

# A review of platelet secretion assays for the diagnosis of inherited platelet secretion disorders

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## Summary

Measurement of platelet granule release to detect inherited platelet secretion disorders (IPSDs) is essential for the evaluation of patients with abnormal bleeding and is necessary to distinguish which granule sub-types are affected and whether there is abnormal granule biosynthesis or secretion. The radioactive serotonin incorporation and release assay, described before 1970, is still considered the "gold standard" test to assess platelet  $\delta$ -granule release, although is unsuitable for clinical diagnostic laboratories. Luciferin-based assays, such as lumiaggregometry, are the most widely performed alternatives, although these methods do not distinguish defects in  $\delta$ -granule biosynthesis from defects in secretion. Platelet  $\alpha$ -granule release is commonly evaluated using flow cytometry by measuring surface exposure

of P-selectin after platelet activation. However, this assay has poor sensitivity for some  $\alpha$ -granule disorders. Only few studies have been published with more recently developed assays and no critical reviews on these methods are available. In this review, we describe the rationale for developing robust and accurate laboratory tests of platelet granule release and describe the characteristics of the currently available tests. We identify an unmet need for further systematic evaluation of new assays and for standardisation of methodologies for clinical diagnostic laboratories.

## Keywords

Platelet pathology, inherited, acquired, secretion, exocytosis, diagnosis management

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Received: November 30, 2014  
Accepted after minor revision: February 20, 2015  
Epub ahead of print: April 16, 2015  
<http://dx.doi.org/10.1160/TH14-11-0999>  
Thromb Haemost 2015; 114: 14–25

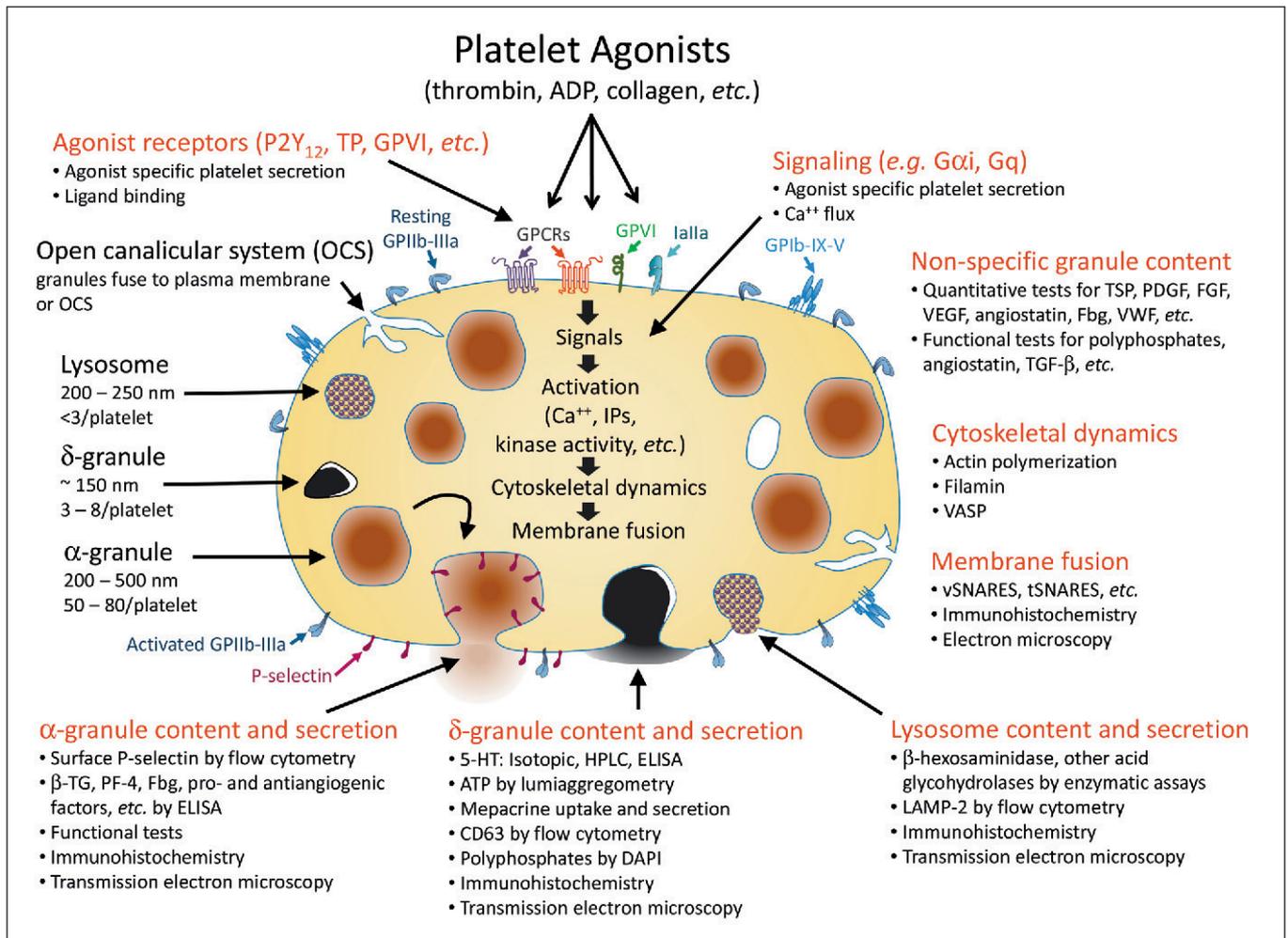
## Introduction

Inherited Platelet Secretion Disorders (IPSDs) are a group of common and heterogeneous platelet function disorders characterised by defective release of the contents of  $\alpha$ -granules, dense ( $\delta$ -) granules or both, during the platelet activation response. The IPSDs include the disorders  $\delta$ -storage pool disease and Gray Platelet Syndrome (GPS), in which there is abnormal biogenesis of platelet granules. The term IPSD also encompasses diverse disorders in which granule secretion is reduced because of abnormal platelet receptors, signalling or granule trafficking (►Figure 1). This group includes primary secretion defects in which reduced secretion occurs in isolation, and disorders in which there are additional defects in platelet function.

