CD41, GPIIb).

Conclusion: Procoagulant platelets are expected to be positive for all 3 markers, while apoptotic platelets are positive for annexin V and the platelet-specific surface receptor(s) but negative for P-selectin.

KEYWORDS
annexin V, apoptosis, flow cytometry, platelet activation, P-selectin

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Emma C. Josefsson and Sofia Ramström contributed equally to this study.

1 | INTRODUCTION

Procoagulant platelets are a subpopulation of highly activated platelets that promote coagulation by phosphatidylserine (PS) exposure. To drive the platelet procoagulant phenotype, a high and sustained level of cytosolic calcium is required, as reviewed in previous studies [1–4]. Membrane ballooning, with the formation of extracellular vesicles (previously referred to as microparticles) [5], is another characteristic feature. Procoagulant platelets contribute to clot stabilization during hemostasis (via local generation of thrombin and fibrin) and may serve as diagnostic, prognostic, or therapeutic biomarkers in thrombotic or bleeding diseases. For example, increased levels of these platelets correlate with transient ischemic attack and stroke [6,7].

In previous publications, procoagulant platelets have been given many names, including sustained calcium-induced platelet morphology [8], ballooned nonspread platelets [9], ballooned and procoagulant spread [10], coated platelets (formerly known as collagen and thrombin [COAT]–activated platelets, as reviewed in a previous study [11]), highly activated platelets [12], high-density bubble-shaped platelets [13], and fibrinogen-capped platelets [14,15]. Furthermore, suggestions have been made that procoagulant platelets, in fact, undergo necrosis [16–18]; however, consensus is yet to be reached on this topic. In a recent review by Agbani and Poole [1], it was suggested that they all should be unified under the term procoagulant platelets.

PS exposure facilitates binding and catalytic efficiency of coagulation factor Va (FVa) and FXa by providing a negative surface, which facilitates thrombin generation [15,19,20]. Subsequently, thrombin converts fibrinogen to fibrin, leading to stable thrombus formation. TMEM16F (ANO6) is a Ca^{2+}-dependent scramblase involved in procoagulant PS exposure [21]. Variants of TMEM16F have been found in patients with Scott syndrome [21–23], a rare bleeding disorder with a defect in phospholipid scrambling activity, wherein activated platelets have decreased PS exposure and deficient thrombin generation [24]. Moreover, TMEM16F-deficient mice often show prolonged bleeding times, and platelets from these mice show a deficiency in Ca^{2+}-dependent PS exposure, procoagulant activity, and membrane blebbing [25–28].

At least 2 distinct pathways lead to platelet PS exposure [29]. The first pathway is procoagulant platelet formation, which can be induced *in vitro* with dual-agonist thrombin (or PAR4 peptide or thrombin PAR1 receptor–activating peptide [TRAP]) and a GPVI agonist (convulxin, collagen, or collagen-related peptide [CRP-XL]). Intracellular signaling includes increased cytosolic Ca^{2+} levels, cyclophilin D–dependent loss of mitochondrial polarization, Ca^{2+}-mediated activation of TMEM16F, and externalization of PS, which promotes thrombin generation and clot stabilization, as reviewed in previous studies [1–4,30,31]. Cyclophilin D is an essential regulator of the mitochondrial permeability transition pore. A study on murine platelets lacking cyclophilin D has shown defects in dual-agonist (thrombin + convulxin)–induced formation of the mitochondrial permeability transition pore, PS exposure, and thrombin generation [12]. The second pathway is intrinsic apoptosis (or mitochondrial apoptosis), which is known to regulate platelet lifespan in circulation [32–35]. A signal such as a BCL-X_L inhibitor (or BH3 mimetic) can trigger this pathway, activating proapoptotic BAK and BAX, causing mitochondrial outer membrane permeabilization, cytochrome C release, and caspase activation. Subsequent activation of a caspase-dependent scramblase is likely to promote externalization of PS with subsequent *in vivo* clearance of platelets. While platelets stimulated to undergo intrinsic apoptosis expose PS and generate thrombin *in vitro* [29], intrinsic apoptosis does not seem to contribute to procoagulant activity (thrombin generation) *in vivo* [36].

