Current status and future prospects for platelet function testing in the diagnosis of inherited bleeding disorders

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Summary
Platelets play a crucial role in haemostasis by preventing bleeding at the site of vascular injury. Several defects in platelet morphology and function have been identified and described over the years. Although a range of methodologies is available to assess platelet function, a significant proportion of subjects with bleeding symptoms and normal coagulation parameters still appear to have normal results on platelet function testing. This might suggest that the reason for bleeding is multifactorial and is due to a combination of several minor defects in platelet function and/or other parts of the haemostatic system or might indicate that the currently available platelet function tests do not provide optimal diagnostic power. This review will summarize the established platelet function tests used for diagnosing inherited platelet abnormalities in adults and children, and discuss the newly developed methodologies as well as unmet challenges and potential areas for further improvement in this field.

Keywords: Platelet disorders, platelet function, platelet activation, platelet aggregation, flow cytometry.

Role of platelets in haemostasis
Platelets play a pivotal role in haemostasis by ensuring the integrity of the vessel wall and initiating primary haemostatic mechanisms when this integrity is compromised. Platelets respond to the endothelial damage by adhering to subendothelial collagen and von Willebrand factor (VWF) via corresponding glycoprotein (GP) receptors on the platelet surface, GPVI, GPIa/IIa and GPIb-IX-V. This initial process of platelet adhesion leads to a series of downstream signalling events, which transform the platelet into its activated state. The latter is characterized by the reorganization of cytoskeleton and platelet shape change to increase the surface area; by the release of platelet granule contents, thromboxane A2 (TxA2) generation, change in GPIIb/IIIa conformation into its high affinity state to bind fibrinogen and VWF and, finally, by the exposure of procoagulant phosphatidylserine (PS) on the platelet surface. Platelet alpha and dense granules contain a range of compounds, which potentiate further platelet activation and platelet plug formation at the site of vascular damage. These include adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin (5HT), catecholamines and calcium in dense granules, and platelet factor 4, VWF, coagulation factor V and fibrinogen in alpha granules. The release of the latter also leads to the exposure of P-selectin on the surface of platelets, which mediates platelet interaction with leucocytes. As a result of the release of all these compounds, more platelets are activated and recruited and a stable platelet aggregate is formed via the binding of fibrinogen to GPIIb/IIIa on adjacent platelets. According to the scope of platelet physiological response a variety of platelet abnormalities have been described that affect different aspects of platelet function (Fig 1).

Abnormalities in platelet function
Inherited platelet function disorders (PFDs) include defects of adhesive receptors and receptors to soluble agonists; granule defects, which include granules deficiency and defective release process (storage pool and primary secretion defects, respectively); and defective cytoskeleton and procoagulant function (Cattaneo, 2003; Jurk & Kehrel, 2007). All these abnormalities are associated with various bleeding severity and prevalence, although the latter is mostly low (Table I). Well-defined disorders, such as Bernard–Soulier syndrome, are very rare, at one in a million, and present with moderate to severe bleeding (Cattaneo, 2013), however, the prevalence of mild bleeding disorders is significantly higher, at 1 in 10 000, and might be underestimated due to complexity and challenges in existing diagnostic procedures (Bolton-Maggs et al, 2006; Watson et al, 2013).

Glanzmann thrombasthenia and Bernard-Soulier syndrome, which are characterized by mutations in glycoprotein

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receptors on platelet membrane, GPIb/IIa and GPIb-IX-V, respectively, are usually identified early in life and are fairly easy to diagnose in clinical laboratories (Cox et al., 2011). Other milder defects of platelet function, such as secretion defects, are more prevalent (Cattaneo, 2003), but some of them are less well understood and thus are more difficult to diagnose, even after extensive laboratory testing that is available only in specialized laboratories (Watson et al., 2013). Additionally, in a significant proportion of patients (40–50%) who present with mild bleeding symptoms characteristic of defects in primary haemostasis and normal coagulation parameters, no platelet defect can be identified using existing platelet function techniques (Quiroga et al., 2007; Dawood et al., 2012).

The severity of bleeding phenotype varies even within one type of PFD and often does not correlate with the severity of platelet function abnormality identified with platelet testing in vitro (Lowe et al., 2013a). It was suggested that a mild defect in platelet function as well as a mild defect in coagulation parameters could act as risk factors, which contribute to bleeding phenotype only when combined together or perhaps with other, yet unidentified, factors (Jurk & Kehrel, 2007; Watson et al., 2013). This could raise a question on the clinical significance of platelet function testing in identifying PFDs, however, every attempt should be made to characterize the underlying defect in subjects who present with bleeding to manage these cases appropriately and prevent bleeding episodes during predictable challenges, such as during surgery or childbirth. Due to the diversity and lack of clear definitions of some PFDs, the diagnostic approach is complex and, most importantly, starts with the careful assessment of the bleeding history, as indicated in previously published suggested diagnostic algorithms (Bolton-Maggs et al., 2006; Gresele et al., 2014a). This review will not address...
the overall diagnostic approach, rather it will summarize most available methods to assess platelet function, discuss their advantages and limitations as well as describe new developments and future prospects of platelet function testing.

