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Assessing platelet function – a basic introduction

The purpose of this newsletter is to provide a basic overview of platelet function and how this can be tested in the routine haematology laboratory

Key words:

Platelet function, bleeding time, light transmission platelet aggregometry, platelet aggregation, agonist

Platelet structure and function

Platelets are produced in the bone marrow. They circulate in the blood and have a life span of about 10 days. Platelets have a very complex structure which facilitates the critical role that they play in the normal physiology of haemostasis.

The major components of the platelet include the surface membrane composed mainly of phospholipids, intracellular granules, surface receptors and the canalicular system. Ordinarily, platelets circulate as 'inert' particles, without interacting with the blood vessel wall or any blood constituent proteins. However, at sites of blood vessel damage, platelets stick to a variety of structures via a multitude of platelet surface receptors.

Von Willebrand factor (vWF) is a plasma protein the function of which is to act as an anchor for platelets at sites of injury. vWF and platelets ordinarily do not interact, but once vWF has bound to subendothelial tissues, it changes its own structure which the platelet then recognizes and binds tightly. The bond takes place via one of the major surface receptors, which in turn triggers multiple reactions inside the platelet which lead to 'activation' of the platelet.

Platelet activation includes several key end points:

- *Shape change*

  Platelets undergo a shape change from disc like to flattened spread out structures with multiple finger-like extensions. This physically assists in plugging the hole in the vessel wall. The platelet is able to do this because it stores an abundance of 'excess membrane' in the form of the canalicular system. This canalicular system is connected to the outside of the platelet and when activated, this excess membrane is pushed to the outside thereby greatly increasing the surface area.

- *Granule release*

  Platelets contain granules that are secreted when platelets are activated. These granules contain a number of key elements, such as ADP, factor V, vWF and calcium which further amplify the haemostatic process by attracting more platelets to the site of injury.

- *Membrane flip-flop*

  Platelet activation results in an irreversible flip-flop of its bilayer membrane resulting in the exposure of phospholipids (especially phospatidylserine) to the

plasma which are normally housed primarily within the inner leaflet of the platelet membrane and hence separated from plasma proteins. This is a critical step in the haemostatic process as phospholipid is an essential element in the support of the coagulation cascade.

- *Fibrinogen receptor*

  Another vital consequence of platelet activation is the induction of a shape change in the main surface receptor for fibrinogen. As the fibrinogen molecule has two identical arms, one fibrinogen molecule is able to bind to fibrinogen receptors on adjacent platelets thereby linking them together.

The overall effect of this is that there is now a physical seal comprising of stretched out platelets which are tethered to collagen in the vessel wall at the site of injury. The primary platelet plug by itself is however only a temporary seal, and the formation of a proper clot is needed in order to seal the vessel wall securely whilst the damaged vessel repairs itself. The exposed phosphatidylserine on the activated platelets surface is ready to support the coagulation cascade which has its substrate fibrinogen right there, holding the platelets together.

Platelet abnormalities

a) Abnormalities of platelet count

The risk of bleeding is directly proportional to the degree of thrombocytopenia. There are numerous causes of thrombocytopenia the topic of which is beyond the scope of this newsletter. Likewise, very high platelet counts, as may be observed in the essential thrombocythemia, carry an increased risk of clotting. Platelet counts are readily measurable on haematology analysers. The standard principle of measurement is impedance which assigns particles to a particular cell class, e.g. platelet or red blood cell, based purely on size. In the event of there being red cell fragments, or extreme microcytosis, or giant platelets present, the platelet count may be erroneous. In such instances, an optical platelet count, as is available by measuring samples in the reticulocyte channel on Sysmex haematology analysers, should be used to provide an accurate platelet count.

b) Abnormalities of platelet function

Qualitative platelet abnormalities are generally divided into acquired or inherited defects. Whilst relatively rare, the latter are generally further classified according to which aspect of platelet function is affected as shown in Table 1.