Granule release by platelets requires regulated granule synthesis but also a multi-step secretion pathway that can be summarised into the following components: 1) agonist interaction with platelet surface receptors; 2) intracellular signalling; 3) changes in intracel-

lular levels of free  $Ca^{++}$ , inositol phosphates and kinases; 4) cytoskeletal remodelling, and, 5) fusion of granule and platelet surface membranes causing release of granule contents (►Figure 1). Defects at any point in this pathway may give rise to an IPSD and lead to impaired platelet activation. However, because of the complexity of this process, abnormal granule release frequently manifest as a wider platelet activation defect that affects multiple laboratory tests of platelet function (1–4). Despite improvements in the understanding of the components of the release pathway (5, 6), the molecular and genetic mechanisms in the majority of patients with an IPSD remain unknown (6).

Laboratory tests that measure the content and release of platelet granules were first introduced more than 40 years ago (7, 8). However, adoption of these tests into clinical diagnostic practice remains inconsistent. This was highlighted by a recent worldwide survey conducted by the International Society on Thrombosis and Haemostasis (ISTH) in which more than half of the 202 responding laboratories did not evaluate platelet  $\delta$ -granule or  $\alpha$ -granule



**Figure 1: Schematic representation of the major pathways required for platelet granule release and the repertoire of diagnostic tests for inherited platelet secretion disorders.** The pathways necessary for platelet granule release are complex and require interactions between arrays of platelet proteins. Consequently, the inherited platelet secretion disorders, which arise from defects in these pathways, are highly diverse and may be causally associated with defects in many platelet genes. The IPSDs include defects in the biogenesis of α-granules (Gray Platelet Syndrome, Arthrogryposis - Renal Dysfunction-Cholestasis), δ granules (Hermansky-Pudlak Syndrome, Chediak-Higashi Syndrome) of both (αδ storage pool disease) and defects in the secretion of these granules. Accurate detection of IPSP requires detailed phenotype testing, usually with assays to examine each of the differ-

ent granule components, using standardised methodologies to ensure reproducibility and diagnostic precision. Abbreviations: 5-HT, serotonin; ADP, adenosine diphosphate; β-TG, beta-thromboglobulin; DAPI, 4',6-diamidino-2-phenylindole; Fbg, fibrinogen; FGF, fibroblast growth factor; GP, glycoprotein; GPCRs, G protein-coupled receptors; HPLC, high performance liquid chromatography; IP<sub>3</sub>, inositol phosphates; LAMP-2, lysosome associated membrane protein 2; PDGF, platelet-derived growth factor; PF4, platelet factor 4; TGF-β, transforming growth factor beta; TP, thromboxane receptor; tSNARE, target soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor; vSNARE, vesicular SNARE; TSP, thrombospondin; VASP, vasodilator stimulated phosphoprotein; VEGF, vascular endothelial growth factor; VWF, von Willebrand Factor.

content or release during investigation of patients with suspected platelet function defects (4). Amongst the laboratories in which granule release was evaluated, the choice of laboratory methods were diverse and methodology was poorly standardised. Together, these observations suggest that there is an unmet need for wider adoption of specific tests to detect IPSPs and for more precise definition of best laboratory practice. This review outlines the different methodologies described to measure the content and release of platelet granules with a focus on tests that are suitable for clinical diagnostic practice.

## Why evaluate platelet secretion in a clinical setting?

Glanzmann thrombasthenia and Bernard Soulier syndrome typically manifest as severe inherited bleeding disorders caused by reduced or absent expression of the platelet α<sub>IIb</sub>β<sub>3</sub> integrin and glycoprotein (GP)Ib-IX-V complex, respectively. These characteristics usually enable straightforward diagnosis using light transmission aggregometry (LTA) in platelet-rich plasma (PRP) and the measurement of GP expression by flow cytometry, without the need to evaluate the platelet secretion response.

**Table 1: Functional types of platelet granules components.**

Granules	Type of granule constituents	Prominent components
Dense bodies ( $\delta$ -granules)	Small molecules	ATP, ADP, serotonin (5-HT), calcium, polyphosphates, pyrophosphate
$\alpha$ -granules	Membrane glycoproteins	GPIb, $\alpha$ IIb $\beta$ 3, GPVI
	Clotting factors	vWF, FV, FXI, FII, fibrinogen, HMWK, (FXIII?) <sup>§</sup>
	Clotting inhibitors	TFPI, protein S, protease nexin-2
	Fibrinolysis components	PAI-1, TAFI, $\alpha$ 2-antiplasmin, plasminogen, uPA
	Other protease inhibitors	$\alpha$ 1-antitrypsin, $\alpha$ 2-macroglobulin
	Inflammatory, pro-atherogenic, wound healing and antimicrobial proteins	P-selectin, PSGP-1, thrombospondin, CD40L, chemokines and cytokines (e.g. PF4, $\beta$ -TG, IL-8, IL-1 $\beta$ , TGF $\beta$ , MCP-1, RANTES), TLT-1 $\P$ , osteonectin, complement components (e.g. C3, C4, C1 inhibitor, protein H)...
	Pro-angiogenic growth factors	VEGF, PDGF, EGF, IGF, FGF, angiopoietin
	Anti-angiogenic growth factors	Angiostatin, endostatin, inhibitors of matrix metalloproteinases, TIMPs, LAMP-2
	Matrix metalloproteinase inducer	EMMPRIN (CD147)
	Ligand for cell surface receptors	Semaphorin 7A
Lysosomes	Membrane proteins*	LAMP3 (lysosome integral membrane protein 3), LAMP-2
	Stored enzymes	Proteases, $\beta$ -galactosidase, cathepsin, $\beta$ -N-acetylhexosaminidase, phosphatases...

\* LIMP-1(CD63), formerly granulophysin, and LAMP-2: also found in  $\delta$ -granule membranes.  $\P$  TLT-1, from TREM family (triggering receptors expressed on myeloid cells). <sup>§</sup> FXIII is a cytoplasmic transglutaminase (125).