**Assessment of platelet number and size**

The peripheral blood platelet count should be evaluated before any functional studies can be performed; low platelet count can itself be the underlying cause of bleeding and would affect the results of all further platelet function tests apart from flow cytometry. Standard automated whole blood counters provide a platelet count based on impedance sizing, which might generate somewhat incorrect results in the case of an abnormal platelet size distribution. For example, in Bernard–Soulier syndrome or MYH9-related disorders (e.g. May–Hegglin anomaly) thrombocytopenia might be overestimated due to large platelets not being correctly distinguished from small or fragmented red cells, while small size platelets

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<td>Reduced secretion and in most cases reduced aggregation responses to several agonists; deficiency of δ-granules on EM</td>
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<td>Moderate</td>
<td>Decreased</td>
<td>Large platelets</td>
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GP, glycoprotein; VWD, von Willebrand disease; VWF, von Willebrand factor; PS, phosphatidylserine; TxA2, thromboxane A2; EM, electron microscopy.
in subjects with Wiskott-Aldrich syndrome could not be well distinguished from the cell debris. Mean platelet volume (MPV) and platelet distribution width (PDW) itself represent important diagnostic parameters, as several platelet defects are characterized by abnormal, mostly increased, platelet size.

Recently, commercial analysers were complemented with light scatter and specific fluorescence parameters to improve the sensitivity of the platelet number assessment. The new analysers also offer the detection of reticulated platelets – immature platelet fraction (IPF), which usually represents the efficiency of thrombopoiesis (Abe et al, 2006) and can be useful in diagnosis and monitoring of patients with thrombocytopenia due to elevated platelet consumption (thrombotic thrombocytopenic purpura) or destruction (autoimmune thrombocytopenia) to predict platelet recovery and hence guide the decisions on prophylactic platelet transfusion.

An alternative method to assess platelet number, which preserves the sensitivity regardless of the platelet size, is immunological counting with flow cytometry (Harrison et al, 2000), which was suggested to be a reference method to assess platelet number (International Council for Standardization in Haematology Expert Panel on Cytometry; International Society of Laboratory Hematology Task Force on Platelet Counting, 2001). Alongside platelet count, it is recommended that platelet morphology is assessed early in the diagnostic workup using blood smears (Gresele et al, 2014a). Once the peripheral blood platelet count is confirmed to be within the normal range (150 – 400 × 10⁹/l), platelet function is then assessed in vitro using available techniques, which could be classified as global tests and specialized assays.

**Global screening tests for PFDs**

Global screening tests should be easy to perform and relatively inexpensive. In theory, they should be helpful in distinguishing between von Willebrand disease (VWD, abnormalities associated with VWF and thus impaired early platelet adhesion) and other specific defects in platelet function; or to exclude a particular platelet abnormality before more detailed testing is performed. However, the two available screening tests, template bleeding time and closure time in the platelet function analyser (PFA-100) were shown to lack the required sensitivity and specificity to detect platelet defects (Podda et al, 2007).

Template bleeding time is the oldest test of platelet function (Duke, 1910) and is, surprisingly, still used quite widely (Jennings et al, 2008; Gresele et al, 2014b), despite being an invasive test with high interoperator variability, poor reproducibility and dependent on several non-haemostatic variables (e.g. skin thickness, age, haematocrit). Accordingly, in two up-to-date guidelines it was recommended not to perform template bleeding time as a screening test for defects in primary haemostasis (Harrison et al, 2011; Gresele et al, 2014a).

An alternative method that was considered to be an *in vitro* replacement of the bleeding time was introduced by Kratzel and Born (1985) and was further developed into a PFA-100 instrument (Kundu et al, 1995). This instrument utilizes a small volume of whole blood (800 µl), which is aspirated at high sheer rates through the membrane coated with collagen and epinephrine (CEPI) or collagen and ADP (CASP) with a small aperture, which becomes occluded as the platelets become activated and adhere on the membrane. The instrument records the time to complete interruption of the blood flow – closure time (CT), which can reach a maximum of 5 min, above this time the result would be reported as >300 s (Kundu et al, 1995).

Technically the PFA-100 is well suited as a screening test, given that it is fairly easy and quick to perform, is non-invasive and does not require specialized technical expertise. Additionally, it uses only a small volume of blood sample, which would make it suitable for paediatric patients, as CTs in children were similar to adults and were somewhat shorter in neonates due to higher haematocrit and VWF levels (Carcao et al, 1998; Israels et al, 2001). However, numerous studies that assessed the PFA-100 as a diagnostic and screening tool showed that the method lacks the required sensitivity and specificity, especially to mild platelet defects (reviewed in Harrison, 2005; Hayward et al, 2006). PFA-100 did demonstrate overall better sensitivity to VWD than the bleeding time (Fressinaud et al, 1998; Cattaneo et al, 1999) and CTs were consistently prolonged in relatively severe platelet defects, such as Bernard-Soulier syndrome and Glanzmann thrombasthenia (Harrison et al, 1999, 2002). However, the sensitivity of the test is insufficient in milder platelet function defects, such as secretion defects (Cattaneo et al, 1999; Quiroga et al, 2004), which represent a significant proportion of platelet abnormalities (Cattaneo, 2003; Dawood et al, 2012).