***Table 1** Classification of inherited disorders of platelet function*

1. Disorders of platelet adhesion (defects in platelet-vessel wall interaction)
   - Von Willebrand disease (vWD) (plasma vWF defect or deficiency)
   - Bernard-Soulier syndrome (GPIb defect or deficiency).
2. Disorders of platelet aggregation (defects in platelet-platelet interaction)
   - Glanzmann's thrombasthenia (GPIIb/IIIa defect or deficiency)
   - Congenital afibrinogenemia (absence of plasma fibrinogen).
3. Disorders of platelet granules
   - Storage pool disorder
   - Quebec platelet disorder.
4. Disorders of platelet signaling
   - Defects in platelet receptor-agonist interaction (e.g. ADP, collagen etc.)
   - Other signaling defects (e.g. calcium mobilization).
5. Defects in platelet cytoskeleton regulation
   - Wiskott-Aldrich syndrome.
6. Membrane phospholipid defects (disorder of platelet membrane clotting factor interaction
   - Scott syndrome.

Acquired platelet disorders, as listed in table 2, in contrast are common.

***Table 2** Common causes of acquired platelet functional disorders*

1. Drugs
   - Therapeutic intent is platelet inhibition (e.g. aspirin, Clopidogrel)
   - Platelet inhibition is a side effect (e.g. cephalosporin antibiotics)
2. Herbal supplements, foods and alcohol
3. Renal failure
4. Haematological disorders
   - Myeloproliferative disorders
   - Myelodysplastic syndromes
5. Cardiopulmonary bypass circuits

Approach to patients with suspected platelet dysfunction

Clinical assessment of bleeding history, family history, review of medication and exposure to any substances with platelet inhibitory properties as well as symptoms of underlying systemic disease and physical examination will guide a clinician to distinguish between primary (platelet and vessel wall) and secondary haemostatic (coagulation) disorders. During this process it is essential to recognise that numerical and/or functional platelet disorders are prevalent amongst patients with abnormal bleeding and may be clinically indistinguishable from other haemostatic disorders, particularly von Willebrand disease (vWD). Platelet disorders can also sometimes co-exist with other coagulation factor defects or vWD. Laboratory investigations of platelet number and function are therefore recommended in any patient where bleeding symptoms are not fully explained by standard clinical laboratory investigations.

Laboratory investigation of suspected platelet abnormalities

The diagnostic evaluation of platelet disorders is complex, poorly standardised and time consuming. This coupled with the wide spectrum of a known range of disorders some of which are very rare, presents a significant challenge to even the best diagnostic laboratory.

Baseline tests should include as a minimum a full blood count (FBC) to evaluate platelet count and to assess general haematological status as well as peripheral smear review to comment on platelet morphology. Even though the history may favour a primary haemostatic defect, baseline coagulation tests such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) should always form part of the baseline investigation of a patient with a bleeding diathesis.

a) Von Willebrand factor (vWF) assays

vWD is the commonest inherited bleeding disorder with highly variable underlying mutations and consequently also highly variable clinical manifestations, ranging from a severe bleeding diathesis to easy bruising syndromes. Although not a platelet disorder, vWF is essential for normal platelet adhesion hence any work-up of a suspected primary haemostatic defect would be incomplete without assays of vWF antigen and activity levels (please see SEED No 11_2011 for details).

b) Bleeding time

The bleeding time is a very basic screening test of primary haemostasis. In the most commonly utilised method (Ivy) a blood pressure cuff is applied to the arm and inflated to 40mmHg. After cleansing the skin, a superficial incision of controlled depth and length is made. This can be done with a sterile blade or scalpel but nowadays most laboratories make use of a disposable template device which controls the size and depth of the cut. Once the cut is made, the time taken to stop bleeding is recorded. From the time of incision until the cessation of bleeding, filter paper is applied to the edge of the cut every 30 seconds. As long as blood is absorbed, active bleeding is still occurring.

The bleeding time is considered prolonged if it exceeds 9 minutes. Causes of prolonged bleeding time include thrombocytopenia, von Willebrand disease, platelet function defects or more rarely abnormalities of collagen (or other structural proteins) compromising the integrity of the blood vessel wall.

