Expert opinion on the use of platelet secretion assay for the diagnosis of inherited platelet function disorders

Mezzano, Diego; Harrison, Paul; Andrew L. Frelinger, III; Mumford, Andrew D.; Noris, Patrizia; Lordkipanidzé, Marie; Gresele, Paolo

DOI:
10.1111/jth.15781

Expert opinion on the use of platelet secretion assay for the diagnosis of inherited platelet function disorders: Communication from the ISTH SSC Subcommittee on Platelet Physiology

Diego Mezzano[1], Paul Harrison[2], Andrew L. Frelinger III [3,4], Andrew D. Mumford[5], Patrizia Noris[6], Marie Lordkipanidzé[7,8], Paolo Gresele[9]

1. Department of Hematology-Oncology, School of Medicine, P. Catholic University of Chile, Santiago, Chile.
2. Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK
3. Division of Hematology/Oncology, Center for Platelet Research Studies, Boston Children's Hospital, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, Massachusetts, USA.
4. Harvard Medical School, Boston, Massachusetts, USA
5. School of Cellular and Molecular Medicine, University of Bristol, Bristol, United Kingdom.
6. Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation and University of Pavia, Pavia, Italy.
7. Faculté de Pharmacie, Université de Montréal, Montréal, Quebec, Canada.
8. Research Center, Montreal Heart Institute, Montréal, Quebec, Canada.
9. Department of Medicine and Surgery, University of Perugia, Perugia, Italy.

Text word count: 3947
Abstract word count: 247
Table: 1
Figure: 1
References: 42

Corresponding author: Diego Mezzano, Lab Thrombosis and Haemostasis, School of Medic, P. Catholic Univ of Chile Portugal 61, First Floor Santiago Centro RM 8330034 dmezzano@med.puc.cl; Phone: +56 9 9533 9636

ABSTRACT

Assessment of platelet secretion is crucial for diagnosing suspected inherited platelet function disorders (IPFD). A previous survey of the SSC on Platelet Physiology of the ISTH and a comprehensive review highlighted that most of the platelet secretion assays (PSA) lack standardization and validation. The aim of this study was to provide expert consensus guidance on the use of PSAs for IPFD diagnosis.

We surveyed 26 experts from 10 different countries using the RAND/UCLA methodology, to attain a consensus on sensitivity, specificity, feasibility, time to readout and cost of most PSAs. Answers were then graded in 3 categories: appropriate, uncertain, and inappropriate. Equivocal or misinterpretable statements required a second and third round survey involving 14 of the original 26 experts. We report here the consolidated results of the entire procedure.

There was uniform agreement on several general statements, including that PSAs should be performed in hemostasis laboratories as first line diagnostic tests even in patients with normal platelet aggregation, and should include a δ-granule secretion marker. Among the specific assays examined, lumiaggregometry, other luciferin/luciferase-based assays, HPLC-methods, radiolabelled-serotonin based assays and whole-mount transmission electron microscopy were rated as appropriate for the measurement of δ-granule release, and platelet P-selectin expression by flow cytometry and released proteins by ELISA for α-granule release. For most of the other PSAs, the expert opinions were widely dispersed.

Lack of expert consensus on many PSAs indicates clearly an unmet need for rigorous standardization, multicentre comparison of results and validation of PSAs for clinical laboratory practice.

Keywords: Expert consensus; Inherited platelet disorders; Platelet function; Platelet granules; Platelet release reaction; Platelet Secretion

1. | INTRODUCTION

Laboratory assays that measure the content and release of platelet granules were introduced in clinical practice more than 50 years ago [1], and are considered a crucial step in the diagnosis of inherited platelet function disorders (IPFD). An international survey showed that less than 50% of 202 specialized laboratories worldwide evaluated either the content or the secretion of specific platelet granule constituents during the diagnostic work-up of a suspected IPFD and the majority performed tests as a second diagnostic step [2]. That survey was followed by a consensus guidance which recommended the assessment of platelet secretion as a first line test in the diagnostic approach [3].

The [14] C-serotonin (5-HT) secretion test, later modified using ^{3}H-5-HT developed by H. Holmsen et al. in 1969 [1], remained for decades the "gold standard" for the diagnosis of platelet secretion disorders (PSDs). It allowed concomitant measurement of platelet aggregation and 5-HT secretion but, despite its advantages, the increasing restriction of radioisotopes from clinical laboratories led the [14] C-5-HT secretion test to fall in disfavor; its current use is restricted to very few laboratories worldwide. Lumiaggregometry (LA), introduced in 1977 [4], is currently the most popular test for measuring δ-granule release. Regarding α-granules, after the initial studies reporting the release of platelet factor 4 (PF4), β-thromboglobulin (βTG), platelet-derived growth factor (PDGF) and fibrinogen [5], a vast number of proteins and assay methods have been described as α-granule biomarkers. Currently, flow cytometric measurement of platelet P-selectin expression is used in most hemostasis laboratories. In contrast, after the early studies reporting the measurement of lysosomal acid hydrolases secretion [6], very few assays have been developed to evaluate lysosomal release. These include measurement of lysosomal associated membrane proteins LAMP-1, 2 and 3 by flow-cytometry, although these are seldom used in routine diagnostics. Many other platelet secretion assays (PSAs) have been reported, but there is a lack of evidence-based information on their overall diagnostic performance for IPFD and of comparative studies [3].

The Subcommittee on Platelet Physiology of the ISTH carried out a comprehensive review on PSAs used for clinical or research purposes and the results showed a wide variety of tests, most of which were developed mainly for research purposes [7]. That overview also underlined that most of the currently used PSAs are poorly standardized, not validated and thus are not endorsed for diagnostic use. The main weaknesses singled out for most of the current tests were insufficient sensitivity, specificity and reproducibility [7]. Altogether these observations reveal an important unmet diagnostic need and highlight the importance of improved standardization of current assays and/or the development of new tests complying with good laboratory practices.