As summarized above, some features of procoagulant platelets are also associated with platelet apoptosis, including PS exposure, *in vitro* thrombin generation, increased membrane permeability, mitochondrial depolarization, and formation of extracellular vesicles. There are, however, important differences between procoagulant and apoptotic platelets. In procoagulant platelets, mitochondrial damage is cyclophilin D dependent, PS exposure is Ca^{2+} dependent by activation of TMEM16F, ballooning occurs, and there is significant α-granule release. In apoptotic platelets, mitochondrial damage is BAK and BAX dependent, cytochrome C is released from damaged mitochondria, PS exposure is caspase-dependent, blebbing occurs, and there is no or limited P-selectin release [35].

There is a need for harmonization in this research area since many of the markers and/or methods used to assess procoagulant platelets are also associated with platelet apoptosis. In this project, our primary panel of experts identified a combination of 3 minimal markers that can distinguish procoagulant platelets from apoptotic platelets by flow cytometry.

2 | METHODS

Primary and secondary panel members were selected from individuals who had expressed interest through online registration. Registration was open from July 9 to September 8, 2021, and all International Society on Thrombosis and Hemostasis (ISTH) members could express interest in participating in this project. During registration, they were given the option to select if they would like to participate in the primary panel, consisting of a short survey, virtual focus group meeting, and reviewing themes/statements that emerged from focus groups (∼5 hours of personal work), or the secondary panel, which was tasked with reviewing themes/statements that had emerged from the focus groups (∼1 hour of personal work). The decision for inclusion in the primary panel was based on expertise, experience, geographic distribution, and gender; only 1 person from each research group was selected. The composition of the primary panel (also referred to as the "ProCOAGulant and APOptotic" (COAGAPO) study group) included 27 experts (15 women and 12 men). Of them, 96% declared expertise in procoagulant platelets and 63% in apoptotic platelets. All had experience working with human platelets, and 52% had experience working with both human and animal platelets. The panel included members residing in North America (29.6%), South America (3.7%), Europe (44.4%), Asia (11.1%), and Oceania (11.1%). Specific countries included the United States, Canada, Chile, Belgium, France, Germany, Italy, the Netherlands, Switzerland, United Kingdom, India, and Australia.

Primary panel members were invited to participate in an electronic survey and a virtual focus group (Zoom) meeting. The primary panel was divided into 3 groups to facilitate discussion and consider differences in time zones. In these 3 meetings, chaired by Emma Josefsson and Sofia Ramström, the main survey results were briefly presented, and potential markers to identify and differentiate procoagulant platelets from apoptotic platelets in a laboratory setting were discussed. A list of single markers had been generated from the survey. The following questions were asked for each marker: does it differentiate procoagulant platelets from apoptotic platelets; advantages vs disadvantages; whether it is an intracellular marker, is permeabilization needed; does this method work well for both human and murine platelets; and is the marker commercially available? Finally, a combination of minimal markers was discussed. Each focus group put forward 2 suggestions that contained 3 to 4 markers each that could be assessed by flow cytometry. This resulted in 5 suggestions as 2 from each of the 2 focus groups ended up being the same. The primary panel members were then invited to vote electronically in order to select their preferred combination. A draft paper was prepared, describing the most selected combination of markers and themes of statements from the focus group meetings, and circulated to all panel members for feedback. A white paper was published on the ISTH Platelet Physiology and Vascular Biology Scientific and Standardization Committee websites for comments prior to submission to the *Journal of Thrombosis and Haemostasis*.

