Guidelines for the Laboratory Investigation of Heritable Disorders of Platelet Function

British Committee for Standards in Haematology

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METHODOLOGY

The guideline writing group was selected to be representative of UK-based medical experts. MEDLINE was systematically searched for publications in English up to the Summer of 2009 using key words platelet, platelet function testing and platelet aggregometry. Relevant references generated from initial papers and published guidelines/reviews were also examined. Meeting abstracts were not included. The writing group produced the draft guideline which was subsequently revised and agreed by consensus. Further comment was made by members of the Haemostasis and Thrombosis Task Force of the British Committee for Standards in Haematology. The guideline was then reviewed by a sounding board of approximately 40 UK haematologists, the BCSH (British Committee for Standards in Haematology) and the British Society for Haematology Committee and comments incorporated where appropriate. Criteria used to quote levels and grades of evidence are as outlined in appendix 1 of this guideline. The objective of this guideline is to provide healthcare professionals with clear guidance on platelet function testing in patients with suspected bleeding disorders. The guidance may not be appropriate to patients receiving antiplatelet therapy and in all cases individual patient circumstances may dictate an alternative approach.

Guideline update

A previous BCSH guideline was published in 1988 (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988) and the new guideline is designed to completely replace this.
1 INTRODUCTION

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. (Bolton-Maggs et al, 2006; Hayward & Favaloro, 2009; Pai & Hayward, 2009; Watson et al, 2010). Many new tests (e.g. use of the PFA-100 and flow cytometry) have become available since the last BCSH guideline (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988) and the bleeding time (BT) is now used less frequently. A number of recent surveys have shown large variations between laboratories in platelet function testing practice and clearly demonstrate that new guidelines are urgently required (Jennings et al, 2008; Moffat et al, 2005; Cattaneo et al, 2009). These surveys have revealed why many types of mild platelet defects, (e.g. primary secretion defects) may be missed. This is not only because of the heterogeneity and rarity of some defects, but is also probably related to the failure to apply certain key platelet tests. This document outlines a new standardised approach which could be adopted by most clinical laboratories for the investigation of heritable platelet bleeding disorders. When the clinical picture and/or laboratory results suggest an inherited platelet disorder, referral to an expert reference centre should also be considered. Platelet function tests used specifically for monitoring antiplatelet drugs and/or detecting platelet hyperfunction will not be discussed in these guidelines.

An evaluation of patients with abnormal bleeding requires objective clinical assessment of bleeding history, any family history and physical examination followed, when appropriate, by laboratory investigations. 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). (Hayward, 2008; Cattaneo, 2003) Platelet disorders can also sometimes co-exist with other coagulation factor defects or VWD. (Daly et al, 2009; Quiroga et al, 2007) 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. Further information on the clinical presentation of patients with platelet disorders and the differential diagnosis
is available in detail elsewhere. (Bolton-Maggs et al, 2006) The current guideline focuses on the laboratory investigation of suspected platelet function disorders that should be performed in UK haematology laboratories. However, laboratory tests should ultimately be interpreted in terms of the clinical information.

2 PRE-ANALYTICAL VARIABLES

2.1 Specimen collection

2.1.1 Venipuncture

Ideally, samples for platelet function studies should only be collected from fasting and resting subjects who have refrained from smoking and caffeine ingestion on the day of testing. If the patient is taking medication known to affect platelet function, e.g. non-steroidal anti-inflammatory drugs (George & Shattil, 1991), testing should, if possible, be deferred for 10-14 days after the last dose. Herbal remedies, garlic, alcohol and certain foods may also cause acquired platelet dysfunction. (George & Shattil, 1991) See table 1 for a list of drugs and other agents that are known to affect platelet function. In normal clinical practice it is difficult to avoid some of these patient related variables and so a pragmatic approach is to consider proceeding with platelet function tests, but if they are abnormal, collecting a fresh sample under more suitable conditions and repeating the tests.

Blood should be collected by experienced phlebotomists using a standardised, atraumatic protocol, from the antecubital fossa, by clean venipuncture using minimum tourniquet pressure. Needles should be 19-21 gauge (butterfly cannulae are suitable, providing blood flow is not restricted) and either evacuated tube systems or plastic syringes may be used. A discard tube should be used before collecting successive citrate tubes. Where tubes with a variety of anticoagulant types are required, the citrate tubes should be collected before EDTA- or heparin-containing tubes wherever possible to avoid the potential for carryover. (Favaloro et al, 2008)
2.1.2 Anticoagulants

Blood should be collected into a 1/10 volume of trisodium citrate (105-109 mmol/L final concentration) for clinical platelet function testing. Buffered citrate solutions that maintain the sample pH are preferred. Care must be taken to ensure that tubes are correctly filled.

2.1.3 Specimen processing

All specimens must be maintained at room temperature (20-25°C) and should not be placed on ice, in a refrigerator or a water bath. Immediately after blood collection, all tubes should be mixed by gentle inversion at least 6 times (and discarded if there is any evidence of clotting). Tubes should be kept capped at room temperature and not subjected to any vibration, shaking, vortexing, continuous mixing or agitation; they should not be transported via pneumatic tube systems. The time delay between collection, transport and analysis should ideally be preferably between 30 minutes and 2 hours but not more than 4 hours.

Recommendations

- A complete record of current medication taken by patients or controls should be taken prior to blood collection to either prevent unwanted drug interference or help interpretation of test results (1A)
- Collect blood using a standardised, atraumatic protocol, with minimal stasis (2C)
- Use needles between 19 and 21 gauge; evacuated tube systems or syringes are acceptable (2C)
- The first 3-5ml of blood should not be used for platelet function tests (2C)
- Use 105-109 mmol/L buffered trisodium citrate tubes (2C)
- Maintain specimens at room temperature (1B)
- Keep tubes upright and capped; do not subject to excessive mixing or agitation; do not use pneumatic transport systems (2C)
• Samples should be tested between 30 minutes and no more than 4 hours from blood collection (2C)