Thus, the SSC on Platelet Physiology of the ISTH decided to undertake a survey among international experts on the clinical indications of PSAs, the most suitable assays for clinical diagnosis of IPFD, and their performance. Our objective was to attain the best possible consensus on current practice based on published evidence and expert opinion to generate an informed up-to-date statement on the best laboratory practice for platelet secretion studies for IPFDs diagnosis.

2. | METHODS

Given that a thorough review of the literature established that there is not sufficient evidence on the relative value of PSAs [7], the working party decided to adopt a formal consensus method (RAND) to develop recommendations on PSAs [8]. The RAND approach requires that expert panel members, blinded to the responses of the others, score each statement from 1 to 9, where 1 is completely inappropriate and 9 is fully appropriate. Each statement is then classified as inappropriate (scores of 1–3), uncertain (scores of 4–6), or appropriate (scores of 7–9).

The working party developed an online questionnaire with 52 items and 163 questions, which was distributed in 2016 to 54 ISTH members chosen for their clinical and laboratory expertise in PSAs. Items covered the types of diagnostic PSAs and their main characteristics (sensitivity, specificity, operating speed, feasibility, cost, and overall satisfaction of each assay) as well as the diagnostic step at which PSAs are indicated.

Questions about type and concentration of agonists to be used were also included (**Suppl. data 1**). Aspects related to the indications or clinical correlations of the tests were not considered in the questionnaire.

We collected 26 replies and the results were presented at the SSC/ISTH meeting in Montpellier in 2016. Given some uncertainties due to possibly unclear or mis-interpretable questions and upon feedback from the audience, a second set of questions was distributed in 2017 to 14 experts and all of them answered (**Suppl. data 2**). Results were presented at the SSC/ISTH Meeting in Berlin, 2017. Some remaining doubts and new questions raised during the presentation prompted us to send a third, short, questionnaire to the same 14 experts to attain the best possible agreement on some remaining controversial issues and 13 of them responded (**Suppl. data 3**). This report summarizes the results of all the steps of this project.

All the replies, anonymized, were entered into a database. Basic statistical analyses were performed, and results are presented as medians and range, fitting with the degree of appropriateness according with the RAND methodology. Outliers were ruled out using Grubbs´ test.

3. | RESULTS

I. General Statements.

All the 26 respondents to the first questionnaire agreed that specialized diagnostic hemostasis laboratories should perform at least one PSA, but only 5/26 (19%) recommended testing at the initial diagnostic step, while 19/26 (76%) suggested performing PSA only after ruling out von Willebrand disease. Other main conclusions were:

a) PSA should be carried out even when light transmission aggregometry (LTA) already strongly suggests an IPFD (median score *9;* range *7-9)*.
b) PSA should be performed also in patients with normal LTA (9, 5-9).
c) PSA should include a δ-granule secretion marker *(9; 3-9)*, while the appropriateness of including an α-granule secretion marker was rated as uncertain (*5; 3-9).* In patients

with clear evidence of δ-granules defect it is appropriate to measure an α-granule secretion marker to rule out α/δ-storage pool disease (*8; 3-9).*

A PSD can be diagnosed only upon confirmation of an abnormal PSA in at least two separate laboratory sessions (*7.5, 3-9).* The diagnosis of a PSD after only one abnormal test *(3.5; 1-7*) or one laboratory session was rated as inappropriate (*3; 1-6)*.

d) The diagnostic utility of measuring the release of lysosomal markers for the diagnosis of PSD was rated as uncertain *(4.5; 1-7).*

II. Evaluation of specific methods.

A. Measurement of δ–granule content and secretion.

Measuring the total content and secreted fraction of either ATP/ADP or serotonin by HPLC in the supernatant of stimulated platelets should be considered the gold standard for the evaluation of platelet dense-body content and secretion *(7; 3-9)*; these tests also distinguish between functional secretion defects from storage pool disease (SPD) or defective granule biogenesis. However, the implementation of these assays in diagnostic laboratories was rated as uncertain due to their complexity and cost *(6; 1-8).*

1. Light transmission-Lumiaggregometry (LT-LA) in PRP measures secreted ATP.
   This test reflects the ability of platelets to release their δ-granule content.
   LT-LA sensitivity and specificity were rated as appropriate when using strong agonists *(7; 4-9 and 7; 1-9, respectively)* but uncertain with weak agonists (*5; 1-9* and *6; 1-7,* respectively). The inter-assay reproducibility, reference range and concordance of LT-LA with LTA were rated as uncertain *(6; 3-9; 6; 5-8, and 6.5; 2-9, respectively)*. There was agreement on the need to run the test in duplicate *(7; 2-9).* Feasibility, time to read-out, and overall satisfaction with the assay were rated as appropriate but with a rather wide range of scores *(7; 3-9; 7.5; 1-9, and 7; 2-8, respectively)*. The assay was considered expensive *(7; 2-9)*.

2. Other luciferin/luciferase-based assays. These tests quantitate secreted ATP and ADP, useful for the diagnosis of SPD. Platelet lysates with or without previous

pretreatment with pyruvate kinase plus phosphoenolpyruvate can then be used for the measurement of total ATP or ATP+ADP content, respectively.
They were rated as sensitive *(7; 5-9)*, specific *(7; 3-9)*, and overall satisfactory *(7; 5-9),* whereas feasibility *(5; 3-8),* time to read-out *(5; 1-8)* and cost *(5; 3-7)* were rated as uncertain.

3. HPLC-based methods measure total and secreted ADP, ATP, and ATP/ADP ratio, thus allowing to differentiate δ-body secretion defects from δ-SPD
   These assays were rated as sensitive *(9; 2-9)* and specific *(8; 3-9),* but feasibility *(3; 1-8),* cost of the test *(5; 1-8),* and time to readout *(5; 1-6)* were considered unsatisfactory.