3 | RESULTS AND DISCUSSION

3.1 | Survey results

Twenty-seven primary panel members were invited to participate in an electronic survey, with a response rate of 66.7%. The first 2 questions of the survey were as follows: 1) what marker(s) and method(s) do you have experience in detecting procoagulant platelets and 2) what marker(s) and method(s) do you have experience in detecting apoptotic platelets? Two lists of markers were provided to select from 17 procoagulant and 15 apoptotic markers, respectively, while additions could be made as free text. The 6 markers that the experts had the most experience in for the assessment of procoagulant platelets were PAC-1 at 94% (antibody [Ab] recognizing activated human platelet integrin $\alpha_{IIb}\beta_3$ [CD41/CD61, GPIIb/IIIa]) [37], annexin V (annexin A5) at 89% (PS surface exposure), P-selectin (CD62P) at 89% (P-selectin surface exposure/degranulation of α-granules), inner mitochondrial membrane potential (Δψm) at 78%, cytosolic Ca^{2+} at 72%, and thrombin generation at 67% (Figure 1). For apoptotic platelets, the most common marker was annexin V at 83%, followed by assessment of caspase activation at 50%, levels of BCL-2 family proteins at 44%, lactadherin at 39% (PS surface exposure), cytochrome C release or retention at 33%, Δψm at 33%, and thrombin generation at 33% (Figure 2). The most commonly used method for identifying procoagulant platelets was flow cytometry. More details on specific methods used for each marker are described below, and additional methods and markers are described in Supplementary Figures S1–S4. Furthermore, agonists and inhibitors used to modulate procoagulant or apoptotic platelet generation are listed in Supplementary Figures 5 and 6. Finally, the panel members were asked to propose a combination of markers and/or methods that, in their opinion, were the best to distinguish procoagulant from apoptotic platelets. The majority suggested 2 to 4 markers by flow cytometry, but no unifying combination could be

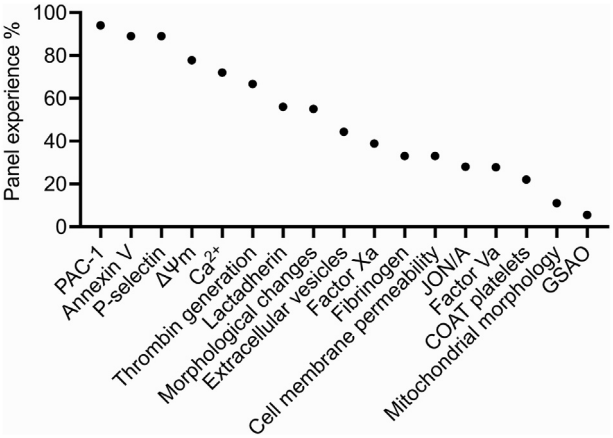

FIGURE 1 Procoagulant platelet markers. Percentage of primary panel members who had experience with the indicated marker for assessment of procoagulant platelets (markers stated as in the survey). PAC-1 and JON/A antibodies recognize activated $\alpha_{IIb}\beta_3$ integrin in human and murine platelets, respectively. Δψm represents inner mitochondrial membrane potential. Eighteen primary panel members (n = 18) participated in the survey. COAT, collagen and thrombin; GSAO, 4-(N-[S-glutathionylacetyl]amino) phenylarsonous acid.

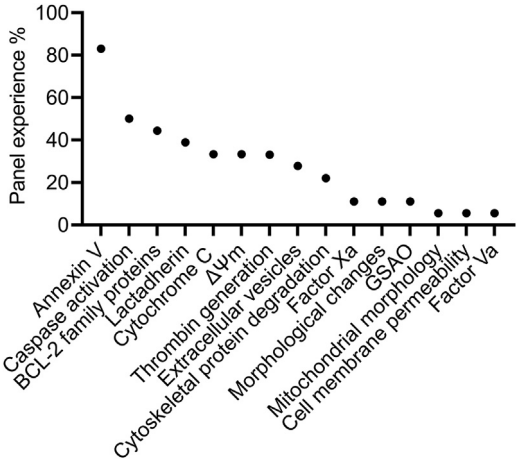

FIGURE 2 Platelet apoptosis markers. Percentage of primary panel members who had experience with the indicated marker for assessment of apoptotic platelets (markers stated as in the survey). Δψm represents inner mitochondrial membrane potential. Cytochrome C stands for cytochrome C release or retention. Eighteen primary panel members (n = 18) participated in the survey. PAC-1 and JON/A antibodies recognize activated $\alpha_{IIb}\beta_3$ integrin in human and murine platelets, respectively. GSAO, 4-(N-[S-glutathionylacetyl]amino) phenylarsonous acid.

identified. Two members suggested immunoblotting (western blotting).