More commonly, inherited platelet function disorders are associated with a milder clinical phenotype that comprises spontaneous mucocutaneous bleeding and abnormal bleeding after trauma, surgery or childbirth, similar to other disorders of primary haemostasis such as von Willebrand disease (VWD) (9). It has been estimated that platelet secretion defects constitute more than 90% of all inherited platelet disorders, and that this group may be more prevalent than VWD (9–11).

The laboratory characteristics of mild platelet function disorders include abnormalities of LTA, such as absent secondary wave, decreased slope, prolonged lag phase or reversible aggregation. These abnormalities result primarily from impaired release of the soluble agonists ADP and 5-HT from  $\delta$ -granules or from defective synthesis or responsiveness to thromboxane A<sub>2</sub>, which

normally provide positive feedback signals during platelet activation and promote irreversible aggregation. Since the pattern and extent of aggregation abnormalities in mild platelet function disorders is highly variable between individuals, it is essential to evaluate platelet granule release to determine whether an IPSD is present, either as a primary defect of platelet biogenesis or as a direct or indirect signalling pathway disorder that affects granule release or platelet activation.

The importance of evaluating granule release is further emphasised by observations that defects in the release of granules, particularly of  $\delta$ -granules is highly prevalent amongst patients with mild platelet function defects (3, 12) and that 10% to 23% of these patients display normal LTA responses in PRP to standard agonist panels (3, 9, 13–15). In order to ensure accurate diagnosis of this important subgroup of IPSDs, measurement of  $\delta$ -granule release alongside LTA, is a necessary investigation early in the diagnostic pathway for all patients with bleeding symptoms (3, 15, 16).

## Platelet granules contents and their secretion sequence

It is estimated that human platelets contain 4,000–4,500 different proteins (17, 18), up to 1,048 of which have been demonstrated in the platelet releasate following  $\alpha$ -granule secretion (19). The  $\alpha$ -granule proteins may be sub-classified as: 1) Proteins such as platelet factor 4 (PF4), beta-thromboglobulin ( $\beta$ -TG) and possibly TLT-1, one of the TREM (triggering receptors expressed on myeloid cells) family of proteins, that are synthesised exclusively in megakaryocytes (MKs) and which are stored and secreted only by platelets; 2) Proteins such as von Willebrand factor (VWF), P-selectin (CD62), thrombospondin-1, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- $\beta$ ) and IL-1 $\beta$  that are synthesised in MKs, platelets and in other tissues; and, 3) Proteins, (like fibrinogen, IgG, albumin and fibronectin), which are synthesised in other tissues, but which are incorporated into  $\alpha$ -granules by MKs or platelets by endocytosis ( $\blacktriangleright$  Table 1). Most  $\alpha$ -granule proteins are soluble and released into the extracellular compartment. These include several coagulation factors that serve an important role in supporting local thrombin generation and fibrin deposition during the hemostatic response. Other proteins are structural components of the  $\alpha$ -granule membrane and remain bound to the platelet surface after secretion. These include proteins that are also expressed on the surface of un-activated platelets such as  $\alpha$ IIb $\beta$ 3, GPVI, Fc receptors, PECAM, GPIb-IX-V complex, tetraspanins and CD36, and also proteins normally confined to the  $\alpha$ -granule membrane such as P-selectin, CD40L, CD63 (lysosome associated membrane membrane protein 3, LAMP3), fibrocystin L, CD109 (19–21). Amongst the soluble  $\alpha$ -granule proteins, the most abundant and easy to quantify are thrombospondin-1,  $\beta$ -TG and PF4, which are present at concentrations of >0.5  $\mu$ g per 10<sup>8</sup> platelets compared to VWF, which has a concentration of 0.05–0.5  $\mu$ g per 10<sup>8</sup> platelets (18).

Platelet  $\delta$ -granules contain predominantly small non-protein molecules such as ADP, ATP, serotonin (5-HT), pyrophosphates

(22), calcium, which is responsible for their electron opacity (23), and the more recently reported polyphosphates (24). Some of them are essential mediators for sustained platelet activation responses, including the weak agonist ADP that alone induces only reversible aggregation. However, once released from dense granules after the stimulation of platelets by other agonists, ADP amplifies platelet activation responses by binding the  $G_i$  purinergic receptor  $P2Y_{12}$ , thereby enabling stable platelet aggregate formation (25). 5-HT accumulation in platelets during their lifespan in circulation (26), is most likely derived from overflow of 5-HT production by enterochromaffin cells of the digestive tract. In humans, 5-HT that is released from  $\delta$ -granules is also unable to activate platelets directly, but instead potentiates the effect of other agonists and also mediates additional vascular effects including both vasodilation and vasoconstriction, often dependent on species and anatomical site (27). Medium-sized chains of polyphosphates in platelet  $\delta$ -granules also have complex effects that include acceleration of factor V and factor XI activation by thrombin, abolition of the anticoagulant function of tissue factor pathway inhibitor, and modification of fibrin clot structure (28).

The integral membrane proteins of  $\delta$ -granules more widely expressed proteins, such as LAMP3 (CD63, granulophysin) and LAMP2, but also proteins with roles in maintaining  $\delta$ -granule content such as the ADP transporter multidrug resistance protein MRP4 (29) and the putative 5-HT transporter VMAT-2 (30). The importance of these transporters is highlighted by observations that defective expression of MRP4 in platelets results in a sub-type of  $\delta$ -storage-pool disease with selected decrease in ADP storage but normal 5-HT (29).

Platelet lysosomes contain enzymes such as  $\beta$ -hexosaminidase and other acid glycohydrolases (31), and membrane bound proteins such as LAMP-1 (CD107a), LAMP-2 (CD107b) and LAMP-3 (32), which are also present in secretory granules of other cell types, and in the platelet  $\delta$ -granules membranes (► Table 1).

Stimulation of platelets with low concentrations of weak agonists causes greater release of  $\alpha$ -granules than  $\delta$ -granules (33, 34). However, strong agonists appear to induce faster secretion of  $\delta$ -granules (35). There is also consensus that release of lysosome contents occurs after the release of  $\alpha$ - and  $\delta$ -granules, and requires greater energy and stronger agonist stimulation (36).