Finally, it should be noted that, similarly to bleeding time, the performance of PFA-100 test is affected by haematocrit and platelet count (Kundu et al, 1995; Harrison et al, 1999) as well as by several pre-analytical variables, such as the choice of anticoagulant and time between specimen collection and analysis (Heilmann et al, 1997). Although the PFA-100 is still used quite widely in the diagnosis of platelet abnormalities (Gresele et al, 2014b), it was recommended not to use the test as a screening tool due to the analytical characteristics and insufficient sensitivity of the test (Gresele et al, 2014a).

Recently, a new cartridge with improved sensitivity to P2Y₁₂ receptor function (INNOVANCE® PFA P2Y*) was introduced and was shown to detect abnormal platelet responses in patients with P2Y₁₂ receptor defects (Scavone et al, 2014), which suggests that it should perhaps be evaluated in a wider population of patients with bleeding disorders to see if this new formulation improves the performance of PFA-100 as a screening tool.
Specialized assays for platelet function

Platelet aggregation in whole blood

Performing platelet aggregation in whole blood offers the advantage of assessing platelet function in a more physiological milieu than platelet-rich plasma (PRP) and avoids variables associated with PRP preparations. One of the widespread and now commercially available methods to measure platelet aggregation in whole blood is Multiple Electrode Aggregometry (MEA, Multiplate®, Roche Diagnostics International Ltd, Rotkreuz, Switzerland), which is an advanced and simplified version of the original impedance aggregometer (Cardinal & Flower, 1980) that utilizes disposable cuvettes and thus disposable electrodes (Tóth et al, 2006). The method monitors the change in impedance between two electrodes immersed in whole blood as stimulated platelets adhere to and aggregate on these electrodes. The method has been used extensively to assess platelet inhibition by antplatelet agents, however, to date only two studies were conducted to assess the performance of Multiplate® in detecting inherited platelet defects. The test was shown to identify defective platelet aggregation in patients with Glanzmann thrombasthenia, similarly to light transmission aggregometry defective platelet aggregation in patients with Glanzmann inherited platelet defects. The test was shown to be capable of identifying other platelet function defects not yet tested yet. Although this test is relatively easy to use and standardized it is quite unlikely to become widely adopted for diagnosing PFDs as it lacks the kinetic information provided by light transmission aggregometry (LTA) – the aggregation curves on Multiplate® do not depict two phase response and platelet disaggregation (Tóth et al, 2006). Therefore the test might not be able to identify mild platelet abnormalities, although this should be evaluated in a separate clinical study.

Platelet aggregation in platelet-rich plasma

Light transmission aggregometry (LTA) is considered the ‘gold standard’ method for assessing platelet function. Although this method was introduced originally in the early 60s (Born, 1962), it is still used essentially in the same way, the only improvement being the development of several new aggregometers that are smaller, have digitized output of aggregation measurement and sometimes semi-automated sample processing [AggRAM® (Helena Biosciences Europe, Gateshead, UK); Chrono-Log (Chrono-Log Corporation, Havertown, PA, USA); PAP-8E (Bio/Data Corporation, Horsham, PA, USA)].

The utility of LTA in diagnosing platelet function defects has been assessed in several studies (Hayward et al, 2009; Dawood et al, 2012) and, indeed, it is currently the most widely used diagnostic test for PFDs (Gresele et al, 2014b). It is based on the assessment of the increase in light transmission through PRP as platelets are stimulated, stirred and aggregate together. LTA is the only method that can register a two-phase aggregation response with initial platelet aggregation induced by the added agonist and the second phase of aggregation by self-potentiation due to the release of dense granules and TxA2 generation. Accordingly, if the amplification mechanisms are compromised, e.g. when there is a defect in ADP P2Y12 receptor or TxA2 receptor or synthesis, the platelet aggregation is not sustained and the method shows subsequent platelet disaggregation as a decrease in light transmission after the initial increase. This dynamic information is perhaps the most useful characteristic of LTA, which is not available with any other aggregation technique.

The optimal number of agonists and the range of concentrations that should be used in order to identify platelet defect most efficiently have been debated. Traditionally, quite a wide range of concentrations of several platelet agonists would be used, however, this increases the volume of blood required to do the testing and the time that is needed to complete it. Therefore several attempts at optimization of LTA testing have been made and, in the study by Hayward et al (2009), it was concluded that the use of five agonists – ADP, arachidonic acid (AA), collagen, epinephrine and TxA2 analogue U46619 – each used at a single concentration provides the optimal diagnostic power for detecting PFDs.