3.2 | Three surface markers by flow cytometry: P-selectin, PS (annexin V), and α_{IIb} or GPIX

Next, we invited the 27 primary panel members to participate in virtual focus group meetings. The panel was divided into 3 groups to allow efficient and fruitful discussions and balance differences in time zones. Twenty-five of the invited primary panel members (93%) took part in these meetings. The main survey results were briefly presented, and potential markers to identify and distinguish procoagulant from apoptotic platelets in a laboratory setting were discussed. A list of single markers had been generated from the survey to provide a starting point. Flow cytometry was suggested as a preferred method because of the following reasons: 1) this was the most commonly used method, 2) it allows identification of different platelet populations, 3) the technology is readily available, and 4) small numbers of platelets are required for phenotyping. Finally, a combination of markers was discussed. Each focus group put forward 2 suggestions that contained 3 or 4 markers to be assessed by flow cytometry. This resulted in 5 suggestions rather than 6 as 2 from each of the 2 focus groups were the same. The primary panel members were then invited to vote in order to select their preferred combination. This resulted in the recommendation to use the combination of 3 surface markers by flow cytometry in order to distinguish procoagulant from apoptotic platelets: P-selectin/CD62P (detected by anti–P-selectin Ab); PS (detected by annexin V binding); and a platelet-specific receptor, either GPIX (CD42a) detected by anti-GPIX Ab or α_{IIb} detected by anti-α_{IIb} Ab. Procoagulant platelets are expected to be positive for all of the 3 markers, while apoptotic platelets are positive for annexin V and the platelet-specific surface receptor but negative for P-selectin. The panel concluded that as procoagulant platelets release α-granules, they are positive for P-selectin surface expression, whereas apoptotic platelets do not markedly release α-granules and, therefore, lack surface P-selectin expression, in agreement with published literature [12,16,35,38]. Of note, P-selectin can be quickly shed from the platelet surface if a shear-induced platelet activation system is used under flow conditions [39]. However, in suspension, P-selectin remains on the platelet surface for at least 15 minutes at room temperature [40,41]. Furthermore, it was discussed that high concentrations or prolonged exposure to fixatives could lead to the risk of platelets being false positive for P-selectin. Due to this problem and the potential difficulties of using fixation related to PS exposure, researchers with a need for fixation should optimize their specific protocol to ensure that it does not cause artefactual results [42].

The platelet-specific receptors discussed as alternatives for identification of platelets were GPIX and α_{IIb}, which are surface receptors and will allow specific gating on platelets when measured by flow cytometry. They are highly expressed and do not get rapidly shed; fluorescently labeled antibodies for these receptors are commercially available for both human and murine platelets. Platelet-specific receptors other than GPIX and α_{IIb} integrin can be employed, and 1 option put forward was GPIbβ (CD42c). However, in terms of other receptors, caution must be taken when it comes to receptor shedding [43,44]. It is preferred to use directly conjugated fluorescent antibodies. Several different clones and fluorophores are commercially available for both the platelet receptors and P-selectin. To allow adaptation to different flow cytometer setups, we have not made specific recommendations here but recommend that users provide plots showing representative staining patterns in relevant positive and negative control samples with the antibodies of choice for their study.