Recent studies also suggest that stimulators and inhibitors of angiogenesis are stored in different  $\alpha$ -granule compartments and are released differentially by distinct agonists, suggesting heterogeneity in  $\alpha$ -granules contents and release sequence (37–39). Subpopulations of  $\alpha$ -granules have also been defined according to expression of membrane proteins (40), morphology (41) and TLR9 content, which defines an  $\alpha$ -granule subgroup termed T granules that do not express P-selectin (42). The notion of distinctive  $\alpha$ -granule compartments and secretion sequences has been disputed, based on experimental evidence suggesting that platelet secretion fits better with a random release model (35, 43). This controversy has important implications for diagnostic granule release assays since if heterogeneity of  $\alpha$ -granules is confirmed, then specific adaptations of assays may be required to fully characterize secretion defects.

## Available tests to evaluate platelet granule release

Laboratory tests to evaluate platelet granule release utilise several different strategies to measure platelet granule content or secretion, many of which are specific to platelet granule sub-types. These may be quantitative or semi-quantitative assays to measure granule components or indirect tests to assess platelet granule release through functional end-point assays. Secreted granule contents may be measured in the platelet supernatant at a single time point after stimulation and then compared to the total platelet content to determine the secreted fraction of granule contents. Alternatively, granule contents can be measured over a time course to determine the kinetics of secretion. Platelet secretion may also be evaluated by measuring granule proteins that remain bound to the platelet membrane after release by flow cytometry. Defective granule number and content can be detected by direct- or immunofluorescence-light microscopy, or by electron microscopy (EM) or whole mount transmission EM (44, 45).

In this review, we focus on tests of granule release that are suitable for the diagnosis of IPSDs in clinical laboratories and which would typically be performed alongside LTA (46). Like LTA, the current tests of platelet granule release need to be performed within few hours of sample collection and therefore are not amenable to being run by an off-site core. For diagnosis of the IPSDs, it is usually sufficient to demonstrate a reduction in the maximum release of a granule marker at a single time point. However, it is also desirable to distinguish whether release defects result from defective granule biogenesis or defective secretion. A summary of current and historical tests for evaluation of platelet secretion is presented in ► Table 2 and shown diagrammatically in ► Figure 1.

### 1. Serotonin (5-HT) secretion from $\delta$ -granules

Virtually all of the 6–8  $\mu\text{mol}$  of 5-HT transported in whole blood circulate within platelet  $\delta$ -granules at a concentration of 400–600  $\text{ng}/10^9$  platelets (47–49). In contrast, the plasma 5-HT concentration reported in 101 studies between 1952 and 2010 varied more than 100-fold from 0.6 to 179  $\text{nmol}/\text{l}$  (50). The high plasma concentrations of 5-HT in many of these studies most likely reflect leakage of platelet 5-HT into plasma during sample handling, since careful platelet processing to minimise leakage (51) yields more reliable estimates of  $\leq 0.6$   $\text{nmol}/\text{l}$ . The high 5-HT concentration within platelet  $\delta$ -granules results from avid uptake of 5-HT from plasma through the ligand-gated ion channel carrier SERT into the platelet cytoplasm, and its translocation to the  $\delta$ -granule by the vesicular monoamine transporter 2 (VMAT2, SLC18A) (52). Although the high content of 5-HT in platelet  $\delta$ -granules and the absence from other blood cells render 5-HT a robust and selective marker of platelet  $\delta$ -granule release (► Table 3), only 16% of 202 ISTH surveyed laboratories utilised a 5-HT-based test as a diagnostic tool.

The uptake of 5-HT into platelet  $\delta$ -granules is exploited by the  $^{14}\text{C}$ -5-HT release assay, in which a trace amount of radioactive 5-HT added to PRP is rapidly and almost completely incorporated

<b>1. Quantitative assays for specific granule components:</b>
<ul style="list-style-type: none"> <li>• ADP, ATP</li> <li>• 5-HT (53, 56, 57, 61, 62, 66, 67, 69, 70)</li> <li>• PF4 (CXCL4); <math>\beta</math>-TG (CXCL7) (87, 88)</li> <li>• Platelet membrane P-selectin measured by FC* (94, 95)</li> <li>• TLT-1 (102)</li> </ul>
<b>2. Quantitative and semi quantitative assays for non-specific granule contents:</b>
<ul style="list-style-type: none"> <li>• sP-selectin<sup>†</sup> (126, 127); CD40L/sCD40L (128); thrombospondin (95, 129); osteonectin (130)</li> <li>• Angiogenic, anti-angiogenic factors (38)</li> <li>• Polyphosphates (DAPI fluorescence) (85)</li> <li>• PDGF (131); fibrinogen (132); vWF (133)</li> <li>• Lysosomes and dense bodies integral membrane proteins LIMP-1 (CD63) (109) and LAMP-2 (110) and lysosomes enzymes (e. g. <math>\beta</math>-hexosaminidase, other acid glycosidases, proteases, phosphatases...) (31, 134)</li> <li>• Mepacrine uptake and secretion<sup>‡</sup> (surrogate test) (59)</li> </ul>
<b>3. Functional testing of secreted platelet components:</b>
<ul style="list-style-type: none"> <li>• Polyphosphates (135)</li> <li>• Other <math>\alpha</math>-granule proteins: angiotatin (136, 137); TGF-<math>\beta</math> (138, 139), among many others</li> </ul>
<b>4. Surrogate, indirect assessment of platelet secretion defects:</b>
<ul style="list-style-type: none"> <li>• Whole mount EM: semi-quantitative measurement of <math>\delta</math>-granules number (45, 83)</li> <li>• Observation of platelets in blood smears allows suspecting GPS</li> <li>• Transmission EM, Immune EM and IF-confocal microscopy (37, 140, 141): identify and estimate number and contents of granules and lysosomes</li> <li>• Mepacrine fluorescence assay: for counting the number of <math>\delta</math>-granules<sup>‡</sup> (59)</li> <li>• <math>\delta</math>-granule 5-HT assessment by FC and immunocytochemical microscopy (63, 65)</li> <li>• Measurement of different platelet components in lysate fractions, i.e. fibrinogen, PF4, <math>\beta</math>-TG and many other proteins, before and after activation</li> </ul>
<p>*: This test measures intrinsic platelet P-selectin, although this protein is not platelet-specific. P-selectin has been also localised in the membrane of <math>\delta</math>-granules (93).  <sup>†</sup>: sP-selectin (soluble P-selectin in plasma) is not platelet-specific.  <sup>‡</sup>: Mepacrine (quinacrine) is a reagent incorporated into the <math>\delta</math>-granules like 5-HT.</p>