4. Radio-labelled serotonin (^{14}C-5-HT or ^{3}H-5-HT) release in PRP measures the ability of platelets to secrete δ-granules. It also allows to suspect defective granule biogenesis when the platelets fail to uptake isotopic 5-HT.
   The assay was still considered the gold standard for platelet secretion measurement *(8; 5-9)*, despite 62.5% of the respondents stating they had never used the test in their laboratories. The test was rated as sensitive *(8; 5-9)*, specific *(8; 3-9)* and overall satisfactory *(7.5; 3-9)*. However, it was rated as of uncertain value regarding time to readout *(6.5; 2-9)*, feasibility (*5; 3-8)*, cost *(5; 1-9) and* safety for the operator *(6; 1-8)*.

5. Fluorometric measurement of 5-HT release using an ortho-pthaldialdehyde assay.
   Sensitivity *(5; 4-9)*, specificity *(5, 4-9)*, time to read out *(5; 1-7)*, feasibility *(5; 1-6)*, cost *(5.5; 4-8)*, safety for the operator *(5, 3-8)* and overall satisfaction with the test *(5; 4-7)* were all rated as uncertain.

6. HPLC-based assays of 5-HT secretion using fluorometric or electrochemical detection. Quantitation of total and secreted 5-HT measures δ-granule secretion and contributes to the diagnosis of SPD.

This methodology was rated as appropriate for sensitivity *(8; 4-9)* and specificity *(7; 3-9).* All the other items were rated as uncertain, including time to readout *(5; 2-6)*, feasibility *(5; 2-7)*, cost *(4; 2-8)* and overall satisfaction *(6; 4-9).*

7. Dense body secretion by flow cytometry (FC) using mepacrine-labeled platelets.
   This assay was rated as simple (7; 4-8) but of uncertain value for all the other items, including sensitivity *(5; 1-7),* specificity *(5; 1-7),* time to readout *(6.5; 4-8)*, feasibility (6; 3-8), cost *(6; 4-7)* and overall satisfaction *(5; 1-7).*

8. ELISA measurement of 5-HT is done in PRP, platelet pellets or washed platelets.
   This assay was rated as appropriate for sensitivity *(7; 4-8)* and specificity *(7; 4-8).* All the other characteristics were rated as of uncertain value, including time to readout *(5; 3-8),* feasibility *(6; 3-9),* cost *(5; 1-7)* and overall satisfaction *(5; 2-8).*

9. Whole mount transmission electron microscopy (WM-TEM) in PRP. This morphological counting of platelet dense bodies helps to confirm the diagnosis of SPD.
   WM-TEM to count δ-body number was rated appropriate for sensitivity *(7; 5-9)* and specificity *(7; 5-9).* This test was instead rated as inappropriate for time to readout *(2.5; 1-5),* feasibility *(3; 1-9)*, and cost *(3; 1-5)*. Overall satisfaction was good *(7; 3-9).*

B. Platelet α-granules secretion assays.

1. Platelet P-selectin expression by flow cytometry (FC)

   P-selectin expression on activated platelets measured by FC in whole blood was considered the first-choice assay to measure α-granule secretion *(8; 5-9).*
   The test was rated as appropriate for sensitivity *(7.5; 7-9),* specificity *(7; 3-9),* time to readout *(7.5; 3-9)*, feasibility *(7; 3-9),* and overall satisfaction *(7; 6-9).* However, the test was rated as of uncertain value for its cost *(6; 3-9).* Also, the correlation of this assay with LTA was rated as uncertain (*4; 1-8*).

FC measurements of P-selectin secretion in PRP or washed platelets were rated as having uncertain value *(5; 1-7,* and *4; 1-9,* respectively).

2. ELISA measurement of specific α-granule proteins (e.g., β-TG, PF-4, thrombospondin) in the supernatant of stimulated PRP or of lysed washed platelet pellets. These tests are important in the diagnosis of α- or α/ δ-granule SPD.
   This kind of assays was rated as appropriate for sensitivity *(8; 4-9)* and specificity *(8; 3-9), but* there was uncertainty on their use as first-line test for α-granule release *(6; 2-9)*. ELISA assays were also rated as of uncertain value with respect to time to readout *(5; 3-8),* feasibility *(6; 3-8),* cost *(4.5; 1-8)* and overall satisfaction *(5; 2-8).*

3. Immunofluorescence assessment of specific α-granule proteins (e.g., β-TG, PF-4) on a blood smear.
   There is uncertainty whether these assays should be carried out in smears of whole blood *(6.5; 1-9)*, of PRP *(5; 2-9)* or of washed platelets *(5; 1-9).* Similarly, sensitivity *(5; 3-8)*, specificity *(6; 3-8)*, time to readout *(5; 2-7)*, feasibility *(6; 3-7)*, cost *(6.5; 5-8)* and overall satisfaction *(5; 3-8)* were rated as uncertain.

   Although many α-granule proteins are characterized and assessed using western blot probes, we did not include questions on these assays regarding their possible use in diagnostic laboratories.

C. Lysosomal release assays.

There was no consensus on the need to measure lysosomal granule release for the diagnosis of PSDs (median score 4.5, range 1-7).

D. Selection of agonists for platelet secretion assays.

1. Collagen

Collagen was rated as the best platelet agonist for PSAs *(8; 7-9)* and should always be used for diagnosis of IPSD *(7; 3-9).* However, we did not include a question about the most suitable type of collagen to be used.

2. Stable thromboxane A_2 analogs and arachidonate
   Thromboxane analogs were rated as preferable over arachidonate for PSAs *(7; 3-9* vs *6; 3-9),* despite the different mechanisms of each one in eliciting platelet secretion.