The bleeding time is however a poorly reproducible test and is operator dependent and influenced by non-haemostatic factors such as skin temperature, age and haematocrit. Consequently the bleeding time is no longer widely used nor recommended as a screening test for primary haemostatic abnormalities as defects are inconsistently detected.

c) Platelet Function Analyser (PFA) closure time

The PFA-100 is a commercially available test system whereby citrated blood is aspirated at high shear rates through single-use cartridge containing an aperture which is coated with collagen and either adrenalin or ADP. These agonists initiate platelet adhesion which triggers platelet aggregation and progressive closure of the aperture. The closing time is recorded. Closing times are influenced by other factors and therefore should not be interpreted without knowledge of platelet count and haemoglobin level. Thrombocytopenia and anaemia can

both cause prolongation of closing time which could be erroneously interpreted as defects of platelet function. Detailed discussion of the PFA is beyond the scope of this newsletter.

d) Platelet aggregation studies

Platelet aggregation studies are the most widely available specific laboratory tests of platelet function. Light transmission aggregometry, which has been used for over 50 years, although considered the gold standard of testing, is a poorly standardized test with wide inter-laboratory variability. However, when conducted under stringent conditions, it provides valuable insights into platelet function.

- **Test principle**

  The test is performed on platelet rich plasma (PRP) obtained from whole blood collected in citrate. Platelet aggregometers measure the change in optical density (or light transmittance) over time of stirred PRP in cuvettes at 37°C after addition of an agonist. The neat PRP sample has platelets in suspension, and much like water droplets suspended in the air in foggy or misty conditions, light shone at the sample will be dispersed and not pass through. However, as platelets are activated in response to the agonist and begin to stick to each other (aggregate), they start to settle and progressively allow light to transmit through. The amount and rate of increase in transmitted light are directly proportional to the reactivity of the platelet to the agonist. The changes in light transmission are recorded as a reaction curve plotted against time. (See figure 1). The principles of aggregation can be viewed as an animated movie at the following link: http://www.platelet-research.org/3/aggregometry.htm

- **Sample preparation**

  Testing is ideally conducted between 30 minutes and 2 hours post sample collection but no older than 4 hours. Citrated whole blood samples are collected and differentially centrifuged to obtain PRP and platelet poor plasma (PPP) respectively. Platelet count should be performed on the PRP. Values of between 150 and 600 x 109/L are acceptable for testing. Adjusting the PRP platelet value to within normal limits with PPP is no longer recommended as this can stimulate platelet