**Table 2: Types of tests used for the assessment of platelet secretion.**

Method	Measurement	Comments
<sup>14</sup> C- or <sup>3</sup> H-5-HT uptake and release from platelets	Fraction of 5-HT secreted by platelets	Current "gold standard". Measures % 5-HT release, not content. Radio-isotopic. Fast, simultaneous aggregation measurement.
Fluorometric detection of o-phthalaldehyde (OPT)-derivatized 5-HT	Platelet total and secreted 5-HT	Fluorometric assay. Not automated. High sensitivity.
5-HT ELISA	Platelet total and secreted 5-HT	High sensitivity. Needs validation.
5-HT Immuno-fluorescence by FC	5-HT in permeabilised platelets	Use anti-5-HT antibodies. Not quantitative. Not validated.
HPLC-Fluorometric or ED Detection	Platelet total and secreted 5-HT	Needs experienced operators. Highly sensitive, specific, accurate and precise.
LC-MS	Platelet total and secreted 5-HT	Could be used as reference standard.

**Table 3: Methods for measuring platelet 5-HT secretion and content.**

and equilibrated with the endogenous 5-HT of  $\delta$ -granules (53, 54). Platelets in PRP are then activated with routine agonists at concentrations similar or higher than those recommended for LTA (3). Then, the <sup>14</sup>C released into the plasma is counted and the released fraction is expressed as a percentage of the total <sup>14</sup>C taken up by platelets. This assay is fast, cheap, quantitative and allows simultaneous measurement of platelet aggregation and  $\delta$ -granule secre-

tion in the same reaction cuvette. One study reported intra-assay correlation coefficients of > 80% for weak agonists and coefficients of variation < 20% for strong agonists, denoting high reproducibility (15). Patients treated with selective serotonin reuptake inhibitors have pronounced decreases in both platelet content and uptake of <sup>14</sup>C-5-HT, but this does not affect significantly the result of the assay since the secreted <sup>14</sup>C is expressed as a proportion of

**Table 4: Methods for measuring platelet adenine nucleotides.**

Method	Measurement	Comments
Luminometry	ATP, ADP	Quantitative, high sensitivity, fast assay.
PRP Lumiaggregometry	ATP	Quantitative, measures secreted and total (in lysed platelets) ATP; measures simultaneously platelet aggregation, fast results, approved for diagnostic use.
Whole blood, impedance lumiaggregometry	ATP	Quantitative, small sample volume, fast processing, not validated test, influenced by platelet count.
HPLC-Fluorometric detection	ATP, ADP, AMP	Needs experienced operators. Highly sensitive, specific, accurate and precise. Slow response. Expensive instrumentation. Employs reference standard.

the total platelet uptake (unpublished observation). The  $^{14}\text{C}$ -5-HT release assay, however, does not allow measurement of the total 5-HT content of platelets, and so is unable to distinguish between defects in  $\delta$ -granule biogenesis or content and defects in the secretion mechanism.

Although the  $^{14}\text{C}$ -5-HT assay is still considered the “gold standard” test for the evaluation of platelet  $\delta$ -granule secretion (55), the requirement for a radioisotope now limits its use in clinical laboratories. An attractive alternative to  $^{14}\text{C}$ -5-HT is *o*-phthalaldehyde (OPT) that forms a fluorophore with 5-HT (56, 57), and which can be detected at pmol concentrations by fluorimetry (29, 58). Although the OPT assay is non-isotopic and low cost, the methodology requires a strong acid medium at boiling temperature and is not currently available in automated form.

Another indirect marker of 5-HT content is the green fluorescent dye mepacrine (quinacrine), which utilises the same SERT transporter as 5-HT and may be used as a lumen marker of  $\delta$ -granules (30). Platelet mepacrine staining and release after agonist stimulation can be detected by flow cytometry and has been used as surrogate marker of platelet  $\delta$ -granules release because it avoids the use of radioisotopes (59, 60). However, this assay has also several disadvantages, including rapid disappearance of fluorescence after platelet staining, variable background staining due to retention of excess mepacrine in the platelet cytoplasm and a semi-quantitative endpoint. To our knowledge, validation of this test for clinical use has not been published.

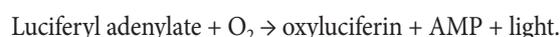
Alternative methods to measure 5-HT release are now available and may be sufficiently sensitive for the diagnosis of IPSDs. These include highly sensitive ELISAs, for which there are at least ten different commercially available kits that utilise poly- or mono-clonal antibodies against 5-HT. To the best of our knowledge there are no direct comparison studies between the ELISA and the gold-standard  $^{14}\text{C}$ -5-HT test. However, ELISA was inferior to HPLC in the measurement of 5-HT release in patients with heparin-induced thrombocytopenia (61), and there was poor agreement between ELISA and LC-tandem mass spectrometry (LC-MS), particularly in the high 5-HT concentration ranges found in platelet releasates (62). ELISA was utilised by only 1/202 laboratories to measure  $\delta$ -granule release in the ISTH survey (4). Before wider adoption of 5-HT ELISA, the development of more rapid and cost effective methods suitable for the study of platelet secretion induced by multiple platelet agonists, and the completion of diagnostic accuracy studies against other quantitative tests are required. The anti-

bodies against 5-HT developed for ELISA may be used for alternative tests, such as immunofluorescence microscopy and flow cytometry (63–65), although these tests have not yet been validated.