3. ADP
   ADP was rated as an appropriate agonist *(7.5; 3-9)*, but only at high concentrations *(7; 1-8).*

4. Epinephrine
   This agonist was rated as not recommended for the study of platelet secretion *(1; 1-9).*

5. PAR-1 and PAR-4 Agonists.
   PAR-1 agonists, like TRAP, were rated as appropriate (*7.5; 3-9),* but there was no consensus in using it routinely for diagnostic purposes *(6; 4-9)*. The use of PAR-4 was rated as of uncertain value *(4.5; 3-8).*

E. Major Recommendations

A summary of the major recommendations rated as appropriate for the study of platelet secretion for the diagnosis of IPFD by expert consensus is shown in **Table 1**. A flow diagram showing the consensus for the study of platelet secretion in specialized hemostasis laboratories is shown in Figure 1.

4. | DISCUSSION

Limitations of current PSAs

The study of platelet granule secretion was initiated more than 50 years ago [1] and we now know that impaired secretion is present in the vast majority of IPFDs, either as a main determinant or important contributor to platelet dysfunction [9]. In fact, a laboratory-confirmed PSD is considered sufficient for diagnosing an IPFD. A myriad of PSAs has been developed in the last decades for clinical diagnosis or research purposes [7].

This survey, distributed to a panel of experts in platelet function studies with experience with PSAs, highlights the wide variety of opinions regarding the main features of the evaluated tests. This emphasizes an unmet need for the systematic evaluation of PSAs, methods of standardisation and consensus on diagnostic criteria for PSDs. On the other hand, there was broad agreement on several important statements which may then be considered as current good laboratory practice for PSAs (**Table 1**).

Our survey shows that more than three quarters of the panelists perform PSAs as a second-line diagnostic step, after ruling out VWD or low von Willebrand factor in their patients. This is probably explained by the belief that VWD is the most frequent inherited hemorrhagic disorder, a notion that has been challenged by several studies in the last 15 years [10, 11]. However, 52% of the panelists perform LTA and PSA concomitantly, in line with the SSC guidelines for the diagnosis of IPFD [3]. Of note, the highest survey consensus was related to the need of carrying out PSAs even when LTA tracings are consistent with PSD, since LTA does not measure granule content secretion, needed to diagnose SPD[12]. Moreover, there was wide agreement that normal LTAs do not rule out PSD [13-15], and only 50%[16] and 52%[17] of the patients with dense granule deficiencies have LTA abnormalities. Accordingly, PSAs should be carried out even when LTA is normal. The expert consensus states that platelet secretion study should start with the measurement of dense-body secretion and that an abnormal result should be confirmed in a repeat laboratory test.

The measurement of an α-granule marker is also recommended after the detection of a δ-granule secretion defect to enable detection of combined α- and δ-storage pool disease.

Critical evaluation of currently available PSAs.

LT-LA, a longstanding platelet function assay [4] that progressively replaced the classical radioactive ^{14}C-5-HT secretion test, was rated by the expert panel members as the most suitable test currently available for measuring δ-granule release. However, the ratings of individual panelists showed a large variability. In the 2014 SSC-ISTH survey, 41% of the 197 participants declared they have LT-LA in their laboratories, but only 21.3% used it as a first diagnostic step [1]. This assay measures continuously platelet ATP release upon agonist stimulation and allows the simultaneous determination of secretion and aggregation in PRP [4]. However, agonist-stimulated LT-LA does not distinguish secretion defects from defects in δ-granule content, although an estimate of the latter can be obtained by the measurement of ATP and ADP in platelet lysates. Recent studies have also raised doubts about LT-LA sensitivity [18], specificity [19], and inter-assay reproducibility [20]. Therefore, this Sub-Committee encourages that these uncertainties are addressed through well-controlled studies that should include definition of the lower threshold concentrations for agonists, the place of weak agonists and refined diagnostic criteria for mild PSDs.

Other luminometry-based assays using luciferin/luciferase to quantitate ADP and ATP were rated as appropriate for sensitivity and specificity and they might be especially suited for the measurement in platelet lysates [21], and thus for the diagnosis of δ-storage pool disease. However, the test was rated as cumbersome and indeed is used by few respondents.

There was consensus that HPLC-based platforms for platelet ADP/ATP and serotonin are highly sensitive and specific [22-24], and should now be considered the “gold standard” for measuring dense bodies secretion instead of the almost abandoned ^{14}C-5-HT release assay. However, they were rated as cumbersome, expensive, and with a long time to read-out and need for skilled operators. In the 2014 SSC-ISTH survey, only 5.5% of the participants had HPLC-based assays running in their laboratories to measure ATP and ADP in the same run and to quantify 5-HT in one single step, either in the platelet supernatant, or in platelet lysates [24, 25]. These methods would be highly valuable to calibrate and validate current or newly developed 5-HT and ADP-ATP assays.

The ortho-phtaldialdehyde test to measure serotonin release, first described in 1974 [26, 27], is currently only sporadically used [28, 29] and was rated of uncertain value for all the characteristics, including safety for the operator.

There was wide consensus that semi-quantitative FC measurement of platelet P-selectin expression in whole blood, but not in PRP or washed platelets, is currently the most appropriate test to measure α-granules secretion [30, 31]. FC is being continually updated and progressively more standardized [32-35], and upon fixation it allows the blood sample to be stored or shipped to reference laboratories [36]. The cost of the assay was rated as uncertain, possibly in part due to differing perceptions of costs and budgets among laboratories. Importantly, the correlation of FC measurement of P-selectin expression with LTA was rated as highly uncertain. Since platelet aggregation and secretion are interdependent and highly correlated functions, this relationship should be further explored by studies with validated controls and cutoff values to establish more precise diagnostic criteria of PSDs. Moreover, agonist-stimulated expression of P-selectin on platelet-surface by FC was reported to be normal in some patients with the Gray platelet-syndrome (GPS), an α-granule disorder [37], suggesting that in rare cases this assay may not detect the α-granule secretion deficit.