Assessment of PS exposure with annexin V requires the presence of Ca^{2+} in the staining buffer, and annexin V may be switched to lactadherin in a setting in which no addition of Ca^{2+} is desired. However, the panel considered lactadherin less specific for procoagulant and apoptotic platelets as it recognizes not only PS but also integrins [45,46]. The results generated when using lactadherin were also considered more variable than those generated when using annexin V. It should also be noted that a high and sustained level of cytosolic Ca^{2+} is required to drive the procoagulant phenotype, which may require extracellular Ca^{2+} to provide sufficient Ca^{2+} entry. If plasma is present in the experimental setup, clotting needs to be prevented by addition of a thrombin inhibitor or, if thrombin is to be used as an agonist, a fibrin polymerization inhibitor [38,47]. An advantage of annexin V compared to lactadherin is that it is commercially available and conjugated to many different fluorochromes, which gives flexibility when combining this marker with other markers. Moreover, annexin V binding can be prevented by omitting Ca^{2+} in the buffer, thereby allowing the possibility of creating a background fluorescence control for positivity gating. As annexin V can block procoagulant function, its use is not recommended if using a functional assay, although it can be used as a tool to assess the PS-specific–dependent procoagulant activity of platelets.

The panel emphasized that time is a factor that differs between procoagulant and apoptotic platelets [4,29,40], at least when generated *in vitro*. Furthermore, murine platelets undergo apoptosis more quickly than human platelets. The classical way of generating procoagulant platelets *in vitro* with dual-agonist thrombin (or PAR1/PAR4 activating peptides) and a GPVI agonist (convulxin, collagen, or CRP-XL) is a rapid process occurring within a few minutes [40,48] even at room temperature. Triggering PS exposure associated with intrinsic apoptosis in platelets with a BCL-X_L inhibitor, such as ABT-737, is a slower process that takes hours (up to 180 minutes at 37 °C for human platelets and ~30-90 minutes for murine platelets) [29,40].

As a complement to the suggested panel of markers, the addition of markers to detect activated platelet integrin $\alpha_{IIb}\beta_3$ or mitochondrial dyes to the mix was discussed for a more extensive analysis. Further details of these markers are discussed below.

3.3 | What do we expect to see with other markers?

There are several other useful markers and/or methods (Table) to apply in conjunction with our proposed panel of 3 markers for flow

TABLE Procoagulant and apoptotic platelet markers.[a]

Marker	Procoagulant platelets	Apoptotic platelets	Comment
P-selectin–positive[b]	Yes	No	Released by α-granules in procoagulant platelets[b]
Annexin V binding[b]	Yes	Yes	Timing differs (see text for details).[b]
GPIX-positive[b]	Yes	Yes	[b]
α_{IIb}-positive[b]	Yes	Yes	[b]
Caspase activation	No or modest	Yes	
Cytochrome C release	No	Yes	
Binding of the marker of activated $\alpha_{IIb}\beta_3$ (eg, PAC-1, JON/A)	No	No	Gradual loss in procoagulant platelets
Δψm loss	Yes	Yes	Timing differs
Ca^{2+} signal	Yes (high)	Modest	A transient, modest signal in apoptotic platelets, followed by depletion of intracellular Ca^{2+} stores
Thrombin generation	Yes	Yes	Timing differs. Shown *in vitro* for apoptotic platelets
Lactadherin binding	Yes	Yes	Timing differs. Reported as less specific than annexin V
Morphologic changes	Yes	Yes	Timing differs
Extracellular vesicles	Yes	Yes	Timing differs
Fibrinogen binding	Yes	No	Fibrinogen is retained at the surface of procoagulant COAT-activated platelets
FXa binding	Yes	Yes?	Apoptotic platelets can generate thrombin *in vitro*, but information on FXa is limited
FVa binding	Yes	Yes?	Apoptotic platelets can generate thrombin *in vitro*, but information on FVa is limited
GSAO-positive	Yes	Yes	Timing differs
Increased cell membrane permeability	Yes	Yes	Timing differs
Caspase-dependent cytoskeletal protein degradation	No	Yes	
Changes in the levels of BCL-2 family proteins	No	Yes	

COAT, collagen and thrombin; GSAO, 4-(N-[S-glutathionylacetyl]amino) phenylarsonous acid.

[a] Markers present (yes) or absent (no) in procoagulant and apoptotic platelets. PAC-1 and JON/A antibodies recognize activated $\alpha_{IIb}\beta_3$ integrin in human and murine platelets, respectively.

