Guidelines for the laboratory investigation of heritable disorders of platelet function

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Summary

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 2010 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 British Committee for Standards in Haematology (BCSH) 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 7 of the Procedure for Guidelines Commissioned by the BCSH [http://www.bcsh-guidelines.com/BCSH_PROCESS/EVIDENCE_LEVELS_AND_GRADES_OF_RECOMMENDATION/43_GRADE.html]. 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.

Keywords: platelets, platelet function, platelet disorders.

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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.

Introduction

The diagnostic evaluation of platelet disorders is complex, poorly standardized 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 platelet function analyser (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 (Moffat *et al*, 2005; Jennings *et al*, 2008; 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 standardized 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

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history and physical examination followed, when appropriate, by laboratory investigations. During this process it is essential to recognize 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) (Cattaneo, 2003; Hayward, 2008). Platelet disorders can also sometimes co-exist with other coagulation factor defects or VWD (Quiroga *et al*, 2007; Daly *et al*, 2009). 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.

Pre-analytical variables

Specimen collection

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 d after the last dose. Herbal remedies, garlic, alcohol and certain foods may also cause acquired platelet dysfunction (George & Shattil, 1991). Table I lists 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 standardized, 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).

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.

Table I. A list of drugs, compounds and dietary components/herbs that can affect platelet function (reprinted and modified with permission from Kottke-Marchant and Corcoran (2002) with permission from *Archives of Pathology and Laboratory Medicine*.

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) Non-steroidal 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) ($P2Y_{12}$)

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
- Vincristine

Psychotropics and Anaesthetics
- Tricyclic antidepressants (imipramine)
- Phenothiazines (chloropromazine)
- 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

Table I. (Continued).

Fish Oil
Tamarind
Turmeric
Willow
Vitamins C and E
Black Tree Fungus ("Chinese mushroom")

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.

Specimen processing. All specimens must be maintained at room temperature (RT; 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 six times (and discarded if there is any evidence of clotting). Tubes should be kept capped at RT 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 min and 2 h but not more than 4 h.

Recommendations

1. **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).**
2. **Collect blood using a standardized, atraumatic protocol, with minimal stasis (2C).**
3. **Use needles between 19 and 21 gauge; evacuated tube systems or syringes are acceptable (2C).**
4. **The first 3–5 ml of blood should not be used for platelet function tests (2C).**
5. **Use 105–109 mmol/l buffered trisodium citrate tubes (2C).**
6. **Maintain specimens at RT (1B).**
7. **Keep tubes upright and capped; do not subject to excessive mixing or agitation; do not use pneumatic transport systems (2C).**
8. **Samples should be tested between 30 min and no more than 4 h from blood collection (2C).**

Tests and assays

Laboratory tests for platelet disorders comprise:

1. Measurement of platelet number and size;
2. Global screening tests of platelet haemostatic function and
3. Specific assays of platelet haemostatic function.

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 mactothrombocytopenia (Harrison *et al*, 2000).

Global tests of platelet haemostatic function

Global tests of platelet function are often used during the investigation of individuals with pathological bleeding. As global tests do not enable a diagnosis of a specific platelet disorder, they are normally performed as the first part of a strategy that requires further testing with more specialized 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 specialized 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 antigen (VWF:Ag), Ristocetin cofactor activity (VWF:RCo) and factor VIII coagulant activity (F:VIII:C)] and measurement of platelet numbers). 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 (Bolton-Maggs *et al*, 2004; Laffan *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) provide global tests of haemostasis and platelet function and are mainly used within the surgical setting (Perry *et al*, 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.

Template bleeding time. The BT, described by Duke (1910) is the oldest test of platelet function. 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.

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 (5000–6000/s) through disposable cartridges containing an aperture coated with either collagen and epinephrine (CEPI) or collagen and ADP (CADP) (Kratzer & Born, 1985; Kundu *et al*, 1995; Jilma, 2001). 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 s. 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 (Moffat *et al*, 2005; Jennings *et al*, 2008).

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) (Heilmann *et al*, 1997; Harrison *et al*, 1999). 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-100® is published elsewhere (Harrison, 2004; Hayward & Eikelboom, 2007; Christie *et al*, 2008; Favaloro, 2009) There are extensive general reviews of the clinical utility of the PFA-100 (Hayward *et al*, 2006; Favaloro, 2008).