Similar conclusions were made when the diagnostic potential of LTA in combination with ATP secretion measurement using luminescence was assessed within the UK-based Genotyping and Platelet Phenotyping (GAPP) study (ISRCTN 77951167), which included subjects with mild bleeding in whom well-defined platelet defects were excluded. It was shown that using the data on aggregation and secretion with six agonists – ADP, AA, collagen, epinephrine, PAR1 agonist and ristocetin at one optimal concentration – there was 90% agreement (kappa = 0.829) with the diagnosis made with the extended panel of agonists at several concentrations (Dawood et al, 2012). The latest International Society on Thrombosis and Haemostasis Scientific and Standardization Committee guidelines for the diagnosis of inherited PFDs recommended that the LTA should be performed with five agonists (ADP, AA, collagen, epinephrine and ristocetin) as a first line testing and if the results suggest an abnormality in platelet responses the panel of agonists should be expanded within the second step of testing to include α-thrombin, PAR1 agonist, U46619, collagen-related peptide (CRP), convulxin, PAR4 agonist, phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23287 (Gresele et al, 2014a). Thrombin can be used only in the presence of glyceryl-L-prolyl-L-arginyl-L-proline (GPRP) to prevent fibrin polymerization, or specific PAR1 and PAR4 receptor agonists could be used instead.

Although informative, the whole LTA procedure involves multiple steps from sample handling and PRP preparation to the actual aggregation measurements, which leads to significant variability in performance of LTA between different laboratories (Jennings et al, 2008; Cattaneo et al, 2009) and
reduces the diagnostic value of the test as the results become incomparable. Recommendations on the standardization of the LTA procedure were recently issued (Cattaneo et al., 2013), which aim to improve reproducibility and comparability of the LTA. Among the most important recommendations is the completion of LTA within a maximum of 4 h after blood sampling and the requirement to perform concurrent testing on the control blood sample obtained from a known healthy donor.

Similarly to most other platelet function tests, LTA provides reliable measurements only when platelet count in PRP is within the limited range – generally it is accepted that the test can be performed if the platelet count in PRP is between 150 and 600 × 10^9/l (Harrison et al., 2011). Previously platelet count used to be standardized to around 250–300 × 10^9/l with autologous platelet-poor plasma (PPP), however it was shown that dilution with PPP can artefactually decrease platelet aggregation responses (Cattaneo et al., 2007; Linne mann et al., 2008), and although in some studies the adjustment of platelet count did not affect the results of LTA testing (Hayward et al., 2009), it is generally concluded that platelet count should not be adjusted using the autologous PPP (Gresele et al., 2014a), but if it is above 600 × 10^9/l it could be adjusted with a buffer rather than with PPP (Harrison et al., 2011).

One of the major limitations of LTA is that the method is time- and labour-intensive and requires a large volume of blood. The latter presents significant challenges when platelet function needs to be assessed in paediatric patients, in whom the volume of blood sample is usually very limited. To overcome these limitations a high throughput method was developed to measure platelet aggregation in PRP on a 96-well plate using lyophilized reagents (Opti mul!), which offers simultaneous assessment of several platelet activation pathways in a smaller volume of PRP using a wide range of concentrations of seven platelet agonists (ADP, AA, collagen, PAR1 peptide, epinephrine, U46619 and ristocetin) (Chan et al., 2011; Chan & Warner, 2012). The diagnostic potential of this novel assay to detect PFDs was evaluated within the GAPP study mentioned above and Optimul demonstrated fairly high level of sensitivity and specificity in detecting mild platelet defects as compared to the traditional LTA even though Optimul is an end-point assay and does not provide kinetic information as LTA (Lordkipanidzé et al., 2014). The test showed high negative predictive value, missing platelet function abnormality in only 2 out of 65 subjects, which suggests that it might be useful in the future as a screening tool that can be available in non-specialized laboratories once this test is validated further and becomes commercially available.

Platelet granule secretion

As indicated above, secretion of platelet granules is an important amplification mechanism that regulates primary haemostasis, and abnormalities in granule secretion, predominantly dense granules, are quite frequent among subjects who present with bleeding (Quiroga et al., 2009; Dawood et al., 2012). Therefore, in the latest guidelines the assessment of platelet granule secretion was included in the first line tests to diagnose a suspected PFD with an assay for nucleotides (ATP/ADP) for dense granules and at least one marker for alpha-granules secretion (Gresele et al., 2014a).