[b] Recommended in combination using flow cytometry by the panel.

cytometric assay. Here, we discuss the advantages and disadvantages of some of them.

Mitochondrial cytochrome C release is a marker of intrinsic (or mitochondrial) apoptosis and occurs during mitochondrial outer membrane permeabilization [35,40]. A third of the panel members had experience with successfully assessing cytochrome C release or retention in apoptotic platelets (Figure 2) by immunoblotting and/or flow cytometry, in agreement with published literature [32,35,40,49], and confirmed that procoagulant platelets do not release cytochrome C [40,49]. However, permeabilization is required when assessing cytochrome C by flow cytometry [40], making this apoptosis marker difficult to combine with other markers as permeabilization is incompatible with the assessment of surface expression of P-selectin and PS. We hope to see future development and validation of research tools for assessment of platelet cytochrome C release that are cell permeable and, hence, would avoid the permeabilization step.

The majority (78%) of the panel members had experience with assessing the inner mitochondrial membrane potential (Δψm) in platelets with markers, including, but not limited to, TMRM, TMRE, JC-1, $DiIC_1(5)$, and DiOC6(3) (Figures 1 and 2, Supplementary Figures S1D and S3F), by flow cytometry or microscopy. Both procoagulant and apoptotic platelets lose Δψm [36,40,49] due to inner mitochondrial membrane disruption, although the timing of Δψm loss is substantially different [40]. It can, however, be a useful marker to differentiate activated platelets from procoagulant platelets. A limitation is that this method only works on live cells, not fixed cells.

Caspase activation is a well-established marker of platelet apoptosis [32,49,50] and was used by 50% of the panel members

(Figure 2). The panel had experience with successfully assessing caspase-3 and caspase-9 activation in apoptotic platelets by immunoblotting and microscopy (Supplementary Figure S3), in agreement with published literature [32,33,49]. However, limitations raised with immunoblotting were that a concentrated platelet sample is necessary and that platelet subpopulations in a mixed sample cannot be readily distinguished. Platelets activated by CRP-XL did not generate detectable caspase activation, detected using immunoblotting, indicating that caspase activation is not required to initiate platelet activation [49]. In addition, the luminescent CaspaseGlo assay works well for detecting platelet caspase activity [36,50,51] but cannot distinguish different populations. Detection of caspase-3 activation in human platelets has also previously been achieved by flow cytometry [48,52], a method that showed a low level of caspase activity in procoagulant platelets. However, this technique often requires permeabilization, and when discussed, the panel considered this method as being difficult and not giving a strong positive signal. Of note, some experts also stated that they had not been successful with the assessment of caspase activity in platelets by flow cytometry.

During platelet apoptosis, caspases cause degradation of cytoskeletal proteins, including gelsolin, spectrin, and filamin [29,33,35,49]. A quarter (22%) of the panel members stated experience with the assessment of caspase-dependent cytoskeletal protein degradation linked to platelet apoptosis (Figure 2), mainly by immunoblotting (Supplementary Figure S3H), and several members considered this as a method of value when used in conjunction with other assays.

Activated platelet integrin $\alpha_{IIb}\beta_3$ can be detected by flow cytometry, for example, with the specific monoclonal antibodies PAC-1 [37] and JON/A [53] for human and murine platelets, respectively. Activated platelets bind to these antibodies, while highly activated procoagulant platelets in late stages have reduced or no binding [12] due to calpain activation [2,54]. Hence, the use of such activation-dependent antibodies will allow identification of activated "aggregatory" vs "procoagulant" platelets by flow cytometry but will not distinguish procoagulant platelets from apoptotic platelets as apoptotic platelets are also negative for activated $\alpha_{IIb}\beta_3$ [35]. Most (94%) of the primary panel members had experience with using PAC-1 and 28% of them had experience with handling JON/A for the assessment of human and murine procoagulant platelets, respectively (Figure 1). Addition of fluorescently conjugated fibrinogen or antibodies to fibrinogen is another way of assessing activation of $\alpha_{IIb}\beta_3$, and 33% of the panel members had experience with these research tools (Figure 1). However, fibrinogen can also bind PAC-1–negative platelets through transglutaminase activity [55].