ELISA methods to measure specific α-granule proteins (e.g., β-TG, PF-4) in the supernatant of activated platelets were rated as appropriate for sensitivity and specificity. However, their time to readout, feasibility, cost, and overall satisfaction were rated as uncertain.

Whole mount TEM (WM-TEM) [38] was rated as appropriate for the diagnosis of storage pool disease but it was considered impractical for diagnostic purposes. Regarding the mepacrine uptake/release tests [39], there was consensus that there is insufficient information to recommend its use. Finally, there was consensus that the role of lysosomal content assays in PFD diagnosis is unclear yet.

Platelet agonists for PSAs.

There was consensus that sensitivity and specificity of PSAs performed with weak agonists or low agonist concentrations are uncertain although this recommendation may have been influenced by the predominant use of LT-LA by survey participants. In line with the expert opinions, 2 studies from the same group found that up to 14% of healthy subjects with no bleeding history present platelet 5-HT secretion below normal cutoffs values with weak or low concentration agonists [15, 40].

PSDs may have different degrees of severity and it remains to be established whether milder cases may be missed using strong agonists and if weaker agonists may have a role in the diagnosis of such cases. Thus, studies to correctly define the diagnostic criteria of PSD in clinical laboratories are highly warranted.

Limitations of the current expert opinion survey

No questions on the type of collagen to be used as agonist or on different TxA_2 analogues were included. Moreover, neither statements linking PSAs with clinical manifestations of IPFD nor the relevance of the ISTH-BAT score for the decision to embark in PSAs were included.

Another limitation of our study is that no questions were included regarding the feasibility of the various PSAs examined in patients with low platelet counts. In some IPFD, like Bernard-Soulier syndrome, it may be possible to obtain PRP with platelet counts sufficiently high for PSAs by spontaneous sedimentation of the blood tube at an angle of 45^{o} and careful collection of the supernatant PRP. More frequently, however, classical LTA and PSA testing are not possible while some of the above-described assays may be performed; for instance, flow cytometry, including platelet P-selectin secretion for the diagnosis of α-granule release defects or mepacrine for the study of uptake and release of platelet dense granule contents. The counting of dense bodies using whole mount electron microscopy, complementing the diagnosis of Storage Pool Disease, may also be performed in samples with low platelet counts. Examination of the blood smear is essential in patients with thrombocytopenias: large, grey or pale platelet appearance due to lack of α-granules are characteristic of Gray platelet syndrome, which is confirmed by transmission electron microscopy and detection of NBEAL2 mutations;

other macrothrombocytopenias, like those characterizing the spectrum of phenotypes associated with MYH9 mutations, are recognized by leukocyte inclusions (Döhle bodies), whereas anaemia and leukopenia in male patients with macrothrombocytopenia orients the diagnosis to GATA-1-related X-linked disorder. Moreover, platelet clumps associated with a mildly increased and unequal platelet size may suggest platelet type-VWD [41]. Finally, labelling blood smears with fluorescent monoclonal antibodies directed towards specific platelet proteins and fluorescence microscopic examination may be a useful tool in a fraction of IPFD [42].

Future directions

Despite decades of use of PSAs in laboratory practice there is still need for adequately designed and powered studies to define the advantages and limitations of current tests, the interpretation of results and diagnostic criteria for PSDs. We believe that the currently most used PSAs should be validated by systematic comparison with an accepted gold standard assay, i.e., HPLC measurement of platelet secreted ATP, ADP or 5-HT. It is also important to explore new ways to measure α-granule secretion, perhaps devising new tests, and to resolve the relationship of FC measurements of platelet secretion with LTA. Finally, it is important to clarify which platelet secretion test better correlates with bleeding severity through prospective clinical studies. This new information may lead to more conclusive and evidence-based recommendations for the use of PSAs for the diagnosis of IPFD and possibly of acquired PFDs.

ACKNOWLEDGMENTS

The authors are grateful to the Platelet Physiology SSC Co-Chairs for their insights and advice. This study was partially funded by ANID-FONDECYT, grant 1181681 (DM). We thank the following expert panellists who contributed to this survey.

Expert panelists:

Marie-Christine Alessi (France), Carlo Brugnara (USA), Marco Cattaneo (Italy), Remi Favier (France), Andrew L. Frelinger III (USA), Kenneth D. Friedman (USA), Ginés Escolar

(Spain), Christian Gachet (France), Andreas Greinacher (Germany), Paolo Gresele (Italy), Johan W. M. Heemskerk (The Netherlands), Martine Jandrot-Perrus (France), Beate E. Kehrel (Germany), Alan D. Michelson (USA), Andrew D. Mumford (U.K.), Patrizia Noris (Italy), Dianne Nugent (USA), Cécile Oury (Belgium), Jaime Pereira (Chile), Fabio Pulcinelli (Italy), A. Koneti Rao (USA), José Rivera (Spain), Analía Sánchez (Argentina), Alvin Schmaier (USA), José-Manuel Soria (Spain), Steve P. Watson (U.K.).

AUTHORS' CONTRIBUTIONS

DM and PG designed the study, identified the expert panelists, conceived the first and secondary survey statements, analyzed data and wrote the manuscript. PH, ALF, AM, PM and ML contributed expertise, provided feedback and critically revised the manuscript. PG and ML chaired the Platelet Physiology SSC and oversaw the project. All authors reviewed and approved the final manuscript for publication.

DISCLOSURES

The authors declare no conflict of interest.

References

1 Holmsen H, Day HJ, Stormorken H. The blood platelet release reaction. *Scandinavian journal of haematology Supplementum*. 1969; **8**: 3-26.

2 Gresele P, Harrison P, Bury L, Falcinelli E, Gachet C, Hayward CP, Kenny D, Mezzano D, Mumford AD, Nugent D, Nurden AT, Orsini S, Cattaneo M. Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey. *J Thromb Haemost*. 2014; **12**: 1562-9. 10.1111/jth.12650.