The simplest and most widely used assay to measure the release of platelet nucleotides from dense granules is lumia aggregometry – the assessment of ATP level by luminescence using luciferin-luciferase reagent that is performed simultaneously with PRP LTA or whole blood impedance aggregation (Miller, 1984; Dawood et al., 2007; Harrison et al., 2011). The addition of simultaneous measurement of ATP release to standard LTA proved to be a valuable tool in detecting platelet secretion defects (Cattaneo, 2009). It increases the diagnostic power of the LTA as subjects with reduced dense granule secretion can still demonstrate practically normal aggregation responses (Nieuwenhuis et al., 1987; Israels et al., 1990; Lowe et al., 2013b). Although this assay is fairly straightforward and easy to perform, a significant degree of variability (Pat et al., 2011) and possible potentiation of platelet aggregation in response to epinephrine by the addition of luciferin-luciferase reagent was demonstrated (Hayward et al., 2012), however, the latter observation was not confirmed in a later study (Lordkipanidzé et al., 2013a).

Alternatively, secretion of dense granule contents could be measured with the uptake and subsequent release of radiolabelled serotonin (5HT), which was shown to provide good reproducibility and sensitivity to secretion defects (Quiroga et al., 2009); however, a significant disadvantage of this assay is the use of radioactive compounds, which limits the availability of this technique to research laboratories. The drawbacks of both described methodologies indicated the need for alternative assays for dense granule secretion and the direct measurement of released 5HT by non-radioactive enzyme-linked immunosorbent assay (ELISA) or High performance liquid chromatography (HPLC) was suggested to be a potential replacement method as in platelets 5HT is only present in dense granules, as opposed to nucleotides that have a significant metabolic pool (Quiroga & Mezzano, 2012). However, both approaches require specialized equipment and expertise, therefore their application would still be restricted to a very limited number of laboratories.

Flow cytometry is increasingly used to assess various aspects of platelet function and dense granule secretion can also be measured by the increase in the surface expression of CD63 protein, which is present in the membranes of lysosomes and dense granules, following platelet stimulation (Nishibori et al., 1993; Wang et al., 1999; Goodall & Appleby, 2004). CD63 seems to be a reliable marker of dense granule secretion and was shown to be reduced in patients with Hermansky–Pudlak syndrome (Nishibori et al., 1993; Dovlatova et al., 2014). Reduced CD63 expression was also demonstrated in patients with bleeding disorders (Quiroga et al., 2009; Dawood et al., 2012). The combination of LTA with CD63 measurement has been suggested as a valuable tool for diagnosis of both PFD and platelet activation in haemostasis (Gresele et al., 2011). Flow cytometry offers several advantages, such as the simultaneous measurement of platelet activation and dense granule secretion, which can be assessed by the surface expression of CD63 and a functional assay for dense granule secretion using the combination of radiolabelled serotonin (5HT) and luciferin-luciferase (Quiroga et al., 2009; Buchtala et al., 2015).
in all patients who presented with mild bleeding and showed reduced dense granule secretion on lumi-aggregometry apart from two subjects with Hermansky–Pudlak syndrome type 2 who had increased level of CD63 even at baseline without platelet stimulation (Dovlatova et al., 2014). This was in accordance with previously published data (Kurnik et al., 2013) and is explained by the impaired sorting of CD63, when it accumulates in the plasma membrane rather than granule membrane (Dell’Angela et al., 1999). This observation suggests that the level of granule proteins by flow cytometry needs to be assessed on both activated and resting platelets.

It should be noted that both lumi-aggregometry and assessment of CD63 by flow cytometry cannot distinguish between storage pool deficiency and primary secretion defects, when the granule number and content is normal and only the release mechanism is affected. Therefore, if either of the described methods indicates reduced granule secretion, the total platelet 5HT or nucleotides should be assessed in platelet lysates (Harrison et al., 2011) or the measurement of uptake and release of mepacrine, which binds selectively to adenine nucleotides in dense granules, could be measured. The latter can be reliably performed with flow cytometry (Gordon et al., 1995; Wall et al., 1995).

Release of α-granule contents could be assessed by measuring the level of α-granule proteins, e.g. β-thromboglobulin or platelet factor 4, with ELISA or Western blotting (Harrison et al., 2011). These techniques are quite cumbersome, therefore currently a more widely used approach to assess the release of α-granules is the measurement of the α-granule marker P-selectin (CD62P) expression on activated platelets by flow cytometry (Goodall & Appleby, 2004; Gresele et al., 2014b).

In all subjects with abnormally low α- and/or dense granule secretion, as detected by either of the described methods, the diagnosis of a storage pool defect should be confirmed with electron microscopy (Clauser & Cramer-Borde, 2009).

Other aspects of platelet function assessed by flow cytometry

Mostly, flow cytometry is used to measure the levels of certain markers on the cell surface and less frequently to measure markers inside the permeabilized cells. The main advantage of this approach is that it can be performed in whole blood and usually requires a very small sample volume. When assessing platelet function in subjects with suspected PFD flow cytometry is most commonly used to quantify the level of glycoprotein receptors, mainly GPIb and GPIIb/IIIa, to exclude or confirm Bernard-Soulier syndrome and Glanzmann thrombasthenia, respectively (Fabris et al., 1989; Harrison et al., 2011). These are now included in the first line tests within the diagnostic workup for PFDs and the measurement of other glycoprotein receptors, e.g. GPIa/IIa, GPIV, GPVI, by flow cytometry should be performed within the second-step testing (Gresele et al., 2014a).