Ca^{2+} influx is required for platelets to become procoagulant [48,56] but not apoptotic. Seventy-two percent of primary panel members had experience measuring cytosolic Ca^{2+} levels in procoagulant platelets (Figure 1). When exploring kinetics, the panel members considered the assessment of Ca^{2+} as a useful tool in distinguishing procoagulant and apoptotic platelets. However, all dyes leak out with time, and this method was not recommended for less-experienced users given its higher technical requirements. Notably, apoptotic platelets stimulated with BH3 mimetics, including ABT-737, have been shown to exhibit a transient yet modest calcium signal, followed by depletion of intracellular Ca^{2+} stores with prolonged treatment [49,57].

Platelet-derived extracellular vesicles (previously known as microparticles) [5] are formed during both procoagulant and apoptotic platelet formation [1, 32]. Many (44%) of the panel members reported experience with the assessment of extracellular vesicles (Figures 1 and 2). However, the majority did not think that the assessment of extracellular vesicles by flow cytometry was a method that could easily be used to differentiate procoagulant platelets from apoptotic platelets. As mentioned previously, morphologic changes detected using microscopy, such as ballooning to characterize procoagulant platelets [1], was a method that 55% of the panel members stated to be experienced with (Figures 1 and 2). Several panel members were in favor of combining the recommended 3-marker flow cytometry assay with microscopic assessment of platelets. Nevertheless, such microscopic analysis can only be performed in research facilities with access to, and training in, advanced microscopy.

Many (44%) of the panel members had experience in assessing the level of platelet BCL-2 family members [32], mainly using Western blotting (Figure 2, Supplementary Figure S3D), to investigate platelet apoptosis, in agreement with published literature [33,35]. A limitation is that as the expression levels of BCL-2 family proteins in platelets are relatively low; therefore, a concentrated platelet sample is required for Western blotting.

The α-granule protein von Willebrand factor (VWF) is often included when assessing procoagulant COAT platelets, defined as a subpopulation of activated platelets that retain a coat of prohemostatic proteins that, in addition to VWF, includes the α-granule proteins FVa, fibrinogen/fibrin, thrombospondin, fibronectin, and α2-antiplasmin, and express PS [11,19,55] plus transglutaminase FXIII activity [58] on their surface. The panel's view was that VWF colocalizes well with P-selectin expression in procoagulant platelets. Of note, VWF binds both activated and procoagulant platelets. The classical way to assess COAT platelets by flow cytometry is to combine VWF, FV, activated $\alpha_{IIb}\beta_3$, and annexin V (Supplementary Figure S2F). The survey results showed that 22% of the panel members had experience of using this method.

Both procoagulant and apoptotic platelets can promote thrombin generation [19,29], a method familiar to 67% of panel members (Figures 1 and 2), even though the timing is thought to differ. Binding of coagulation factors FXa and FVa is an established feature of procoagulant platelets [19] but is less studied in apoptotic platelets. While some panel members stated that apoptotic platelets bind coagulation factors, others were of the view that apoptotic platelets do not bind coagulation factors. Hence, further research is needed to shed light on this topic. Nevertheless, coagulation factors FXa and FVa were considered useful for assessing procoagulant platelets by several panel members to separate them from activated platelets. There is thought to be a good overlap between FXa, FVa, and annexin V binding. Of note, if using washed platelets, addition of FXa is required, but not FV, which is released from platelet α-granules. The panel considered that the availability of commercial research tools for assessment of FXa was better than that for the assessment of FVa.