3 TESTS AND ASSAYS

Laboratory tests for platelet disorders comprise:

i. Measurement of platelet number and size

ii. Global screening tests of platelet haemostatic function

iii. Specific assays of platelet haemostatic function

3.1 Platelet number, size and morphology

Performance of the modern “Full Blood Count” investigation on whole blood is an essential investigation in patients with abnormal bleeding. The measurement of platelet number and size using automated cell counters and blood film analysis is highly sensitive and specific for numerical platelet disorders and is therefore valuable early in the investigation. Normal results will eliminate thrombocytopenia and anaemia as potential causes of bleeding and ensure that subsequent platelet function tests are not going to be affected by low platelet counts. Low platelet counts indeed affect most platelet function tests discussed below except flow cytometry. Thrombocytosis, which may underlie abnormal bleeding, will also be revealed. If abnormalities in either platelet count, size (mean platelet volume, MPV) or distribution are flagged by the instrument then it is recommended that a blood film be examined to look for abnormalities in platelet number, size and/or granule content. (Briggs et al, 2007) (Althaus & Greinacher, 2009) More recently, multiple light scatter parameters and/or fluorescence, rather than impedance sizing alone have been introduced into commercial analysers. This has improved their ability to distinguish large platelets from red cells and can sometimes provide more accurate counts (e.g. in samples from patients with macrothrombocytopenia where counts are usually underestimated). (Harrison et al, 2000) Immunocounting by flow cytometry should also be considered when accurate counts are required in macrothrombocytopenia. (Harrison et al, 2000)
3.2 Global tests of platelet haemostatic function

Global tests of platelet function are often used during the investigation of individuals with pathological bleeding. Since global tests do not enable a diagnosis of a specific platelet disorder, they are normally performed as the first part of a strategy which requires further testing with more specialised assays of platelet function. (Zeidan et al, 2007) (Harrison & Mumford, 2009) Normal test results may therefore theoretically be used to exclude the diagnosis of platelet function disorder so that further specialised testing can be avoided. For this reason, global platelet function tests are usually performed at the same time as global assays of coagulation pathway function (prothrombin time (PT) and activated partial thromboplastin time (aPTT), von Willebrand Factor (VWF) screening tests (VWF:Ag, VWF:RCo and F:VIII:C) and measurement of platelet number). Guidelines for the systematic investigation of patients with suspected VWD and other coagulation factor deficiencies have recently been published elsewhere and are not discussed further in this review. (Lafran et al, 2004; Bolton-Maggs et al, 2004) The most widely performed tests for screening platelet function disorders are currently the template BT and the Platelet Function Analyser (PFA-100®, Siemens Diagnostics) closure time. Other commercial platelet function assay systems are also available, including those designed to measure the effect of antiplatelet drugs. (Harrison et al, 2007) Thromboelastography (TEG) and Rotational Thromboelastometry (ROTEM) ROTEM and TEG provide global tests of haemostasis and platelet function and are mainly used within the surgical setting. (Perry, 2010) The utility of most of these assay systems including TEG/ROTEM for the screening and diagnosis of platelet function defects has not yet been examined systematically and their use for this application is therefore not currently recommended.

3.2.1 Template bleeding time

The bleeding time described by Duke in 1910 is the oldest test of platelet function. (Duke, 1910) Although the BT was previously recommended as a clinically useful test of platelet function, (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988) and surprisingly it remains in wide
use in the UK (Jennings et al, 2008), there is considerable variation in methodology between laboratories.

The BT is highly dependent on operator technique, is subjective and is influenced by patient variables unrelated to haemostasis, such as age, gender, haematocrit, vascular pattern, skin thickness and skin temperature. (Rodgers & Levin, 1990; Peterson et al, 1998) The BT therefore has poor reproducibility, sensitivity and specificity, as well as being invasive; for these reasons it is not recommended.

3.2.2 Closure time by the Platelet Function Analyser

Assay principle

The PFA-100® device is a test system in which citrated whole blood is aspirated at high shear rates (5000-6000s⁻¹) through disposable cartridges containing an aperture coated with either collagen and epinephrine (CEPI) or collagen and ADP (CADP). (Kundu et al, 1995; Jilma, 2001; Kratzer & Born, 1985) These agonists trigger platelet adhesion, activation and aggregation leading to rapid occlusion of the aperture and cessation of blood flow. (Kundu et al, 1995) The end-points for each test are time to occlusion of blood flow (closure time [CT]) or non-closure if the CT exceeds 300 seconds. The PFA-100® assay system requires small quantities of citrated venous blood (0.8 mL per cartridge) and is therefore useful for studying paediatric samples. Accordingly, the PFA-100® device is widely used as a screening tool to measure global platelet haemostatic function. (Jennings et al, 2008; Moffat et al, 2005)

Factors that influence PFA-100® closure time

The choice of anticoagulant, specimen collection and transportation techniques and time between sampling and analysis (see Specimen Collection section above for guidance) all have critical effects on CT results. (Jilma, 2001) (Harrison et al, 1999; Heilmann et al, 1997). Recent evidence reinforces the need for a discard tube during blood collection for PFA-100 testing (Kunicki et al, 2009). It is important that each laboratory establishes a reference range preferably within 105-109 mmol/L buffered trisodium citrate tubes. Further guidance for the quality control of the PFA-
is published elsewhere. (Christie et al, 2008; Harrison, 2004; Hayward & Eikelboom, 2007) (Favaloro, 2009) There are extensive general reviews of the clinical utility of the PFA-100. (Favaloro, 2008) (Hayward et al, 2006)

Knowledge of the full blood count is critical for interpreting CT results from the PFA-100®. Thrombocytopenia (< 100 x 10⁹/l) and anaemia (< 20% haematocrit) often results in prolongation of the CT. (Kundu et al, 1995; Harrison et al, 1999) CT also correlates inversely with plasma VWF activity in normal subjects and may therefore be longer in patients with blood group O. (Lippi et al, 2001) The Collagen/Epinephrine (CEPI) CT, but not the Collagen/ADP (CADP) CT, is usually prolonged by COX-1 inhibitors such as aspirin. (Jilma, 2001)

**PFA-100® CT and VWD**

Abnormal CT on both cartridges are typical for types 2A, 2B, 2M and 3 VWD with a sensitivity of > 98%. (Franchini, 2005) When type 1 VWD is included, the overall sensitivity of CT to VWD is reported to be lower (85-90%), (Favaloro, 2006) but there is a clear relationship between VWF level and CT, (Moeller et al, 2001). Type 2N gives normal results. The PFA-100® may also be useful for monitoring desmopressin therapy in VWD patients. (Cattaneo et al, 1999; Favaloro et al, 2001; Franchini et al, 2002; Hayward et al, 2006; van Vliet et al, 2008)