Knowledge of the full blood count is critical for interpreting CT results from the PFA-100®. Thrombocytopenia (<100 $\times 10^9$/l) and anaemia (<20% haematocrit) often results in prolongation of the CT (Kundu *et al*, 1995; Harrison *et al*, 1999). The 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 (Mammen *et al*, 1998; Harrison *et al*, 1999; Harrison, 2005; Hayward *et al*, 2006). 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 (Harrison *et al*, 2002; Hayward *et al*, 2006). 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 because most reported studies comprise small patient numbers, with varying mixtures of these defects (Harrison, 2005; Hayward *et al*, 2006). 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 (Cattaneo, 2004; Quiroga *et al*, 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, 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 have been provided by various international and national organizations (Bolton-Maggs *et al*, 2006; Hayward *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

1 **Perform a full blood count on all patients (1A).**
2 **In samples with abnormalities in platelet count or size distribution (as indicated by an automated analyser), a blood film should be examined (1B).**
3 **The bleeding time is not recommended (1B).**
4 **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).**
5 **Both PFA-100 CADP and CEPI cartridges should be used for screening (1B).**

Specific assays of platelet function

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 standardized and there are wide variations in laboratory practice (Moffat *et al*, 2005; Jennings *et al*, 2008; Cattaneo *et al*, 2009). Guidelines specific for LTA have also recently been published (Christie *et al*, 2008; Hayward *et al*, 2010; http://isth.org/default/assets/File/SSCMinutes/2010_MINUTES.pdf).

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 min in a swing-out rotor at RT without application of the brake. Autologous PPP is prepared by centrifugation (after removal of PRP or using whole samples) at a minimum of 1500 ***g*** for at least 15 min at RT (Christie *et al*, 2008). At the end of the centrifugation steps a plastic pipette should be used to separate the top two-thirds of PRP or PPP, which 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 min 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 $>600 \times 10^9$/l, the platelet count should not be adjusted using PPP, as this may cause artefactual inhibition of platelet aggregation (Cattaneo *et al*, 2007; Linnemann *et al*, 2008). Analysis of PRP with a platelet $< 150 \times 10^9$/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 II) (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 (TRAPs), Collagen-Related Peptide (CRP), 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 II). Most laboratories perform dose response curves for ADP (0·5–20 μmol/l), collagen (1·0–5·0 μg/ml) and epinephrine (0·5–10 μmol/l). 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) (Dawood *et al*, 2007; Hayward *et al*, 2009a). A recommended baseline example panel therefore comprises: 2·5 μmol/l ADP, 1·25 μg/ml collagen, 5 μmol/l epinephrine, 1·2 mg/ml ristocetin, and 1·0 mmol/l 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 glycoprotein (Gp)Ib defect. If arachidonic acid aggregation is abnormal then further testing should be performed with 1·0 μmol/l U46619 to test for any

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Table II. 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

Agonist	Recommended starting concentrations in PRP	Range of final concentrations in PRP	Receptor targets
Baseline panel			
ADP	2·5 μmol/l	0·5–20 μmol/l	$P2Y_1$ and $P2Y_{12}$
Epinephrine	5 μmol/l	0·5–10 μmol/l	Adrenoreceptors
Arachidonic acid	1 mmol/l	0·5–1·0 mmol/l (single dose)	Testing Thromboxane generation and TX receptor
Collagen (type I tendon)	1·25 μg/ml	1·0–5·0 μg/ml	GpVI and GpIa/IIa receptors
Ristocetin	1·2–1·5 mg/ml	1·2–1·5 and 0·5–0·7 mg/ml (single doses)	GpIb/VWF axis
Extended panel			
Gamma-thrombin		50–200 ng/ml	Thrombin receptors but without clotting
U-46619		1·0 μmol/l single dose	Thromboxane receptor (TPα)
Collagen-related peptide Convulxin		10–1000 ng/ml 1–1000 ng/ml	GpVI stimulation
TRAP peptides		SFLLRN (PAR-1) 10–100 μmol/l AYPGKF (PAR-4) 100–500 μmol/l	PAR-1 and PAR-4
Calcium ionophore – A23187		1·25–10 μmol/l	Calcium mobilization and procoagulant function
Phorbol 12-myristate 13 acetate		30 nmol/l	Protein kinase C

thromboxane receptor abnormalities. An extended panel of tests (usually only available within more specialized centres) could also be considered including gamma thrombin (which does not cause clotting), PAR-1 (SFLLRN) and PAR-4 (AYPGKF) TRAPs (if gamma thrombin is abnormal) CRP, calcium ionophore and phorbol 12-myristate 13 acetate if abnormalities in the thrombin receptors, GpVI, calcium mobilization and protein kinase C respectively, are suspected.