Platelet procoagulant function is also assessed using flow cytometry. Following platelet stimulation with a strong agonist, such as collagen in combination with thrombin, CRP or calcium ionophore, the anionic phospholipids, e.g. phosphatidyserine (PS), translocate from the inner to the outer layer of platelet membrane, transforming it into a negatively charged surface, which facilitates the assembly of enzyme complexes of coagulation factors for efficient thrombin generation. The level of PS exposure can be measured using specialized probes, with Annexin V being the most commonly used (Thiagarajan & Tait, 1990). However, its binding to PS is calcium-dependent, which is not ideal as blood samples are often collected into calcium chelating anticoagulant; therefore for optimal binding the samples need to be recalcified, which can be easily done only with washed platelets isolated from plasma. An alternative probe, lactadherin, was identified, which binds to PS with higher affinity and in a calcium-independent manner (Dasgupta et al., 2006; Alhany et al., 2009). The assessment of PS exposure is essential for the diagnosis of Scott syndrome, which is characterized by significantly impaired PS externalization and reduced shedding of microparticles, although the prevalence of this defect is very low (Satta et al., 1997). Patients with bleeding phenotype and isolated decrease in microparticle generation have also been described (Castaman et al., 1997).

Upon activation in whole blood in vivo or in vitro platelets adhere to leucocytes to form heterotypic aggregates, which can also be readily detected using flow cytometry in samples with lysed red cells (Michelson et al., 2001) or by using fluorescent triggering when only the events positive for leucocyte-specific marker are registered (Li et al., 1999). The latter approach offers the advantage of minimal manipulations with the blood sample.

A method of assessing platelet aggregation in whole blood using flow cytometry was also developed (Fox et al., 2004). The method is based on measuring the decrease in the number of single platelets as they form aggregates in stimulated and stirred whole blood. The platelet number can be measured in small sub-samples removed from the test tube at different time points to provide kinetic information on the platelet aggregation. Platelets are labelled with platelet-specific antibody and the number of red cells is used as a reference for counting individual platelets. The method is sensitive to microaggregate formation and could provide information on platelet disaggregation, although this approach is quite elaborate. Recently a variation of aggregation measurement by flow cytometry in whole blood was described, in which platelets were labelled with two platelet-specific markers (one type of antibody conjugated with two different fluorochromes) and mixed together; the increase in events characterized by both fluorescent labels was representative of aggregate formation (De Cuypere et al., 2013). To date, the performance of this method in identifying platelet defects has been assessed in patients with Glanzmann thrombasthenia and demonstrated absent aggregation in...
response to PMA. However, this technique is quite elaborate – it involves dividing blood into two parts, labelling platelets separately with different markers, then washing away the excess of antibodies and reconstituting cells in a buffer with plasma prior to the actual assessment of aggregation. Although it proves to be a powerful research tool, it would be difficult for diagnostic laboratories to easily and widely adopt this assay for detection of PFDs.

We attempted to modify the original method of single platelet counting by Fox et al (2004) and applied it to a 96-well plate in order to simplify the test and perform assessment of several pathways of platelet activation using only a very small volume of blood (Lordkipanidzé et al, 2013b). In this test, whole blood is directly activated with agonists and stabilized with fixing solution AggFix (Platelet Solutions Ltd., Nottingham, UK) after which the remaining single platelets and aggregates are stabilized for at least 9 days. As before, platelets are labelled with a single platelet-specific marker and the aggregation is assessed as a decrease in the number of single platelets. The method proved reliable and easy to use, generating full dose-response curves to ADP, AA, collagen and PAR1 agonist using 1-2 ml of whole blood, which makes this method appealing for paediatric patients when the blood sample volume is limited. However, similarly to Optimum method for PRP, it is an end point assay and lacks the kinetic information available with LTA. This might explain why the assay showed limited agreement with LTA in identifying platelet abnormalities in the early assessment in 15 patients from the GAPP study population (Lordkipanidzé et al, 2013b). All these approaches to measure aggregation in whole blood by flow cytometry are promising tools to assess platelet function and require further evaluation in wider population of subjects with suspected PFDs.

Additionally, the assessment of vasodilator-stimulated phosphoprotein (VASP) phosphorylation, a surrogate marker for cyclic nucleotides, by flow cytometry (Schwarz et al, 1999) was shown to identify reduced functionality of P2Y$_{12}$ receptor in patients with homozygous defects in P2Y$_{12}$ receptor and disfunctional signal transduction via P2Y$_{12}$, although the technique was not sensitive to P2Y$_{12}$ abnormality in the subject with heterozygous defect (Zighetti et al, 2010). This is a specialized approach and could be used only as a second line test to confirm an abnormality in P2Y$_{12}$ receptor.