**PFA-100® CT and diagnosis of heritable platelet function disorders**

Greater abnormalities in CT in both cartridges occur with the severe platelet function defects, such as Glanzmann thrombasthenia (GT), Bernard–Soulier syndrome (BSS) and platelet type or pseudo-VWD in which non-closure is typical. (Harrison et al, 1999; Harrison, 2005; Hayward et al, 2006; Mammen et al, 1998) In many less severe platelet function defects, the CT may be either normal or prolonged; abnormal results are more frequently reported with the CEPI than the CADP cartridge. (Hayward et al, 2006; Harrison et al, 2002) There are rare reports of abnormal CADP but with normal CEPI CTs suggesting that the CEPI cartridge cannot be used exclusively as a screening test. It is not currently possible to accurately determine the sensitivity of
the PFA-100® for most mild, heritable platelet function defects since most reported studies comprise small patient numbers, with varying mixtures of these defects. (Hayward et al, 2006; Harrison, 2005) The PFA-100® CT exhibits poor sensitivity for mild platelet defects in a small number of prospective studies in patients with an unequivocal personal and family history of mucocutaneous bleeding. (Quiroga et al, 2004; Cattaneo, 2004; Podda et al, 2007) Other retrospective cohort studies of patients with previously diagnosed platelet function defects indicate sensitivities up to >80% for prolonged CT, although many of these studies included subjects with severe phenotypes (e.g. GT, BSS) and VWD. (Harrison et al, 1999; Harrison et al, 2004; Kerenyi et al, 1999; Posan et al, 2003) A recent meta-analysis concluded that the overall sensitivity and specificity of the CEPI cartridge for disorders in primary haemostasis was 83% and 89% respectively. CADP sensitivity was lower at 67% with an equivalent specificity of 86%. (Karger et al, 2007). The PFA-100® has shown good sensitivity (> 90%) in screening patients with menorrhagia for VWD and platelet function defects. (James et al, 2004; Philipp et al, 2005; Acharya et al, 2008)

Guidelines on the utility and practice of using the PFA-100 for clinical assessment of platelet disorders are provided by various international and national organisations. (Hayward et al, 2006; Bolton-Maggs et al, 2006; Christie et al, 2008).

It is reasonable to use normal PFA closure times to rule out a significant platelet defect in patients who have a low clinical suspicion of such a defect, however if the clinical suspicion of a platelet defect is high, then a normal PFA result should not be used to rule out this possibility and specific assays of platelet function are indicated.

Recommendations

- Perform a full blood count on all patients (1A)
- In samples with abnormalities in platelet count or size distribution (as indicated by an automated analyser), a blood film should be examined (1B)
- The bleeding time is not recommended (1B)
• The PFA-100 provides an optional screening test, but this must be interpreted with caution and in the context of the clinical background, as the test is not diagnostic or sensitive for mild platelet disorders (1B)

• Both PFA-100 CADP and CEPI cartridges should be used for screening (1B)

4 SPECIFIC ASSAYS OF PLATELET FUNCTION

4.1 Light transmission aggregometry

Light transmission aggregometry (LTA) was invented in the early 1960s and is still regarded as the gold standard for platelet function testing. Despite its widespread use, the test is poorly standardised and there are wide variations in laboratory practice (Jennings et al, 2008; Moffat et al, 2005; Cattaneo et al, 2009). Guidelines specific for LTA have also recently been published (Christie et al, 2008; Cattaneo et al, 2011; Hayward et al, 2010)

Sample preparation for LTA

Citrated blood samples obtained as described above are centrifuged to prepare platelet rich plasma (PRP) and platelet poor plasma (PPP). To prepare PRP, whole blood tubes should be centrifuged at 170-200 g for 10 minutes in a swing-out rotor at room temperature (RT) without application of the brake. Autologous PPP is prepared by centrifugation (after removal of PRP or using whole samples) at least 1500 g for at least 15 minutes at RT (Christie et al, 2008). At the end of the centrifugation steps a plastic pipette should be used to separate the top 2/3rds of PRP or PPP should be carefully removed without disturbing the buffy coat layer and red cells. PRP or PPP should then be transferred into separate polypropylene tubes capped and stored upright at RT. The PRP should then be left for at least 30 minutes prior to testing. Visual inspection of the samples is important as icteric, lipaemic, red cell contaminated and haemolysed samples should not be tested. A platelet count should be performed on the PRP and unless it is greater than 600 x 10⁹/L, the platelet count should not be adjusted using PPP, as this may cause artefactual inhibition of platelet aggregation (Linnemann et al, 2008; Cattaneo et al, 2007). Analysis of PRP with a
platelet count <150 x 10^9/L is possible, but the results should be treated with caution (ideally a normal control should be analysed, where the PRP count is adjusted to equal that of the test, by dilution with buffer instead of PPP) (to prevent artefacts). PRP with low counts can still be tested to exclude severe platelet disorders such as BSS and type 2B and platelet type VWD.

**Agonists for LTA**

ADP, epinephrine, collagen (type I, tendon), arachidonic acid and ristocetin are the traditional baseline panel of agonists for LTA. (see Table 2) (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988) An extended panel of agonists can include gamma Thrombin, Thrombin Receptor Activating Peptides (TRAP), Collagen-Related Peptide, endoperoxide analogue U46619 and calcium ionophore A23187, which may all be useful when a more detailed investigation of the exact nature of the defect is required (see Table 2). Most laboratories perform dose response curves for ADP (0.5 – 20 μM), collagen (1.0 – 5.0 μg/ml) and epinephrine (0.5 -10 μM). Although this provides a detailed pharmacological approach, more recent evidence supports the use of single doses of a panel of agonists, which significantly increases the likelihood of detecting a platelet defect (odds ratio 32). (Hayward et al, 2009b) (Dawood et al, 2007) A recommended baseline example panel therefore comprises: 2.5 μM ADP, 1.25 μg/ml collagen, 5 μM epinephrine, 1.2 mg/ml ristocetin, and 1.0 mM arachidonic acid (all final concentrations in PRP). If the initial aggregation results with ADP, collagen or epinephrine are abnormal then retesting should be performed at higher concentrations of the agonist(s) and even to supranormal concentrations to confirm a specific defect. It should be noted that a significant proportion of normal samples may not always give a full aggregation response to epinephrine (due to natural variations in adrenoreceptor numbers) and have no related platelet defect. If the aggregation to ristocetin is normal then retesting should additionally be performed with low dose (0.5-0.7 mg/ml) ristocetin to check for hyperfunction or gain of function (associated with Type 2B and platelet type VWD). If results with 1.2 mg/ml ristocetin are absent then retesting can be performed with addition of an external source of VWF (e.g. cryoprecipitate or a VWF concentrate) to confirm either a VWF or Gplb
defect. If arachidonic acid aggregation is abnormal then further testing should be performed with 1.0 µM U46619 to test for any thromboxane receptor abnormalities. An extended panel of tests (usually only available within more specialised centres) could also be considered including gamma thrombin (which does not cause clotting), PAR-1 (SFLLRN) and PAR-4 (AYPGKF) TRAP peptides (if gamma thrombin is abnormal) Collagen-Related Peptide (CRP), calcium ionophore and PMA (Phorbol 12-myristate 13 acetate) if abnormalities in the thrombin receptors, GpVI, calcium mobilisation and protein kinase C respectively, are suspected.