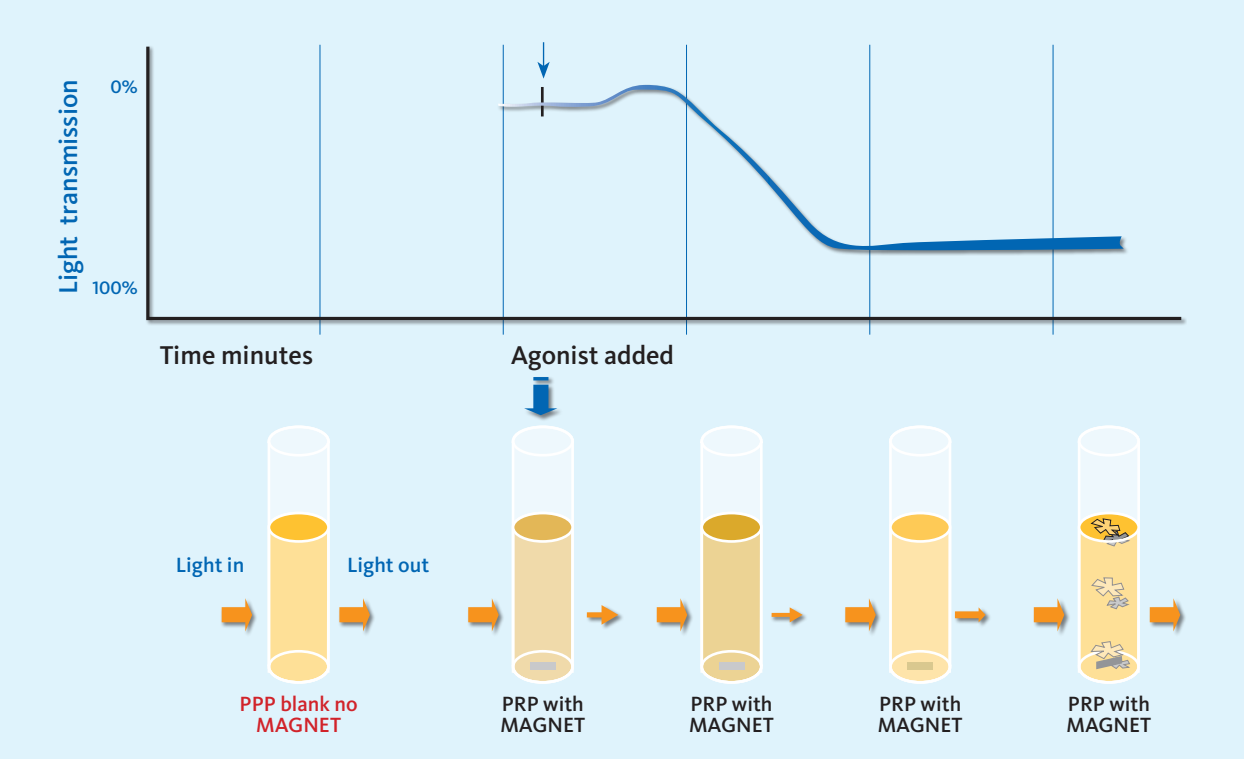

Fig. 1 The principle of optical platelet aggregometry

activation. Normal control samples are prepared in the same way.

The presence of lipaemia may interfere with light transmission hence it is recommended that patients fast prior to blood collection. Samples should also not be refrigerated as cooling may temporarily inhibit platelet responsiveness.

- **Platelet agonists**

The traditional agonists used for baseline testing are shown in table 3.

Table 3 *Common causes of acquired platelet functional disorders*

Agonist	Receptor target
ADP	P2Y1 and P2Y12
Adrenalin (epinephrine)	Adrenoreceptors
Arachidonic Acid	Tests Thromboxane generation and TX receptor
Collagen	GPVI and GPIa/IIa
Ristocetin	GPIb (and vWFs function)

Addition of a platelet agonist to the PRP leads to platelet activation, which manifests as a change in their shape from discoid to spiny spheres which is associated with a transient increase in optical density. The only exceptions to this are ristocetin which causes platelet agglutination rather than aggregation i.e. there is no binding of fibrinogen and adrenalin which does not induce a shape change.

There are two types of agonists:

- *Weak Agonists* e.g. ADP and adrenalin: These induce platelet aggregation without inducing secretion of platelet granules.
- *Strong Agonists* e.g. collagen, thrombin, thromboxane (TxA2): These directly induce platelet aggregation, TxA2 synthesis and platelet granule secretion.

Strong agonists, when used at low concentrations, may act like weak agonists, but weak agonists even at high concentrations will not act as strong agonists.

The aggregation response to an agonist is amplified by the production of TxA2 from platelet membrane phospholipids and by the secretion of ADP from the platelet dense granules. ADP and TxA2 are agonists, which, by binding to their specific receptors, amplify the aggregation response of the platelet.
Platelet secretion can sometimes follow aggregation induced by a weak agonist. This requires the synthesis of endogenous TxA2 by the platelet that can be triggered by the close platelet-to-platelet contact that occurs during platelet aggregation.

With some weak agonists, like ADP and adrenaline, at critical concentrations, the platelet aggregation curve has a biphasic appearance: an initial wave of aggregation (primary wave), followed by a secondary wave of aggregation, which is usually irreversible. Secondary wave aggregation may not occur and the primary wave may disaggregate. At higher agonist concentrations (except with adrenalin) the two waves of aggregation combine and only a single wave is seen and the biphasic waveform is absent. Figure 2 shows a schematic representation of a typical biphasic platelet aggregation reaction curve.