Several HPLC methods for measuring 5-HT have been reported, including HPLC coupled to electrochemical (66–68) or fluorescence (69–71) detection systems. LC-MS enables ultrasensitive measurement of 5-HT which, when combined with internal standards (62), is an attractive reference method for other techniques. HPLC and LC-MS offer high selectivity, sensitivity, and precision for 5-HT measurement (62, 66, 70) and have rapid turnaround and low ongoing costs, but require expensive hardware and experienced operators. Both tests enable quantitation of total 5-HT content and the secreted fraction after agonist stimulation, required for the diagnosis of  $\delta$ -storage pool disease. HPLC and LC-MS were available for diagnostic use in only 10/202 (5%) of respondents to the ISTH survey.

## 2. ADP-ATP secretion from $\delta$ -granules

Platelets contain approximately 5–6  $\mu\text{mol}$  ATP per  $10^{11}$  platelets and 3.0–3.5  $\mu\text{mol}$  ADP per  $10^{11}$  platelets (ratio of ATP/ADP 1.5–2.0) (72). Approximately one third of the total platelet ADP/ATP is in a metabolically active cytoplasmic pool, comprising mostly ATP. The remaining two-thirds are stored in  $\delta$ -granules, where the ATP/ADP ratio is 0.65–0.78. (73) Therefore, more ADP than ATP is normally released from platelets during  $\delta$ -granule secretion (73). ATP/ADP ratios  $>2$  (usually  $>4$ ) in the releasate denote decreased  $\delta$ -granules content, and support a diagnosis of  $\delta$ -storage pool disease (47). There are several methods available to measure ATP and ADP (► Table 4). Amongst them, the most widely used are based on a bioluminescent reaction catalyzed by firefly luciferase:



This reaction may be utilised in a simple two-step luminescence assay in which the whole platelet ATP content, comprising the  $\delta$ -granule and metabolic pools, are first measured in platelet lysates using a luciferase assay. The ADP derived from  $\delta$ -granules is then converted to ATP by a phosphoenolpyruvate–pyruvate kinase reaction before re-measuring ATP to enable calculation of platelet

ADP by subtraction. A similar approach can be used to measure the ATP content in the supernatant of washed or gel-filtered platelets after stimulation. The luminescence assay can also be adapted to measure the nucleotide release in PRP by fast platelet fixation with EDTA-formaldehyde after the release reaction, to inactivate plasma ATP- and ADP-ases. This degradation can be also prevented by the presence of 5 mM EDTA in the PRP (72). Single endpoint luminescence assays were used by 16/97 of the laboratories that quantified platelet adenine nucleotide content or release in the ISTH survey.

The lumiaggregometry assay, which also utilises luciferase and was developed more than three decades ago (74) is largely used as it was performed by 74% of the respondents in the ISTH survey. For 43% of respondents lumiaggregometry was a first-line test (4). This assay allows the continuous quantitative measurement of ATP release after agonist stimulation by using an ATP standard, and allows the simultaneous measurement of ATP release and aggregation in PRP (75, 76). However, lumiaggregometry does not distinguish failures of the secretion mechanisms from defects in  $\delta$ -granule content, although an estimate of the latter may be obtained by the measurement of ATP in platelet lysates. This test uses expensive reagents and intra- and inter-individual variability in ATP release is high, particularly when low concentrations of weak agonists are used (76, 77). For example, one study demonstrated that the measurement of  $\delta$ -granule release in response to 5  $\mu$ M ADP was not informative because the normal reference interval included 0% ATP release (76). One further recent study found wide reference intervals of ATP released in response to 4 and 20  $\mu$ M ADP in healthy controls and an abnormally high rate of diagnoses of  $\delta$ -granule secretion defects using ADP as a stimulus (77). Moreover, one study found enhanced platelet aggregation to some agonists in tests performed by lumiaggregometry compared to LTA. This was attributed to the presence of  $Mg^{++}$  in the lumiaggregometry reagents (78), but this observation was not confirmed in a later study (79). A similar assay that measures aggregation by electrical impedance and ATP release by bioluminescence has been utilised to measure platelet  $\delta$ -granule release in whole blood samples (80). Although this offers theoretical advantages for diagnostic testing, this assay is used in very few laboratories worldwide, and has not been validated for clinical use.

ADP and ATP content and release can also be measured by HPLC, usually with fluorescence detection methods (81, 82). HPLC is highly sensitive, selective and precise, readily distinguishes ATP from ADP enabling greater accuracy for diagnosis of  $\delta$ -granule defects, and reagent and running costs are low. In common with other diagnostic tests using HPLC, hardware costs are high and require skilled operators. Only 6/97 (6%) laboratories utilised HPLC tests to evaluate platelet secretion.

Whole mount transmission EM allows visualising directly the platelet  $\delta$ -granules, and enables a qualitative or semi-quantitative measurement of  $\delta$ -granule number, but not release. This apparently simple technique requires placement of PRP on grids, which are then washed with sterilised water, air-dried and examined by electron microscopy without fixation or staining (45, 83, 84). In the ISTH survey, 57/202 (28%) of the responding laboratories

quantified  $\delta$ -granules by EM, although in almost all cases as part of a broader assessment of platelet ultrastructure. Release of  $\delta$ -granule polyphosphates by activated platelets represents an attractive target for evaluating  $\delta$ -granule granule secretion, since semi-quantitative measurement of this marker in PAGE gels using the fluorescent dye 4',6-diamidino-2-phenylindol (DAPI) is highly sensitive and could be adapted to measure poly-P in platelet releasates (85).

### 3. Alpha granule secretion

The soluble platelet  $\alpha$ -granule proteins PF4 and  $\beta$ -TG are secreted during platelet activation and were initially measured by radioimmunoassay (86), later replaced by ELISA (87, 88), to evaluate  $\alpha$ -granule release from platelets after agonist stimulation. Since PF4 and  $\beta$ -TG are platelet-specific, they offer greater specificity than other soluble  $\alpha$ -granule proteins such as soluble P-selectin (sPS), which is mostly derived by cleavage from platelet membranes, but also from endothelial cells (89).