**Performing aggregometry**

A maximum of 1/10 volume of agonist is added to PRP to initiate aggregation and the final concentration of agonist within PRP is recorded (taking into account the 10 fold dilution factor). It is imperative that new batches of agonist are checked against the previous batch for performance, using normal control samples.

Platelet aggregometers measure the change in optical density (or light transmittance) over time of stirred PRP in cuvettes at 37°C after addition of the agonists. They are calibrated for transmission using autologous PPP (100%) and PRP (0%). A stir speed of 1000 - 1200 rpm is normally recommended. It is important that samples are pre-incubated for at least 5 minutes at 37°C prior to assay to obtain stable baseline traces. The appropriate agonists must then be added directly to the PRP and not pipetted onto the side of the tube. It is important that no air bubbles are introduced at any stage of the procedure as these can interfere with transmission measurement. The aggregation tracing should be observed for at least 5 minutes, but preferably 10 minutes to monitor the lag phase, shape change (negative deflection), primary and secondary aggregation and any delayed platelet responses e.g. reversible or spontaneous aggregation. The assay is then terminated and results printed and stored for visual inspection.

It is recommended that local, normal cut-off values are established, using non-parametric statistics. However, it is recognised that this is not possible for most clinical laboratories due to the inherent variability of the test and the large number of subjects that would be required (i.e. >40). For this reason, most clinical laboratory staff subjectively evaluate the shape of the aggregation curves. The following
parameters should always be considered: lag phase, maximal amplitude, primary aggregation slope, and disaggregation, for each commonly used agonist concentration. (Hayward et al, 2009b; Hayward et al, 2008). It is important that the overall shape of the aggregation responses obtained with each agonist are fully described and interpreted by experienced staff (e.g. is the response fully reversible and is there a significant lag phase; what is the maximal amplitude of the response). See Figure 1 for typical examples of normal and abnormal aggregometry curves in various classical defects.

Recommendations

- **Platelet counts** >600x10⁹/L (1B) in platelet rich plasma should be diluted.

- **Repeat all unexpected, abnormal light transmission aggregometry tests with a fresh sample, in parallel with a normal control sample** (2C)

- **Only experienced individuals should interpret tracings and results** (1C)

- **Assess performance of new batches of agonists by comparison with a previous batch** (1A)

**4.2 Flow cytometry**

The most commonly used flow cytometry tests relevant to platelet function are the quantification of glycoprotein receptor density in the diagnosis of defects such as GT and BSS, and detecting their heterozygous states. Flow cytometry can also be used to measure the collagen (GpIa/IIa and GpVI) and PAR-1 receptor densities if LTA testing suggests any abnormalities in these receptors. There are also tests available to measure: platelet activation in response to classical agonists, dense granule content, and exposure of anionic phospholipids As flow cytometry is expensive, time consuming and requires specialised training, only those patients with an appropriate clinical history and/or abnormalities of other platelet function tests should be assessed for receptor defects. Guidelines and protocols on flow cytometry of
platelets have been published elsewhere. (Schmitz et al, 1998; Goodall & Appleby, 2004; Michelson et al, 2007) It is recommended that analysis should be performed using fresh, citrated whole blood, to avoid platelet activation and loss of platelet subpopulations during centrifugation. If measuring platelet activation and function it is important to control for ex-vivo activation caused by delays in analysis from blood sampling for example. Fluorescently labelled antibodies are added to the blood samples and after incubation at room temperature, in the dark, the samples are diluted to a final volume of between 1-2 ml with buffer (e.g. Hepes buffered saline, pH 7.4), or a mild fixative before analysis. All buffers must be filtered (e.g. using a 0.2 µm filter) and tubes should not be vortexed, but mixed gently by tapping, otherwise platelet aggregation will occur. Matched isotype control fluorescent antibodies should be tested at the same time in control tubes. It is recommended that normal positive control samples are analysed in parallel to verify assay performance and that the antibodies are efficiently binding to their respective receptors particularly if a receptor is completely absent in GT or BS for example. Some commercial assays are now available that can give absolute quantification of the copy number of individual receptors of interest. Normal ranges can be established for either fluorescence or copy number of individual glycoproteins. Neonates may also have significantly lower receptor densities than adults. The lower limit of detection if ~500 receptors/platelet so the test cannot always be used reliably to detect low copy number receptors. It is possible to measure platelet procoagulant activity, apoptosis (and microparticles) by incubating samples with high affinity probes against phosphatidyl serine (e.g. Annexin-V) and activating the cells with calcium ionophore, collagen-related peptide or combinations of thrombin and collagen. This enables the diagnosis of Scott syndrome and related disorders although these defects are indeed very rare.

**Recommendations**

- Flow cytometry should be used in the investigation or confirmation of Glanzmann thrombasthenia, Bernard–Soulier Syndrome (1B) and Scott syndrome (1C); and may also be used to investigate abnormalities in the collagen (GpVI and Gpla/IIa) and thrombin receptors (PAR-1) (1B)
- Whole blood platelet assays are preferable although PRP can be used for BSS diagnosis (1B)
- Analyse normal controls in parallel with test samples (1A)

4.3 Measurement of total and released nucleotides

The measurement of total and/or released adenine nucleotides provides an important additional diagnostic tool usually in conjunction with aggregometry for determining whether there is any specific deficiency in dense granule numbers or their content (e.g. storage pool disease), or specific defect(s) in degranulation (e.g. release defects). There is evidence to suggest that these defects can be misdiagnosed if relying on platelet aggregometry alone.(Nieuwenhuis et al, 1987;Israels et al, 1990)(Hayward et al, 2009b)(Cattaneo, 2009) It is therefore recommended that laboratories perform an independent measurement of the release reaction. However, although nucleotide measurement is very straightforward and normally involves measuring ATP by simple bioluminescent assays (using firefly luciferin/luciferase assays), recent surveys indicate that many laboratories do not measure platelet nucleotides.(Jennings et al, 2008;Moffat et al, 2005) This suggests that many platelet storage and secretion defects are potentially being underdiagnosed with current practice.