Despite several advantages, such as identification of multiple parameters of platelet morphology and function in a small sample volume, flow cytometry is often performed on unfixed samples. It means that the analysis, which requires specialized and expensive laboratory equipment and significant expertise, has to be performed within a limited time after a blood sample was taken. Therefore, similarly to the majority of other techniques for testing platelet function, the application of flow cytometry is currently restricted to specialized laboratories. To overcome this drawback at the University of Nottingham we have developed two fixing solutions (Platelet Solutions Ltd., Nottingham, UK), which can stabilize whole blood samples for up to 9 days, so that after a simple procedure of platelet stimulation at the point of blood sampling, the samples can be fixed and analysed remotely at a central laboratory. One solution (AggFix) is designed to stabilize platelet aggregates, as described above, and platelet-leucocyte conjugates (Algahtani et al, 2014) and the other solution (PAMFix) was developed to stabilized platelet activation markers (Fox et al, 2009). We have evaluated the performance of this remote platelet function testing (RPFT) approach for diagnosing PFDs, which included the measurements for P-selectin and CD63 expression in response to activation of several pathways in fixed whole blood samples (Dovlatova et al, 2014). The test demonstrated fairly good agreement with lumi-aggregometry in detecting platelet abnormalities with CD63 expression being a marker of dense granule secretion and P-selectin – a marker of alpha granule secretion and of overall platelet reactivity via several activation pathways. This initial evaluation suggests that a similar test, potentially with several added measurements, could be developed to serve as a screening pre-test in patients with suspected PFDs.

Platelet function testing in children

Assessment of platelet function in children and neonates is even more challenging than in adults due to significant limitation of the blood volume available for testing and the absence of well-defined reference ranges (Israels, 2009; Knöfler & Streif, 2010). Only in a limited number of studies platelet function was assessed in a healthy paediatric population to establish the normal ranges and it was demonstrated that, in healthy children older than 1 year, platelet aggregation in PRP and whole blood and nucleotide release did not significantly differ from the adult population (Knöfler et al, 1998; Bonduel et al, 2007). However, platelet function seems to be slightly decreased in newborns (Mull & Hathaway, 1970; Knöfler et al, 1998), which is hypothesized to be a transient compensatory mechanism for increased coagulation status and VWF levels (reviewed in Haley et al, 2014). A diagnostic approach for platelet disorders in children has been proposed recently and mainly relies on the same methodologies as used in adult subjects (Israels et al, 2011).

Discussion

Inherited platelet function disorders, especially mild forms, present a significant diagnostic challenge for clinicians as the available methodologies to assess platelet function differ between laboratories, the results of testing are quite variable and are often difficult to interpret. Currently, the assessment of platelet function requires specialized equipment and experienced personnel to perform the tests, which limits the diagnostic evaluation of suspected PFDs to specialized laboratories. This, together with the fact that blood samples need to be processed within a limited time after venipuncture
leads to the clustering of diagnosed cases of PFDs around such specialized centres, which might suggest that the prevalence of PFDs among general population is underestimated (Knöfler & Streif, 2010).

The diagnosis of PFDs mainly relies on traditional platelet function testing, which includes LTA or lumi-aggregometry, but even these methods were reported to be performed only in 59% and 21% of laboratories, respectively (Gresele et al., 2014b). Significant progress has been made recently with the publication of international guidelines for LTA itself and diagnostic approach to PFDs (Cattaneo et al., 2013; Gresele et al., 2014a), which might improve the overall diagnosis of PFDs. However, both LTA and lumi-aggregometry are very time- and labour-intensive, require a relatively large volume of blood and include multiple steps, which increases the assay variability and limits the application of this technique. In addition, only a proportion of subjects (50–60%) presenting with mucocutaneous bleeding and suspected PFD actually receives a confirmation of abnormal platelet function using these methodologies (Quiroga et al., 2007; Dawood et al., 2012). This suggests that performing lengthy elaborate techniques on every patient with suspected PFD may not be the most optimal approach or may not be even possible. However, the existing screening tests, bleeding time and PFA-100, did not demonstrate the desired sensitivity and specificity for platelet defects and thus are not recommended for use (Harrison et al., 2011; Gresele et al., 2014a).

Overall, it seems that the future developments of platelet function assessment should seek progress in two directions. Firstly, more widely accessible, easy-to-use and standardized techniques need to be developed and clinically validated to provide better access to subjects who require testing and to select those who need to undergo further elaborate testing in specialized laboratories. And secondly, the range of available techniques in these specialized centres needs to be expanded to cover various aspects of primary haemostasis, perhaps even beyond platelet function and include, for example, the assessment of fibrinolytic activity. These developments might help to increase the number of patients in whom specific diagnosis of a PFD can be reached. In this respect, the novel tests described here could serve as a starting point for such new developments. Certainly, flow cytometric-based techniques hold promise as they require a small volume of whole blood and can cover multiple parameters of platelet function (Table II), with many of them being measurable in fixed blood samples. These characteristics can not only improve the accessibility of platelet function testing, but also provide much better standardization than what could be achieved with traditional techniques. Additionally, these techniques could be useful in subjects with low platelet count and in children, as they require only a small volume of whole blood.