Several  $\alpha$ -granule membrane proteins fuse with the platelet plasma membrane and remain exposed there after granule release, and may be used as potential markers of  $\alpha$ -granule secretion (90). The most widely adopted of these is P-selectin which may be measured in non-stimulated platelets as a marker of baseline platelet activation, or after stimulation to measure  $\alpha$ -granule release. The measurement by flow cytometry of platelet surface P-selectin is fast and has low reagent costs, but requires experienced operators, and stringent controls and data analysis. However, adding a fixative to the samples can stabilise them for shipping, thereby allowing flow cytometric analysis at a remote site eliminating the requirement for expensive equipment, as has been done in multi-centred clinical trials of anti-platelet agents (91, 92). In contrast to soluble P-selectin, which is in part derived from other tissues, P-selectin exposed on the platelet surface is a specific platelet-activation marker since it is only derived from platelet granules. Currently, platelet P-selectin is considered a specific  $\alpha$ -granule membrane protein. However, there is also good evidence from an early study this adhesive glycoprotein also localises in  $\delta$ -granules membranes (93). Measurement of platelet surface P-selectin is usually performed as a semi-quantitative test, in which the proportions of P-selectin positive platelets are compared before and after agonist stimulation (94, 95). Recently, remote analysis of platelet surface P-selectin (and CD63, also a marker of  $\delta$ -granules and lysosomes) by flow cytometry has been evaluated as a screen for potential platelet secretion defects and showed good agreement with lumiaggregometry for the identification of platelet function defects (96). Quantitative measurement of platelet  $\alpha$ -granule P-selectin for diagnosis of secretion defects is seldom performed, even in specialised laboratories.

Coagulation factors that are stored within  $\alpha$ -granules (► Table 1) are also a potential target for measurement of granule release. These include platelet FV, that is either endocytosed (97, 98) or synthesised (99) by megakaryocytes, and which may be the primary source of FV for haemostasis (100, 101). Platelet secretion of FV can be measured by prothrombinase or thrombin generation

assays performed in PRP or washed platelet suspensions. TLT-1, a protein of the TREM family, is expressed exclusively in platelet  $\alpha$ -granules and is up-regulated during inflammation. The soluble form (sTLT-1), which may be measured by immunoblotting, increases in sepsis and may enhance actin polymerisation, platelet aggregation and adherence to the endothelium (102).

Alpha-granule proteins include also growth factors, cytokines, proteins that stimulate and inhibit angiogenesis, inflammatory and innate immunity proteins (103), etc. Mostly based on empirical observations, this vast body of heterogeneous proteins is being currently exploited for clinical use in wound healing, plastic and orthopaedic surgeries and dental interventions. The overall results are far from conclusive and this procedure does not seem to have solid scientific support yet (104–106).

Alternative approaches to assess the  $\alpha$ -granule secretion of specific or non-specific proteins, include protein functional assays, ELISAs, flow cytometry, immunofluorescence or confocal microscopy, immunoblotting assays and transmission and immune-EM (► Table 5). These have been developed principally as research tools and are not discussed further.

#### 4. Release of platelet lysosome contents

Strong platelet agonists, such as thrombin, induce the secretion of lysosomal enzymes and the expression of lysosome-associated proteins on the platelet surface (107). Amongst the soluble lysosome enzymes, cathepsin D,  $\beta$  galactosidase and  $\beta$ -N-acetylhexosaminidase can be measured by enzymatic assays (31, 107, 108). Alternatively, lysosomal membrane protein such as LAMP-1 and LAMP-2, can be detected on the platelet surface by flow cytometry after agonist activation. Surface exposure of LAMP-1 (CD63) and LAMP-2 have been used to evaluate the quality of platelet concentrates in blood banks (109). However, these proteins are not completely lysosomal-specific, as they are also found in the membranes of  $\delta$ -granules and are decreased or absent in HPS (110, 111).

### Diagnosis of some disorders of platelet secretion associated with other complex inherited defects

Although the laboratory tests described above offer reliable diagnosis of most disorders of  $\alpha$ - and  $\delta$ -granule release, they have also diagnostic value in complex genetic disorders in which platelet release defects are part of wider multi-system syndromic disorders.

#### Hermansky-Pudlak Syndrome (HPS)

HPS is a group of recessive multisystem disorders associated to date with defects in nine human genes that encode subunits of the protein complexes BLOC-1, BLOC-2 and BLOC-3 (biogenesis of lysosome-related organelle complexes) or protein-3 complex, AP3 (112). These defects lead to abnormal biogenesis of lysosome-re-

**Table 5: Methods for measuring  $\alpha$ -granule secretion.**

<b>1. Measurements of <math>\alpha</math>-granular soluble proteins:</b>
A. Quantitative assays: in platelet lysates and releasates
<ul style="list-style-type: none"> <li>• Specific <math>\alpha</math>-granule components: ELISA for PF4 and <math>\beta</math>-TG</li> <li>• Non-specific <math>\alpha</math>-granule components: many other <math>\alpha</math>-granule proteins</li> </ul>
B. Semiquantitative assays for multiple $\alpha$ -granular proteins:
<ul style="list-style-type: none"> <li>• IF, Confocal Microscopy, FC of permeabilised platelets, IP, WB, Immuno-EM</li> </ul>
C. Functional testing of secreted proteins:
<ul style="list-style-type: none"> <li>• Angiostatin, PDGF, TGF-<math>\beta</math>, PAI-1, TAFI, Factor V</li> </ul>
<b>2. Measurements of membrane proteins of <math>\alpha</math>-granules by quantitative or semi-quantitative assays in resting or activated platelets:</b>
<ul style="list-style-type: none"> <li>• P-selectin (CD62P): FC; plasma sP-selectin: ELISA</li> <li>• CD40L (CD154): flow cytometry; plasma sCD40: ELISA</li> <li>• TLR9: FC, Confocal Microscopy, apparently located in T platelet granules</li> </ul>

lated organelles, which include  $\delta$ -granules of platelets, but also melanosomes in melanocytes and granules in cytotoxic T and natural killer (NK)-lymphocytes. Failure of synthesis of these lysosome related organelles results in several clinical features that invariably include oculocutaneous albinism or dyspigmentation and mild bleeding caused by absent synthesis of platelet  $\delta$ -granules. Distinguishing HPS from other genetic causes of oculocutaneous albinism currently requires demonstration of absent platelet  $\delta$ -granules using the assays described above.