The simplest assay of released platelet nucleotides can be performed in real time with a Lumi-Agregometer (either LTA or whole blood aggregometry, WBA). (Dawood et al, 2007; Christie et al, 2008; Watson et al, 2010) These instruments provide a rapid assessment of ATP levels during platelet aggregation and normally demonstrate release of ATP during the secondary aggregation phase in LTA. The amount of ATP released is easily calibrated using commercially available ATP standards analysed in the same channels of the aggregometer. However, it is impossible to distinguish between storage and release defects using this approach.

Many laboratories therefore determine the total platelet content of both ADP and ATP with lysed platelet preparations (at standardised platelet counts) and sometimes after a degranulation step to induce release. Adenine nucleotides are measured in platelet lysates using either luminometers (Summerfield et al, 1981 or by HPLC (Greaves & Preston, 1985), with conversion of ADP to ATP (using pyruvate kinase).
Calibration is performed using an ATP standard. These assays have the advantage that samples can be frozen and shipped to more specialised laboratories that regularly perform nucleotide measurements.

There are two nucleotide pools within the platelet: the metabolic pool and the dense granular/storage pool, the latter comprising about 60% of the total content. The ratio of ATP:ADP is therefore of fundamental diagnostic importance as there are pronounced differences between the relative concentrations in the two pools. Any storage defects are associated with a decrease in the amount of stored and released ADP with an increased ratio of ATP:ADP. Normal ADP levels and ATP:ADP ratios but decreased ADP release are indicative of a release-defect.

Normal ranges should be established locally, but typical values are 19-38 and 41-61 nMol/10⁹ platelets for total ADP and ATP respectively (ATP:ADP ratio 1.24-2.56). Typical normal ranges for released nucleotides are: 18-28 and 8-20 nMol/10⁹ platelets for ADP and ATP respectively (ATP:ADP ratio 0.43-0.79) (Chanarin, 1989).

Serotonin (5-HT) is actively taken up and stored within the platelet dense granules and it is possible to measure the uptake and release of radiolabelled serotonin into and from the platelets with standardised assays. (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988; Zhou & Schmaier, 2005) ELISA assays for platelet serotonin content are also available. Mepacrine uptake and release by the dense granules can be measured by flow cytometry (Wall et al, 1995; Gordon et al, 1995).

**Recommendations**

- **When there is a high clinical suspicion of a platelet function defect,** adenine nucleotides should be measured even if the aggregation is normal (1B)

- **If aggregation results suggest storage pool disease or a release defect,** measure stored and released nucleotides (1B)
4.4 Whole blood aggregometry

In impedance aggregometry, whole blood is stirred at 37°C and aggregation is detected by the accretion of platelets to the surface of two fine, precious metal, wire electrodes. (Fritsma, 2007) Adherent platelets increase the electrical impedance between the electrodes, which can be displayed as a wave of aggregation. (Cardinal & Flower, 1980) Impedance aggregation measurements in whole blood may be influenced by: haematocrit (>0.35 L/L), platelet count, and elevated white cell count, while the agonist responsiveness differs from LTA. (Mackie et al, 1984; Ingerman-Wojenski et al, 1983; Sweeney et al, 1989) Impedance and LTA methods show similar dose responsiveness to equine tendon collagen, but higher ADP concentrations are required to induce aggregation by the impedance technique and low doses (e.g. 1 μM) give no impedance response. Reversible aggregation and biphasic responses to ADP cannot be demonstrated by whole blood impedance, (Mackie et al, 1984; Ingerman-Wojenski et al, 1983) while epinephrine responses tend to be absent or very weak, (Mackie et al, 1984; Swart et al, 1984). A study by UK NEQAS surveyed 169 haemostasis centres, (119 UK and 50 non-UK) and found that only 4/88 performed whole blood platelet aggregation studies. (Jennings et al, 2008) Some of the technical problems of whole blood impedance aggregation have been overcome by the development of disposable electrodes, standardised reagents and the availability of a 5 channel multiple electrode platelet aggregometer (Dynabyte, Munich, Germany)). (Toth et al, 2006) There is very sparse peer review literature comparing impedance and LTA methods and a lack of clinical validation in the diagnosis of heritable platelet function defects.

5 OTHER TESTS

Platelet alpha granule proteins (e.g. Platelet Factor 4 (PF4) and Beta-Thromboglobulin (βTG)) can be measured by ELISA, RIA or western blotting and may be helpful for the diagnosis of Quebec platelet disorder (Kahr et al, 2001). Electron microscopy has also proven very useful for defining ultrastructural abnormalities associated with a variety of platelet defects. (Clauser & Cramer-Borde, 2009). The simpler whole mount electron microscopy technique has proven useful for confirming dense granule defects (Hayward et al, 2009a).
Molecular genetic diagnosis of heritable platelet disorders may offer valuable confirmation of diagnosis in affected individuals, in family members where phenotypic testing of platelets is impractical and for ante-natal diagnosis. Molecular diagnosis is most feasible in GT and BSS where the number of candidate genes is small and there are already accessible databases containing large patient groups to help confirm that observed nucleotide variations are pathogenic (http://www.bss.org/1.html and sinaicentral.mssm.edu). Clinical diagnostic services for GT and BSS by direct sequencing of PCR-amplified genomic DNA are now offered in a small number of clinical genetic laboratories in the UK. For mild platelet function or platelet number disorders individual candidate genes can occasionally be identified using clinical and laboratory phenotypic features (e.g. MYH9 related disorder, CAMT, TAR, WAS) or by laboratory phenotype alone (e.g. thromboxane and P2Y12 ADP receptor defects, GPVI defects). (Nurden et al, 2009; Watson et al, 2010). However, molecular genetic analysis of these disorders is currently available only in research laboratories. Since there is limited repertoire of reported mutations, it is usually difficult to assign pathogenicity to observed nucleotide variations without expression studies. In some patient populations where a specific pathogenic mutation is prevalent (e.g. 16bp deletion in HPS1 in Peurto Rican descent patients with Hermansky-Pudlak syndrome), allele specific mutation detection strategies may enable rapid molecular diagnosis of selected disorders. In all cases when molecular genetic diagnosis is considered, families should undergo careful genetic counselling and provide written consent in accordance with current best practice guidelines. (Ludlam et al, 2005).