Cell viability can be assessed with markers that either leak out or enter the cell when the cell membrane becomes permeabilized. Limitations are that nuclear dyes commonly used to differentiate necrotic from early apoptotic cells (eg, propidium iodide) cannot be applied to anucleate platelets and the same issue relates to a classical apoptotic marker, such as terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Furthermore, 4-(N-[S-glutathionylacetyl]amino) phenylarsonous acid is a cell death marker that enters the cytoplasm as cell membrane integrity is lost and binds a cytoplasmic ligand in both procoagulant and apoptotic platelets. It has successfully been used to distinguish procoagulant and apoptotic platelets when combined with P-selectin [16,59,60]. However, 4-(N-[S-glutathionylacetyl] amino) phenylarsonous acid was not recommended by the panel since it is not currently commercially available but only obtainable through research collaboration, and hence, only 11% of the primary panel members had experience in using this marker (Supplementary Figures S1 and S2).

Procoagulant platelet–bound tissue factor [61–63] was brought up in 1 of the panel discussions, but as this is a controversial topic [64,65] and no evidence was provided in support of using this marker to distinguish procoagulant from apoptotic platelets, this was not further considered for inclusion in this report.

In summary, the panel proposes a combination of 3 surface markers, P-selectin, PS (annexin V), and GPIX or integrin α_{IIb}, which are easily assessed in the same test tube using flow cytometry and applicable worldwide in less-specialized laboratories, in order to differentiate procoagulant platelets from apoptotic platelets. Procoagulant platelets are expected to be positive for all 3 markers, while apoptotic platelets are positive for annexin V and the platelet-specific surface receptor but negative for P-selectin.

APPENDIX

The members of the ProCOAGulant and APOptotic platelets (COAGAPO) study group, also referred to as the primary panel, are as follows: Ejaife O. Agbani (Calgary, Alberta, Canada), Lorenzo Alberio (Lausanne, Switzerland), Tamam Bakchoul (Tuebingen, Germany), Beth A. Bouchard (Burlington, Vermont, USA), Marina Camera (Milan, Italy), Vivien Chen (Sydney, New South Wales, Australia), Fabrice Cognasse (St Etienne, France), Judith M.E.M. Cosemans (Maastricht, The Netherlands), Rutvi G. Dave (Vellore, Tamil Nadu, India), Frederik Denorme (Salt Lake City, Utah, USA), Dorothée Faille (Paris, France), Alison H. Goodall (Leicester, UK), Matthew T. Harper (Cambridge, UK), Johan Heemskerk (Maastricht, The Netherlands), Shawn M. Jobe (East Lansing, Michigan, USA), Lacey Johnson (Sydney, New South Wales, Australia), Andaleb Kholmukhamedov (San Diego, California, USA), Saptarshi Mandal (Jodhpur, India), Meganathan Kannan (Tamil Nadu, India), Diego Mezzano (Santiago, Chile), Nicola Mutch (Aberdeen, UK), Margaret L. Rand (Toronto, Ontario, Canada), Yana Roka-Moiia (Tucson, Arizona, USA), Claudia Tersteeg (Kortrijk, Belgium), Kimberly A. Thomas (Denver, Colorado, USA), Dina Vara (Ridge Herts, UK), and Yuping Yuan (Sydney, New South Wales, Australia).

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AUTHOR CONTRIBUTIONS

E.C.J. and S.R. developed the study and survey forms, led the focus group discussions, drafted the manuscript, and coordinated the revision process. M.L. and J.T. contributed to the study design and provided intellectual input. The study group authors participated in the surveys and focus group discussions and/or they provided intellectual input for revisions of the draft.

DECLARATION OF COMPETING INTERESTS

J.H. is an adviser of Synapse Research Institute, Maastricht, The Netherlands. T.B. has a pending patent on using flow cytometry to detect procoagulant platelets in heparin induced thrombocytopenia. The work of L.A. and his research group on procoagulant collagen and thrombin platelets is currently supported by grants from Dr Henri Dubois-Ferrière Dinu Lipatti Foundation, Novartis Foundation for Medical-Biological Research (#18B074), the Swiss Heart Foundation (FF19117), and the Swiss National Science Foundation (320030-197392). The other authors report no conflicts of interest.

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SUPPLEMENTARY MATERIAL

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