3 Gresele P, Subcommittee on Platelet Physiology of the International Society on T, Hemostasis. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. *J Thromb Haemost*. 2015; **13**: 314-22. 10.1111/jth.12792.

4 Feinman RD, Lubowsky J, Charo I, Zabinski MP. The lumi-aggregometer: a new instrument for simultaneous measurement of secretion and aggregation by platelets. *J Lab Clin Med*. 1977; **90**: 125-9.

5 Kaplan KL, Broekman MJ, Chernoff A, Lesznik GR, Drillings M. Platelet alpha-granule proteins: studies on release and subcellular localization. *Blood*. 1979; **53**: 604-18.

6 Holmsen H, Setkowsky CA, Lages B, Day HJ, Weiss HJ, Scrutton MC. Content and thrombin-induced release of acid hydrolases in gel-filtered platelets from patients with storage pool disease. *Blood*. 1975; **46**: 131-42.

7 Mumford AD, Frelinger AL, 3rd, Gachet C, Gresele P, Noris P, Harrison P, Mezzano D. A review of platelet secretion assays for the diagnosis of inherited platelet secretion disorders. *Thromb Haemost*. 2015; **114**: 14-25. 10.1160/th14-11-0999.

8 Brook RH, Chassin MR, Fink A, Solomon DH, Kosecoff J, Park RE. A method for the detailed assessment of the appropriateness of medical technologies. *Int J Technol Assess Health Care*. 1986; **2**: 53-63. 10.1017/s0266462300002774.

9 Watson SP, Lowe GC, Lordkipanidze M, Morgan NV, consortium G. Genotyping and phenotyping of platelet function disorders. *J Thromb Haemost*. 2013; **11 Suppl 1**: 351-63. 10.1111/jth.12199.

10 Mezzano D, Quiroga T. Diagnostic challenges of inherited mild bleeding disorders: a bait for poorly explored clinical and basic research. *J Thromb Haemost*. 2019; **17**: 257-70. 10.1111/jth.14363.

11 Oved JH, Lambert MP, Kowalska MA, Poncz M, Karczewski KJ. Population based frequency of naturally occurring loss-of-function variants in genes associated with platelet disorders. *J Thromb Haemost*. 2021; **19**: 248-54. 10.1111/jth.15113.

12 Dupuis A, Bordet JC, Eckly A, Gachet C. Platelet δ-Storage Pool Disease: An Update. *J Clin Med*. 2020; **9**. 10.3390/jcm9082508.

13 Nieuwenhuis HK, Akkerman JW, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on one hundred six patients. *Blood*. 1987; **70**: 620-3.

14 Israels SJ, McNicol A, Robertson C, Gerrard JM. Platelet storage pool deficiency: diagnosis in patients with prolonged bleeding times and normal platelet aggregation. *Br J Haematol*. 1990; **75**: 118-21.

15 Quiroga T, Goycoolea M, Matus V, Zuniga P, Martinez C, Garrido M, Aranda E, Leighton F, Panes O, Pereira J, Mezzano D. Diagnosis of mild platelet function disorders. Reliability and usefulness of light transmission platelet aggregation and serotonin secretion assays. *Br J Haematol*. 2009; **147**: 729-36. 10.1111/j.1365-2141.2009.07890.x.

16 Woods GM, Kudron EL, Davis K, Stanek J, Kerlin BA, O'Brien SH. Light Transmission Aggregometry Does Not Correlate With the Severity of δ-Granule Platelet Storage Pool Deficiency. *J Pediatr Hematol Oncol*. 2016; **38**: 525-8. 10.1097/mph.0000000000000638.

17 Brunet JG, Iyer JK, Badin MS, Graf L, Moffat KA, Timleck M, Spitzer E, Hayward CPM. Electron microscopy examination of platelet whole mount preparations to quantitate platelet dense granule numbers:

Implications for diagnosing suspected platelet function disorders due to dense granule deficiency. *International journal of laboratory hematology*. 2018; **40**: 400-7. 10.1111/ijlh.12801.
18 Pai M, Wang G, Moffat KA, Liu Y, Seecharan J, Webert K, Heddle N, Hayward C. Diagnostic usefulness of a lumi-aggregometer adenosine triphosphate release assay for the assessment of platelet function disorders. *Am J Clin Pathol*. 2011; **136**: 350-8. 10.1309/AJCP9IPR1TFLUAGM.
19 Lotta LA, Maino A, Tuana G, Rossio R, Lecchi A, Artoni A, Peyvandi F. Prevalence of disease and relationships between laboratory phenotype and bleeding severity in platelet primary secretion defects. *PloS one*. 2013; **8**: e60396. 10.1371/journal.pone.0060396.
20 Badin MS, Graf L, Iyer JK, Moffat KA, Seecharan JL, Hayward CP. Variability in platelet dense granule adenosine triphosphate release findings amongst patients tested multiple times as part of an assessment for a bleeding disorder. *International journal of laboratory hematology*. 2016; **38**: 648-57. 10.1111/ijlh.12553.
21 Holmsen H, Dangelmaier CA. Measurement of secretion of adenine nucleotides. *Methods Enzymol*. 1989; **169**: 195-205.
22 D'Souza L, Glueck HI. Measurement of nucleotide pools in platelets using high pressure liquid chromatography. *Thromb Haemost*. 1977; **38**: 990-1001.
23 Leoncini G, Buzzi E, Maresca M, Mazzei M, Balbi A. Alkaline extraction and reverse-phase high-performance liquid chromatography of adenine and pyridine nucleotides in human platelets. *Anal Biochem*. 1987; **165**: 379-83.
24 Anderson GM, Hall LM, Yang JX, Cohen DJ. Platelet dense granule release reaction monitored by high-performance liquid chromatography-fluorometric determination of endogenous serotonin. *Anal Biochem*. 1992; **206**: 64-7.
25 Flachaire E, Beney C, Berthier A, Salandre J, Quincy C, Renaud B. Determination of reference values for serotonin concentration in platelets of healthy newborns, children, adults, and elderly subjects by HPLC with electrochemical detection. *Clin Chem*. 1990; **36**: 2117-20.
26 Drummond AH, Gordon JL. Letter: Rapid, sensitive microassay for platelet 5HT. *Thrombosis et diathesis haemorrhagica*. 1974; **31**: 366-7.
27 Holmsen H, Dangelmaier CA. Measurement of secretion of serotonin. *Methods Enzymol*. 1989; **169**: 205-10.
28 Jedlitschky G, Cattaneo M, Lubenow LE, Rosskopf D, Lecchi A, Artoni A, Motta G, Niessen J, Kroemer HK, Greinacher A. Role of MRP4 (ABCC4) in platelet adenine nucleotide-storage: evidence from patients with delta-storage pool deficiencies. *Am J Pathol*. 2010; **176**: 1097-103. 10.2353/ajpath.2010.090425.
29 Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML. Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores: further evidence that some cases of platelet 'primary secretion defect' are heterozygous for a defect of P2CYC receptors. *Arterioscler Thromb Vasc Biol*. 2000; **20**: E101-6. 10.1161/01.atv.20.11.e101.
30 Johnston GI, Pickett EB, McEver RP, George JN. Heterogeneity of platelet secretion in response to thrombin demonstrated by fluorescence flow cytometry. *Blood*. 1987; **69**: 1401-3.
31 Tschoepe D, Spangenberg P, Esser J, Schwippert B, Kehrel B, Roesen P, Gries FA. Flow-cytometric detection of surface membrane alterations and concomitant changes in the cytoskeletal actin status of activated platelets. *Cytometry*. 1990; **11**: 652-6. 10.1002/cyto.990110515.
32 Psaila B, Bussel JB, Frelinger AL, Babula B, Linden MD, Li Y, Barnard MR, Tate C, Feldman EJ, Michelson AD. Differences in platelet function in patients with acute myeloid leukemia and myelodysplasia compared to equally thrombocytopenic patients with immune thrombocytopenia. *J Thromb Haemost*. 2011; **9**: 2302-10. 10.1111/j.1538-7836.2011.04506.x.
33 Frelinger AL, 3rd, Rivera J, Connor DE, Freson K, Greinacher A, Harrison P, Kunishima S, Lordkipanidze M, Michelson AD, Ramstrom S, Gresele P. Consensus recommendations on flow cytometry for the assessment of inherited and acquired disorders of platelet number and function: Communication from the ISTH SSC Subcommittee on Platelet Physiology. *J Thromb Haemost*. 2021; **19**: 3193-202. 10.1111/jth.15526.

34 Pedersen OH, Nissen PH, Hvas AM. Platelet function investigation by flow cytometry: Sample volume, needle size, and reference intervals. *Platelets*. 2018; **29**: 199-202. 10.1080/09537104.2017.1353684.
35 van Asten I, Schutgens REG, Baaij M, Zandstra J, Roest M, Pasterkamp G, Huisman A, Korporaal SJA, Urbanus RT. Validation of flow cytometric analysis of platelet function in patients with a suspected platelet function defect. *J Thromb Haemost*. 2018; **16**: 689-98. 10.1111/jth.13952.
36 Dovlatova N, Lordkipanidze M, Lowe GC, Dawood B, May J, Heptinstall S, Watson SP, Fox SC, Group UGS. Evaluation of a whole blood remote platelet function test for the diagnosis of mild bleeding disorders. *J Thromb Haemost*. 2014; **12**: 660-5. 10.1111/jth.12555.
37 Gresele P, Falcinelli E, Bury L. Laboratory diagnosis of clinically relevant platelet function disorders. *Int J Lab Hematol*. 2018; **40 Suppl 1**: 34-45. 10.1111/ijlh.12814.
38 Hayward CP, Moffat KA, Spitzer E, Timleck M, Plumhoff E, Israels SJ, White J, Deficiency NWGoPDG. Results of an external proficiency testing exercise on platelet dense-granule deficiency testing by whole mount electron microscopy. *Am J Clin Pathol*. 2009; **131**: 671-5. 10.1309/AJCPYMFXZNSNRZ55.
39 Cai H, Mullier F, Frotscher B, Briquel ME, Toussaint M, Massin F, Lecompte T, Latger-Cannard V. Usefulness of Flow Cytometric Mepacrine Uptake/Release Combined with CD63 Assay in Diagnosis of Patients with Suspected Platelet Dense Granule Disorder. *Semin Thromb Hemost*. 2016; **42**: 282-91. 10.1055/s-0035-1564836.
40 Quiroga T, Goycoolea M, Panes O, Aranda E, Martinez C, Belmont S, Munoz B, Zuniga P, Pereira J, Mezzano D. High prevalence of bleeders of unknown cause among patients with inherited mucocutaneous bleeding. A prospective study of 280 patients and 299 controls. *Haematologica*. 2007; **92**: 357-65.
41 Gresele P, Bury L, Mezzasoma AM, Falcinelli E. Platelet function assays in diagnosis: an update. *Expert review of hematology*. 2019; **12**: 29-46. 10.1080/17474086.2019.1562333.
42 Greinacher A, Pecci A, Kunishima S, Althaus K, Nurden P, Balduini CL, Bakchoul T. Diagnosis of inherited platelet disorders on a blood smear: a tool to facilitate worldwide diagnosis of platelet disorders. *J Thromb Haemost*. 2017; **15**: 1511-21. 10.1111/jth.13729.

Table 1. Major recommendations on platelet secretion assays for diagnosis of inherited platelet disorders.