6 DIAGNOSTIC FEATURES

Typical clinical and laboratory findings of platelet function tests in many different platelet defects are detailed in Table 3. For more diagnosis and treatment of all platelet disorders including the inherited thrombocytopenias see Bolton-Maggs et al, 2006(Bolton-Maggs et al, 2006).
7 DIAGNOSING PLATELET FUNCTION DISORDERS IN INFANTS AND SMALL CHILDREN

Severe platelet disorders such as GT and BSS usually present in infancy or early childhood but the diagnosis of these disorders in the very young is more challenging than in older children or adults for a number of reasons, mainly pre-analytical. Junior paediatricians commonly take blood using heelpricks, fingerpricks or a standard venipuncture needle inserted into a vein, collecting the drops from the end of the needle. None of these methods is appropriate for assessment of platelet function which should be done on a free-flowing venepuncture sample though in practice indwelling arterial and central venous catheters have also been used to facilitate getting the necessary volumes from small children. The minimum volume of blood required for full platelet LTA and nucleotide testing is usually 20 ml; this may be 8-10% of the blood volume in neonates and could cause hypovolaemic symptoms. The usual needle gauge for blood sampling in infants and smaller children is 23G as the recommended size of 19-21G can be too big for small peripheral veins and is also more likely to cause trauma to subcutaneous tissues which may be significant if a severe platelet disorder is present. It is therefore recommended that the control sample is also taken with a 23G needle to ensure that the patient sample is processed along with a similarly taken sample.

Validation of platelet function tests in age-matched normal control populations of infants and children is rarely possible due to ethical issues of taking large volumes of blood from healthy normals. Although there are scanty data on LTA in ‘normal’ neonates and infants, there are no known comprehensive studies looking at nucleotide values at different ages through the 1st year of life. The available literature suggests that in infancy the platelets are generally hyporeactive (except to ristocetin and variably collagen) but beyond infancy reactivity of platelets, both aggregation and nucleotide release reactions, is very similar to that in adults (Knofler et al, 1998; Bonduel et al, 2007). Therefore the usual and pragmatic approach is to assess platelet function in children > 1 year of age using adult controls and normal ranges (Hayward et al, 2010). Family testing may also be useful not only to confirm a given defect but to discern the potential inheritance pattern. As there is few data, results of investigations taken in the first year should be viewed with more caution and should always be repeated, particularly if the apparent abnormalities are
relatively subtle and if the putative diagnosis is one of the usually milder disorders such as a granule or secretion defect. Results of investigations in infants with the severe function defects – GT or BSS – are usually very clear-cut at all ages and it could be argued that the safest way to diagnose these disorders in an infant is to limit the investigations to those that can be performed on relatively small volumes of blood; with a full blood count, PFA-100® (which will reliably show non-closure with both cartridges in both GT and BSS), and flow cytometry. Flow cytometry if performed carefully (see above section) can also be utilised to study platelet function/activation in small volumes of blood by determining responsiveness to various agonists at differing concentrations. Confirmatory LTA can then be done when possible but demonstration of a severe defect of primary haemostasis using the PFA-100® in combination with absent or very low levels of the affected receptor, and macro-thrombocytopenia in BSS, is highly suggestive and enough to guide appropriate treatment for bleeding. Conversely, both GT and BSS can effectively be excluded in infants if the PFA-100® shows normal closure times – this can be of crucial clinical use in unexplained severe bleeding such as intracranial haemorrhage when there is a query as to whether this is an inflicted injury or it is due to ‘spontaneous’ bleeding in association with a severe bleeding diathesis. A detailed diagnostic approach to platelet disorders in children has recently been published (Israels et al, 2011)
TABLE 1

Cyclo-oxygenase (COX)-1 inhibitors (irreversible)
Aspirin and all proprietary or over-the-counter preparations containing acetylsalicylic acid.

COX-1 and COX-2 inhibitors (reversible) (Nonsteroidal anti-inflammatory drugs (NSAIDs)
Ibuprofen
Indomethacin, naproxen
Mefenamic acid

Inhibitors of Platelet Receptors
Abciximab, tirofiban, eptifibatide (αIIbβ3)
Ticlopidine, clopidogrel, prasugrel (irreversible), cangrelor (reversible), ticagrelor (reversible) (P2Y12)

Phosphodiesterase Inhibitors
Dipyridamole
Cilostazole

Anticoagulants
Heparinoids, vitamin K antagonists and direct thrombin inhibitors may indirectly influence platelet function due to inhibition of thrombin.

Cardiovascular Agents
β-adrenergic blockers (propranolol)
Vasodilators (nitroprusside, nitroglycerin)
Diuretics (furosemide)
Calcium channel blockers

Antimicrobials
β-lactams (penicillins, cephalosporins)
Amphotericin (antifungal)
Hydroxychloroquine (antimalarial)
Nitrofurantoin

Chemotherapeutic agents
Asparaginase
Plicamycin
Vinristine

Psychotropics and Anaesthetics
Tricyclic antidepressants (imipramine)
Phenothiazines (chlorpromazine)
Local and general anaesthesia (halothane)

**Thrombolytic Agents**
- Streptokinase
- Urokinase
- Tissue Plasminogen Activator (TPA)

**Miscellaneous**
- Clofibrate
- Dextrans
- Guaifenesin (expectorant)
- Radiographic contrast media

**Food/Herbs (at high concentrations)**
- Alcohol
- Caffeine (methylxanthine)
- Cumin
- Dong quai
- Fenugreek
- Garlic, onion, ginger
- Ginseng
- Fish Oil
- Tamarind
- Turmeric
- Willow
- Vitamins C and E
- Black Tree Fungus (“Chinese mushroom”).