Table II. Summary of the traditional and new approaches to measuring platelet function.

<table>
<thead>
<tr>
<th>Platelet function tested</th>
<th>Traditional methodology</th>
<th>Recently developed test</th>
<th>Advantages of new techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet aggregation in PRP</td>
<td>Light transmission aggregometry</td>
<td>Optimul (96-well plate assay)</td>
<td>High throughput, easy to perform</td>
</tr>
<tr>
<td>Platelet aggregation in whole blood</td>
<td>Impedance aggregometry</td>
<td>Single platelet counting (96-well plate assay) - flow cytometry</td>
<td>Lower volume of PRP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dual-labelled platelet aggregates - flow cytometry</td>
<td>Multiple activation pathways simultaneously</td>
</tr>
<tr>
<td>Dense granule secretion</td>
<td>Lumi-aggregometry Mepacrine assay Radiolabelled 5HT release</td>
<td>CD63 exposure - flow cytometry (RPFT)</td>
<td>Small volume of whole blood</td>
</tr>
<tr>
<td>Alpha granule secretion</td>
<td>PF4 or β-thromboglobulin by ELISA or Western blotting</td>
<td>P-selectin exposure - flow cytometry (RPFT)</td>
<td>Sample fixation and remote analysis</td>
</tr>
<tr>
<td>Procoagulant function</td>
<td>Binding of Annexin V - flow cytometry</td>
<td>Binding of Lactadherin - flow cytometry</td>
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</tr>
</tbody>
</table>

SHT, serotonin; PF4, platelet factor 4; ELISA, enzyme-linked immunosorbent assay; RPFT, remote platelet function test; PRP, platelet rich plasma; PS, phosphatidylserine.

References


probe lactadherin: a comparison study with annexin V. Transfusion, 49, 99–107.

N. Dowlatova

Harrison, P. (2005) The role of PFA-100 testing in the investigation and management of haemostat-


lyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function.
*Journal of Thrombosis and Haemostasis*, 4, 312–319.

Hayward, C.P., Pai, M., Liu, Y., Moffat, K.A., Seecharan, J., Webert, K.E., Cook, R.J. & Hed-

Hayward, C.P., Moffat, K.A., Castilloux, J.F., Liu, Y., Seecharan, I., Tasneem, S., Carlino, S., Cor-

tems for platelet function analysis by the PFA-100 system. *Thrombosis Research*, 87, 159–164.

International Council for Standardization in Haem-
matology Expert Panel on Cytometry; Interna-


tion Medicine and Hemotherapy*, 34, 6–19.

cal and laboratory diagnosis of inherited platelet function disorders in children. *Transfus Medici-
eone and Hemotherapy*, 37, 231–235.

Knöfler, R., Weissbach, G. & Kuhlisch, E. (1998) Platelet function tests in childhood. Measur-
ing aggregation and release reaction in whole blood. *Seminars in Thrombosis and Hemo-


lyzer-PFA-100. *Seminars in Thrombosis and Hemo-
sta*, 21, 106–112.

Kurnik, K., Bartsch, I., Maul-Pavicic, A., Ehl, S., Sandrock-Lang, K., Bidlingmaier, C., Rombach, M., Nishibori, M., Cham, B., McNicol, A., Shalev, A., Jain, N. & Gerrard, J.M. (1993) The pro-
pulsive marker of platelet activation than

Li, N., Goodall, A.H. & Hjemdahl, P. (1999) Effi-

Linnemann, B., Schwonberg, I., Mani, H., Proch-
now, S. & Lindhoff-Last, E. (2008) Standardiza-


Lowe, G.C., Sánchez-Guiu, I., Chapman, O., Rive-
za, J., Lordkipanidzé, M., Dowlatova, N., Wilde, J., Watson, S.P., Morgan, N.V. & UK GAPP col-

erative (2013b) Microsatellite markers as a rapid approach for autozygosity mapping in Hermansky-Pudlak syndrome: identification of the second HP57 mutation in a patient present-
ing late in life. *Thrombosis and Haemostasis*, 109, 766–768.

Michelson, A.D., Barnard, M.R., Krueger, L.A., Va-
leri, C.R. & Furman, M.I. (2001) Circulating monocyte-platelet aggregates are a more sensi-
tive marker of in vivo platelet activation than platelet surface P-selectin: studies in abo-


Nichibori, M., Cham, B., McNicol, A., Shalev, A., Jain, N. & Gerrard, J.M. (1993) The protein CD63 is in platelet dense granules, is defi-
cient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulo-

Pai, M., Wang, G., Moffat, K.A., Liu, Y., Seecha-

Pai, M., Wang, G., Moffat, K.A., Liu, Y., Seecha-

testing in the diagnostic screening of patients with suspected abnormalities of hemostasis: comparison with the bleeding time. *Journal of Thrombosis and Haemostasis*, 5, 2393–2398.