General Statements	*PSA should be performed in specialized hemostasis laboratories. (100%) *PSA should be performed after ruling out VWD (76%) *PSA should be performed in patients with pathological bleeding even if they have normal platelet aggregation. (*9; 7-9)*. *PSA should be performed also in patients with normal LTA *(9, 5-9).* *Initially, testing should include a δ-granule PSA, usually done in citrated PRP. *(9; 3-9)*. *Diagnosis must be established after confirming a PSD by repeated laboratory testing. *(7.5, 3-9).*
Secretion of δ-granules	*Lumiaggregometry in PRP is the most frequently used test, despite cost and uncertainties about sensitivity and specificity with weak agonists. *High sensitivity and specificity of HPLC-based measurements of ADP/ATP or 5-HT make these assays new gold standards. However, high cost, need of expertise and slow response limit their feasibility for use in clinical laboratories. *Dense body counting by whole mount TM using citrated PRP is sensitive and specific as supportive diagnostic test. *Other reported assays are rated in the uncertain category.
Secretion of α-granules	*Platelet P-selectin expression measured by flow cytometry in whole blood drawn in citrate, is currently the best and most frequently used assay. *(8;5-9)* *ELISA methods to quantify secreted α-granule proteins are sensitive and specific, but expensive and not sufficiently feasible for routine use.
Agonists	*Collagen, thromboxane analog, ADP in high concentration and PAR-1 agonists are appropriate agonists to elicit and measure platelet secretion. Epinephrine is not recommended. *(1; 1-9)*

Between brackets: percentage of experts agreeing with the statement or median and range of score

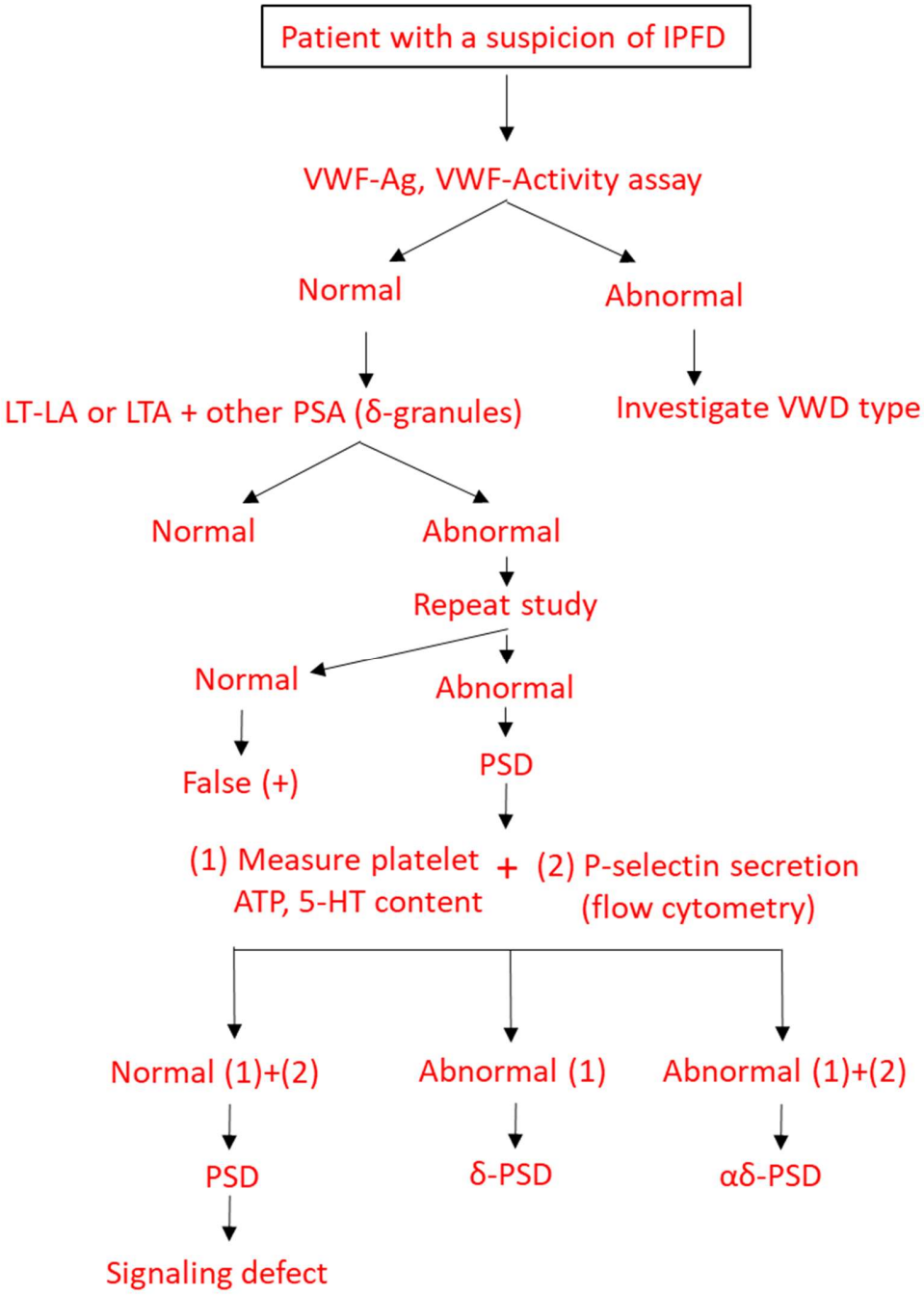

Figure 1. Suggested flow diagram for the study of platelet secretion defects. This diagram does not include specific phenotype patterns which may orient the study to specific disorders before starting the platelet secretion testing. For example, albinism in Hermansky-Pudlak disorder (δ-SPD), blood smears with leukocyte inclusions (i.e., Chediak-Higashi syndrome) or large platelets with gray appearance (Grey platelet syndrome), among others.