**N.B.:** This is only a partial list and many other agents are also known to affect platelet function. A full drug and relevant dietary history should always be taken for each subject tested for platelet function. If abnormal results are obtained then retesting can confirm if any defect is transiently acquired or not.
TABLE 2

A list of the basic and extended panels of platelet agonists used for LTA recommended starting concentrations and the range of final concentrations (after dilution into the PRP) normally used, the receptor target and effect of defects on the aggregation response

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Recommend Starting concentrations In PRP</th>
<th>Range of Final concentrations in PRP</th>
<th>Receptor Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline Panel</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>2.5 μM</td>
<td>0.5 – 20 μM</td>
<td>P2Y₁ and P2Y₁₂</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>5 μM</td>
<td>0.5 -10 μM</td>
<td>Adrenoreceptors</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>1 mM</td>
<td>0.5- 1.0 mM (single dose)</td>
<td>Testing Thromboxane generation and TX receptor</td>
</tr>
<tr>
<td>Collagen (type I tendon)</td>
<td>1.25 μg/ml</td>
<td>1.0 – 5.0 μg/ml</td>
<td>GpVI and GpIa/IIa receptors</td>
</tr>
<tr>
<td>Ristocetin</td>
<td>1.2-1.5 mg/ml</td>
<td>1.2-1.5 and 0.5-0.7 mg/ml (single doses)</td>
<td>GpIb/VWF axis</td>
</tr>
<tr>
<td><strong>Extended Panel</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma-thrombin</td>
<td>50-200 ng/ml</td>
<td></td>
<td>Thrombin receptors but without clotting</td>
</tr>
<tr>
<td>U-46619</td>
<td>1.0 μM single dose</td>
<td></td>
<td>Thromboxane receptor (TP₂)</td>
</tr>
<tr>
<td>Collagen-related peptide</td>
<td>10-1000 ng/ml</td>
<td></td>
<td>GpVI stimulation</td>
</tr>
<tr>
<td>Convulxin</td>
<td>1-1000 ng/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAP peptides</td>
<td>SFLLRN (PAR-1) 10-100 μM</td>
<td></td>
<td>PAR-1 and PAR-4</td>
</tr>
<tr>
<td></td>
<td>AYPGKF (PAR-4) 100-500 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium ionophore – A23187</td>
<td>1.25-10 μM</td>
<td></td>
<td>Calcium mobilisation and procoagulant function</td>
</tr>
<tr>
<td>PMA (Phorbol 12-myristate 13 acetate)</td>
<td>30 nM</td>
<td></td>
<td>Protein kinase C</td>
</tr>
</tbody>
</table>
### TABLE 3

Minimal Diagnostic Criteria for various platelet defects. The pattern of expected platelet function test results are listed for the most common platelet disorders. GT – Glanzmann Thrombasthenia, BSS - Bernard Soulier Syndrome. PCI – Prothrombin Consumption Index. ETP – Endogenous Thrombin Potential. The result for a potential P2Y1 defect are hypothetical as none described yet.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Platelet Count/Morphology</th>
<th>PFA</th>
<th>LTA Pattern</th>
<th>Nucleotides</th>
<th>Flow Cytometry</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1, 2A and 3 VWD</td>
<td>Normal</td>
<td>CADP/CEPI both equally prolonged. Very prolonged in 2A and 3.</td>
<td>Defective response to high dose ristocetin (correctable by VWF source).</td>
<td>Normal</td>
<td>Normal</td>
<td>VWF panel will confirm subtype</td>
</tr>
<tr>
<td>Platelet type or type 2B VWD</td>
<td>Normal</td>
<td>Both abnormal</td>
<td>Platelets aggregate on addition of plasma or cryoprecipitate Gain of function with low dose ristocetin</td>
<td>Normal</td>
<td>Increased VWF binding to platelets</td>
<td>Abnormal VWF panel in Type 2B VWD/ Loss of high MW VWF in platelet type VWD</td>
</tr>
<tr>
<td>GT</td>
<td>Normal</td>
<td>CADP/CEPI both very prolonged</td>
<td>Profound impairment to all agonists except high dose ristocetin</td>
<td>Normal</td>
<td></td>
<td>Significantly reduced copy number of ( \text{IIb} )( \text{IIIa} ) (variants, heterozygotes or defective functioning can be investigated)</td>
</tr>
<tr>
<td>BSS</td>
<td>Mild to moderate macrothrombocytopenia</td>
<td>CADP/CEPI both very prolonged</td>
<td>Defective aggregation to high dose ristocetin (not correctable by addition of VWF source)</td>
<td>Normal to high levels</td>
<td></td>
<td>Significantly reduced copy number of GpIb (heterozygotes can also be measured)</td>
</tr>
<tr>
<td>Dense Granule Defects</td>
<td>Low to normal count Reduced electron dense granules by whole mount EM</td>
<td>CADP normal CEPI sometimes prolonged</td>
<td>Decreased secondary aggregation to ADP and epinephrine</td>
<td>Increased ATP:ADP ratio with reduced ADP level. Reduced ATP release</td>
<td>Reduced Mepacrine uptake and release</td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Normal but with defective release, reduced ATP release by luminoaggregometry</th>
<th>Normal mepacrine uptake but defective release</th>
<th>Retest or defer for 10 days if patient taking aspirin or NSAIDs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secretion defect</strong></td>
<td>Normal</td>
<td>CADP normal CEPI normal, sometimes prolonged</td>
<td>Decreased secondary aggregation to ADP and epinephrine</td>
<td>Normal</td>
<td>Retest or defer for 10 days if patient taking aspirin or NSAIDs</td>
</tr>
<tr>
<td><strong>Aspirin-like defect</strong></td>
<td>Normal</td>
<td>CADP normal CEPI normal, sometimes prolonged</td>
<td>Absent arachidonic acid response but normal to U46619. Decreased secondary aggregation to ADP and epinephrine</td>
<td>Normal</td>
<td>Retest or defer for 10 days if patient taking aspirin or NSAIDs</td>
</tr>
<tr>
<td><strong>Thromboxane receptor defect</strong></td>
<td>Normal</td>
<td>CADP normal CEPI normal, sometimes prolonged</td>
<td>Absent Arachidonic Acid and U46619 response</td>
<td>Normal</td>
<td>Retest or defer for 10 days if patient taking aspirin or NSAIDs</td>
</tr>
<tr>
<td><strong>Giant platelet syndrome</strong></td>
<td>Macrothrombocytopenia</td>
<td>Sometimes abnormal</td>
<td>Normal response to ristocetin</td>
<td>Normal</td>
<td>Retest or defer for 10 days if patient taking aspirin or NSAIDs</td>
</tr>
<tr>
<td><strong>Collagen receptor defects</strong></td>
<td>Normal</td>
<td>Both abnormal</td>
<td>Decreased Collagen aggregation. Decreased CRP response if GpVI defect</td>
<td>Normal</td>
<td>Retest or defer for 10 days if patient taking aspirin or NSAIDs</td>
</tr>
<tr>
<td><strong>P2Y₁₂ defect</strong></td>
<td>Normal</td>
<td>Normal</td>
<td>Decreased ADP aggregation. Reversible response at high doses.</td>
<td>Normal</td>
<td>Retest or defer for 10 days if patient taking aspirin or NSAIDs</td>
</tr>
</tbody>
</table>