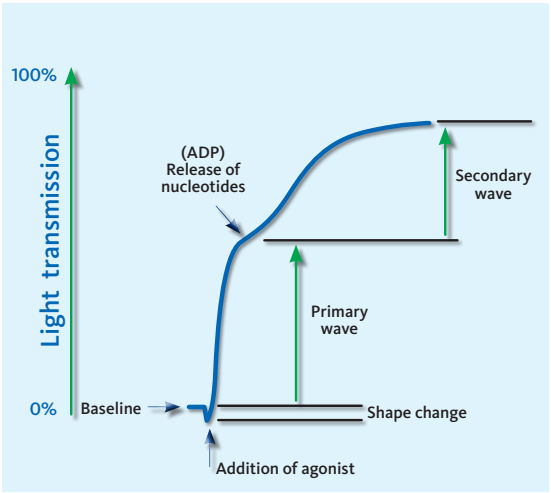

Fig. 2 *Schematic representation of a biphasic platelet aggregation reaction curve*

- **Test method**

  Traditional platelet aggregometers require manual operation. The aggregometer is calibrated prior to analysis of a patient sample. The PRP and PPP are used to define 0% and 100% light transmission respectively. Once the analyser is set, a cuvette with the appropriate volume of PRP is placed into the heating block and warmed to 37°C, the correct volume and concentration of agonist added, and the reaction recorded. This process is repeated for each agonist and for different concentrations of the same agonist.

- **Interpretation**

  The reaction curves expected for the different agonists at varying concentrations are predictable. Deviation from the standard curve or absence of response is indicative or platelet dysfunction (with the proviso that the platelet count is adequate and the sample was appropriately prepared and timeously measured).

  Historically the percentage of maximal aggregation was considered when interpreting platelet aggregation studies. This is the ratio of the height of the maximal aggregation of PRP in response to any given agonist relative to the height of the PPP light transmission (100%). Nowadays as each agonist has a specific target, interpretation is more focused on the patterns observed for the panel of agonists used as this aids in honing in on the source of the platelet function abnormality. (See figure 3)

  The commonly described patterns observed on light transmission aggregometry are listed in Table 4. It should be noted however that many of these platelet function defects are rare. The most commonly observed abnormalities will be vWD (detected by an absent response to ristocetin) as well as drug related platelet inhibition. The latter is variable and depends on the drug and dosage thereof. In this regard a careful drug history is paramount for meaningful interpretation of all assays of platelet function. As platelets have an average lifespan of 7-10 days, any drug ingested over the past 10 days may still have a residual effect.