#### Chediak-Higashi Syndrome (CHS)

CHS is also a rare autosomal recessive disorder affecting the vesicle trafficking and biogenesis of lysosome-related organelles (112), but with the additional feature of giant granules in myeloid cells that is pathognomonic of this disorder. In common with HPS, CHS is associated with absent or reduced numbers of platelet  $\delta$ -granules that may manifest as a mild bleeding disorder. However, other clinical features are usually more prominent and include hypopigmentation, neurological dysfunction and immunodeficiency from neutropenia, impaired leukocyte migration and decreased NK lymphocyte function. CHS is caused by loss of function defects in the *LYST* gene, which encodes the BEACH domain lysosomal trafficking protein *LYST* (113, 114).

#### Gray Platelet Syndrome (GPS)

Patients with GPS display some heterogeneous phenotypes, but a hallmark of the defect is the deficiency of specific  $\alpha$ -granule proteins, such as PF4 and  $\beta$ -TG. However, the evaluation of patients should include analysis of platelet number, morphology and size (21). Also, molecular testing can be used for diagnosis of GPS, since recent reports have recognised causal genetic defects in *NBEAL2* (115–117) and *GFI1B* (118, 119) in this disorder. P-selectin may be present in the membranes of  $\alpha$ -granules of GPS

platelets when determined by immune-electron microscopy (IEM) or immunofluorescence-confocal microscopy (120), and even may translocate normally to the external membrane after platelet stimulation, as demonstrated by flow cytometry (90).

### Quebec Platelet Disorder (QPD)

QPD is a rare  $\alpha$ -granule disorder in which there is a ten-fold increase in platelet expression of urokinase-type plasminogen activator (uPA) mRNA and protein, caused by a tandem duplication mutation of the uPA gene (*UPLA*). This leads to increased intra-platelet plasmin generation and degradation of  $\alpha$ -granule proteins such as factor V, multimerin 1, thrombospondin 1, von Willebrand factor, fibrinogen, fibronectin, osteonectin, and P-selectin. The increase in secreted uPA also accelerates the lysis of clots formed in the vicinity of platelets, thus leading to bleeding. The  $\alpha$ -granule number and secretion are typically normal in QPD, so diagnosis requires the measurement of total uPA and platelet uPA activity alongside with detection of the causal *UPLA* gene defect (121).

### Future developments

Next generation sequencing (NGS) technology to identify causal genetic variants is used increasingly as a diagnostic tool for heritable disorders. NGS has largely replaced previous approaches such linkage analysis studies, which need large informative pedigrees and candidate gene sequencing that are time consuming and require precise phenotypic description of affected cases to select a gene target. The NGS analysis of platelet gene panels, whole exomes or whole genomes promises to advance the diagnosis of platelet disorders, including IPSPD, by the rapid identification of known genetic defects and through new gene discovery. However, rigorous data analysis and statistical genetic approaches are required to distinguish single causal variants for IPSPDs from bystander genetic variations (122). Analysis of the entire platelet proteome (17, 18, 123), the platelet „secretome“ releasate and, possibly, platelet „lipidomics“ are alternatives or complements to genomics that are emerging as promising tools to improve diagnosis (19, 124). However, it should be emphasised that although ‘-omics’ approaches offers great promise for the diagnosis of IPSPDs, selection of candidate genes, proteins or lipids can only be successful after precise definition of the platelet phenotype using the laboratory tests described in this review.

### Conclusions

Most laboratory tests of platelet granule release used in the diagnosis of IPSPDs were developed more than 30 years ago while newer methods are scarce and insufficiently validated for clinical practice. The  $^{14}\text{C}$ -5-HT release assay remains the „gold standard“ test of platelet  $\delta$ -granule release, but it is unsuitable for clinical laboratories because it requires use of a radioisotope. The luciferase-

based lumiaggregometry test is performed more widely in clinical laboratories despite its several limitations.

Quantitative tests such as HPLC-based assays and chemical-fluorometric tests, and luminescence methods to quantify ADP-ATP are highly specific and sensitive, but are currently of limited practical use for their complexity and cost. However, these methods are invaluable to calibrate and validate newly developed assays for 5-HT and ADP-ATP measurement.

The current preferred test to detect defects in  $\alpha$ -granule release is semi-quantitative measurement of agonist-induced P-selectin exposure on the platelet surface using flow cytometry. However, it may be insensitive for some disorders. Finally, the measurements of lysosome contents and release are currently performed in few laboratories and have an unknown role for the diagnosis of IPSPD.

The recent ISTH survey on platelet diagnostic practice (4) shows that there is poor homogeneity amongst diagnostic laboratories in assays employed, hardware, reagents and interpretation of test results. This has created uncertainty about the minimum laboratory criteria necessary for diagnosis of IPSPDs. Therefore, there is a major need for studies to evaluate objectively new methodologies for detection and characterisation of platelet secretion defects and for expert recommendations for standardisation of the available tests.

### Acknowledgment

All the authors are the current members of the Platelet Subcommittee on Platelet Physiology of the International Society on Thrombosis and Haemostasis (ISTH).

### Author contributions

ADM edited, provided original ideas and approved the final version of the manuscript. ALF contributed with valuable ideas, corrected, approved the final version of the manuscript and designed and made the ► Figure 1. CG and PN reviewed, introduced important ideas to improve the manuscript and approved its final version. PH and PG provided the input to trigger this project and followed closely its advance providing new ideas and approving the final version of the manuscript. DM wrote the first draft, coordinated the whole project and approved all of the consecutive corrections and improvements.

### Conflicts of interest

None declared.

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