Higashi syndromes are autosomal recessive and associated with oculocutaneous albinism.
<table>
<thead>
<tr>
<th></th>
<th>Reduced secondary responses</th>
<th>other anti-P2Y12 drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P2Y&lt;sub&gt;1&lt;/sub&gt; defect</strong></td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Scott syndrome</strong></td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>
FIGURE 1: Illustration of how LTA patterns can be used to diagnose a range of rare platelet defects.

Note that these are generalised illustrations and actual patterns may differ slightly between patients with similar defects.

The 0% baseline (bottom of y axes) has been set with undiluted PRP and the 100% aggregation (top of y axes) limit set with autologous PPP. After establishment of a stable baseline for a few minutes the following agonists were added to these final concentrations, 10 μM ADP, 2 μg./ml collagen, 5 μM epinephrine, 1.0 mM arachidonic acid and low (0.5-0.7 mg/ml) and high dose (1.2-1.5 mg/ml) ristocetin. Aggregation was then monitored for up to 10 minutes (x axes from left to right) Example tracings are shown for a normal subject, a patient with Glanzmann thrombasthenia (GT), patients with VWD or Bernard Soulier Syndrome (BSS), Type 2B VWD/pseudo-VWD, GpVI deficiency, P2Y₁₂ deficiency, an aspirin-like defect, storage pool and release defects and a P2Y₁ defect. Normals will give an initial shape change (slight negative deflection) followed by a rapid and irreversible aggregation response to high concentrations of ADP. Lower doses can be used to determine the threshold for secondary aggregation. High dose collagen will give a characteristic lag phase followed by a shape change (slight negative deflection) followed by a rapid and irreversible aggregation. Epinephrine will give the classical biphasic response (primary and secondary aggregation) with no shape change. Arachidonic acid will give a shape change followed by full aggregation. Only high dose ristocetin will give a normal agglutination response in normals. The GT patient shows no aggregation to any agonist except high dose ristocetin (which is reversible). Flow cytometry and molecular biology can then be used to confirm the defect in αIIbβ3. Patients with BSS and VWD do not respond to ristocetin but VWD samples (but not BSS) will be correctable after addition of a source VWF to the plasma. VWD should be confirmed with a VWF panel of tests/molecular biology and BSS confirmed by flow cytometry and molecular biology. Type 2B and pseudo-VWD show the gain of function with a response to low dose ristocetin. A VWF panel/molecular biology will confirm type 2B VWD and molecular biology will confirm the diagnosis of pseudo-VWD in the GpIb gene. Gp VI deficiency gives no response to collagen which can be confirmed using CRP, by flow cytometry (see extended panel of agonists) or molecular biology. A P2Y₁₂ defect shows a reduced and
reversible response to ADP. In a homozygous P2Y$_{12}$ defect, only minimal primary aggregation to all concentrations of ADP will be present. In a heterozygous P2Y$_{12}$ defect there will be absence of secondary aggregation but disaggregation tends to begin < 10 μM ADP with a biphasic response to epinephrine. An aspirin-like defect will show reduced secondary aggregation responses to ADP and epinephrine and absent arachidonic acid aggregation. A defect in the thromboxane receptor can then be checked for using U46619 (see extended panel of agonists). Patients with storage pool disease or release defects will give an identical pattern as the aspirin-like defect except giving a good primary aggregation response to arachidonic acid. The P2Y defect pattern is based upon in vitro response to anti-P2Y$_1$ antagonists as no patients with a P2Y$_1$ defect have ever been described. This figure was significantly modified with permission from Figure 12.3, page 115 in Chapter 12 – Diagnostic Assessment of platelet function by P. Nurden and A. Nurden in Quality in Laboratory Hemostasis and Thrombosis edited by Kitchen S, Olson JD & Preston FE and published by Wiley-Blackwell in 2009.
(Figure 1)
(Figure 1 cont...)
Appendix 1: Grading of recommendations and levels of evidence:
The GRADE Nomenclature:

STRENGTH OF RECOMMENDATIONS:

**Strong (grade 1):** Strong recommendations (grade 1) are made when there is confidence that the benefits do or do not outweigh harm and burden. Grade 1 recommendations can be applied uniformly to most patients. Regard as 'recommend'.

**Weak (grade 2):** Where the magnitude of benefit or not is less certain a weaker grade 2 recommendation is made. Grade 2 recommendations require judicious application to individual patients. Regard as 'suggest'.

QUALITY OF EVIDENCE
The quality of evidence is graded as high (A), moderate (B) or low (C). To put this in context it is useful to consider the uncertainty of knowledge and whether further research could change what we know or our certainty.

**A** (High) Further research is very unlikely to change confidence in the estimate of effect. Current evidence derived from randomised clinical trials without important limitations.

**B** (Moderate) Further research may well have an important impact on confidence in the estimate of effect and may change the estimate. Current evidence derived from randomised clinical trials with important limitations (e.g. inconsistent results, imprecision - wide confidence intervals or methodological flaws - e.g. lack of blinding, large losses to follow up, failure to adhere to intention to treat analysis), or very strong evidence from observational studies or case series (e.g. large or very large and consistent estimates of the magnitude of a treatment effect or demonstration of a dose-response gradient).

**C** (Low) Further research is likely to have an important impact on confidence in the estimate of effect and is likely to change the estimate. Current evidence from observational studies, case series or just opinion.

More information on the GRADE nomenclature can be found at [http://www.gradeworkinggroup.org/index.htm](http://www.gradeworkinggroup.org/index.htm)
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David Keeling (Chair), Henry Watson (Secretary), Mike Laffan, Andrew Mumford, Ian Jennings, Isobel Walker, Elaine Gray, Campbell Tait, Mike Makris
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