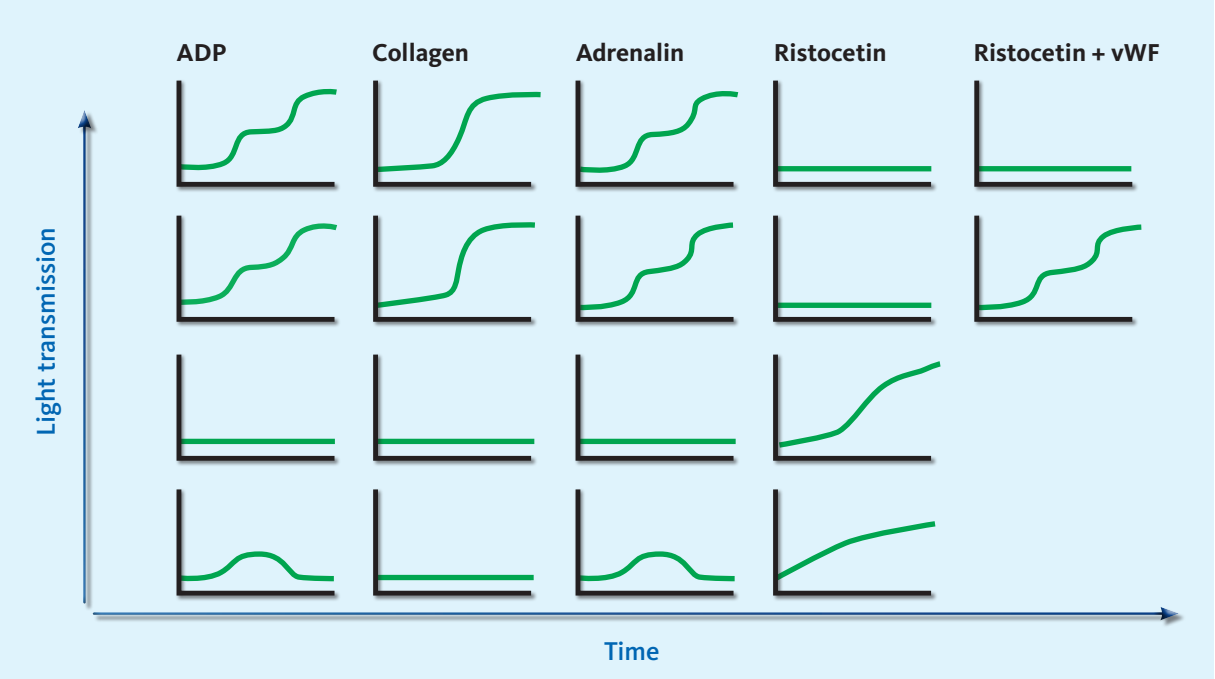

Fig. 3 *Schematic representation of platelet aggregation reaction curves for a panel of agonists. A. Bernard-Soulier disease; B. Von Willebrand disease; C. Glanzmann's thrombasthenia; D. storage pool disease (also aspirin ingestion, TxA2 deficiency)*

Table 4 *Characteristic findings of platelet aggregation studies*

Findings	Disorder	Comment
Absent response to ristocetin	Bernard-Soulier syndrome	Absent GPIb demonstrated on flow cytometry
	Von Willebrand disease	vWF deficiency
Absent or markedly impaired response to all agonists except ristocetin	Glanzmann's thrombasthenia	Absent GpIIb/IIIa demonstrated on flow cytometry
	Afibrinogenemia	Markedly reduced plasma fibrinogen
Primary aggregation only with ADP, adrenalin and collagen. Only partial ristocetin response	Storage pool disorder Platelet release defect	These findings suggest a deficiency of platelet granules or a failure to release granules upon activation
Absent aggregation to arachidonic acid. Decreased or absent aggregation with collagen.	Aspirin effect	Retest after a 10 day washout period (if possible)
	Cyclooxygenase pathway defect	
Absent response to ADP	Clopidogrel (antiplatelet drug)	
Aggregation in response to low dose ristocetin (0.5mg/ml)	Type 2b vWD	Normal platelets are unresponsive to low dose ristocetin. These conditions can be distinguished by conducting specific tests for vWD and by mixing patient platelets with plasma of healthy donor and vice versa.
	Pseudo (Platelet-type) vWD	

Challenges with platelet aggregation studies

Interpretation of platelet aggregation studies is complex. This is complicated further by the lack of standardization from amongst laboratories which makes comparisons difficult. Furthermore, the use of manually operated aggregometers with the need to "blank" each reaction channel with the patient's PPP before analyzing the reactivity of PRP to each agonist, makes this test very labour intensive and unsuitable for routine analysis outside of specialized haemostasis laboratories.

Automation of optical platelet aggregometry

The Sysmex CS series of automated coagulation analysers (CS-2000i, CS-2100i, CS-2400, CS-2500 and CS-5100), besides clot-based, chromogenic and immunoturbidometric based testing, also have a dedicated measurement channel for platelet aggregation. Studies have shown that the results from the automated assay compare very well to conventional platelet aggregometry conducted by experienced personnel in a reference facility. Automation allows for standardization, a faster throughput with walkaway functionality and a significant reduction in the volume of PRP sample required per analysis. This is particularly valuable for the evaluation of paediatric patients in who inherited platelet abnormalities are most likely to manifest. Automation of testing therefore facilitates expanded use of platelet aggregation for the evaluation of platelet function disorders to a wider group of patients and to facilities that may not be currently offering this test.

Take home message

- A thorough bleeding history including family history and detailed review of medication is essential prior to commencing platelet function tests.
- Baseline coagulation tests and an FBC are essential to rule out more common haemostatic abnormalities as well as thrombocytopenia.
- Sample collection, preparation and analysis must be very carefully conducted to minimise interfering variables.
- Platelet aggregation studies should include a panel of standard agonists at different (pre-defined) concentrations.
- Interpretation is based on recognising patterns expected for each agonist and combinations thereof.
- Prior to making a definitive diagnosis and labelling a patient, testing should be repeated, and where possible family members should be included if an inherited disorder is suspected.
- Further testing such as flow cytometry or electron microscopy of platelets may be required.
- Automation of platelet aggregation on the CS-series analysers (except CS-1600) makes testing more standardised, hence reproducible and accessible to more patients.

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