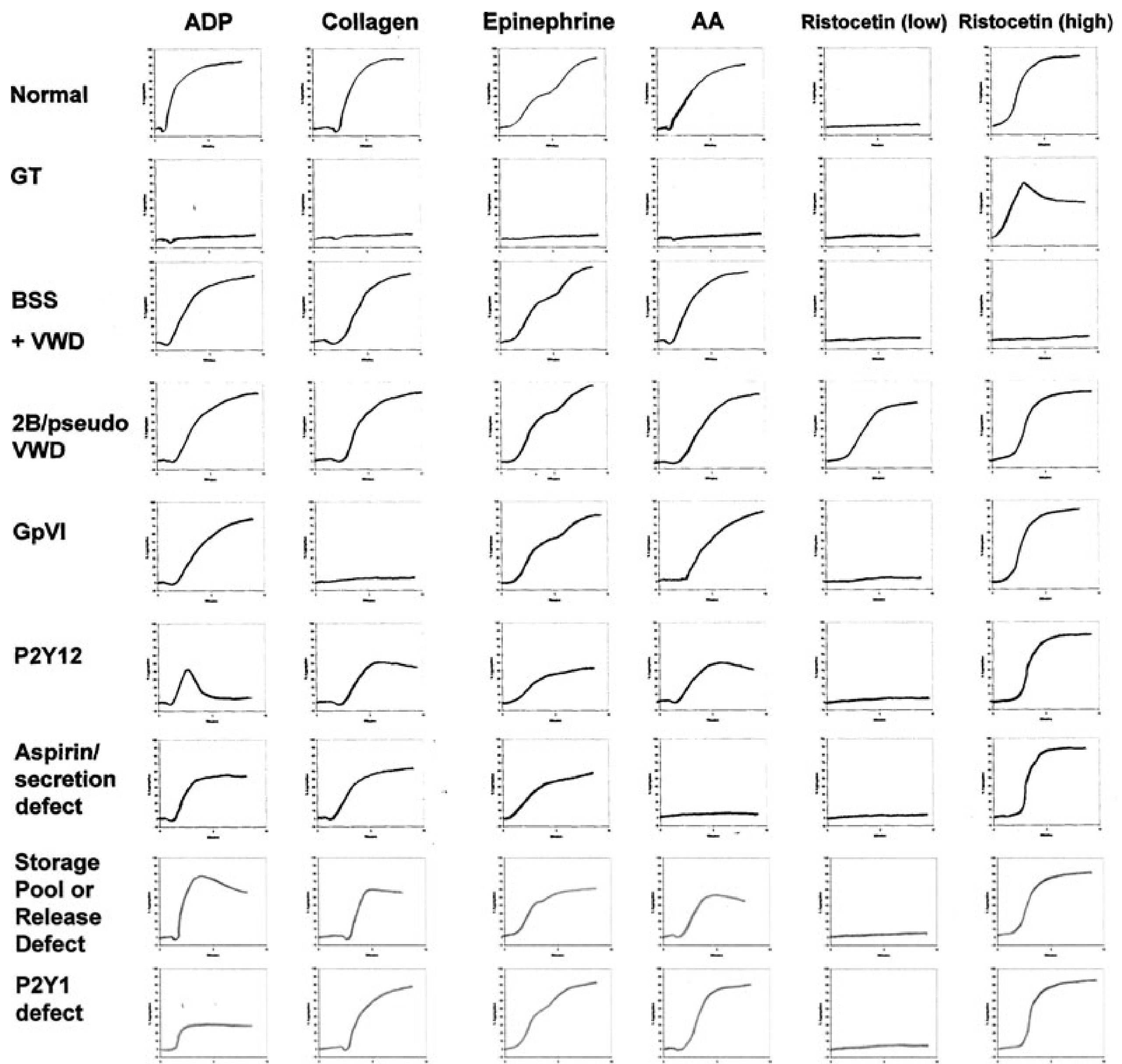
Performing aggregometry. A maximum of 1/10 volume of agonist is added to PRP to initiate aggregation and the final concentration of agonist within PRP is recorded (taking into account the 10-fold dilution factor). It is imperative that new batches of agonist are checked against the previous batch for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

Platelet aggregometers measure the change in optical density (or light transmittance) over time of stirred PRP in cuvettes at

Fig 1. Illustration of how LTA patterns can be used to diagnose a range of rare platelet defects. Note that these are generalized 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 μmol/l ADP, 2 μg/ml collagen, 5 μmol/l epinephrine, 1·0 mmol/l 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 min (*x* axes from left to right) Example tracings are shown for a normal subject, a patient with Glanzmann thrombasthenia (GT), patients with von Willebrand disease (VWD) or Bernard Soulier Syndrome (BSS), Type 2B VWD/pseudo-VWD, GpVI deficiency, $P2Y_{12}$ deficiency, an aspirin-like defect, storage pool and release defects and a $P2Y_1$ 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 *GP1BA/B* gene. GpVI deficiency gives no response to collagen, which can be confirmed using collagen-related peptide (CRP), by flow cytometry (see extended panel of agonists) or molecular biology. A $P2Y_{12}$ 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 μmol/l 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 Fig 12.3, page 115 in Chapter 12 – Diagnostic Assessment of platelet function by Nurden and Nurden (2009) in Quality in Laboratory Hemostasis and Thrombosis edited by Kitchen S, Olson JD and Preston FE and published by Wiley-Blackwell.

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 min 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 min, but preferably 10 min, 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 recognized 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*, 2008, 2009a). 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 Fig 1 for typical



examples of normal and abnormal aggregometry curves in various classical defects.

Recommendations

1 **Platelet counts > 600 × 10^9/l (1B) in platelet rich plasma should be diluted.**
2 **Repeat all unexpected, abnormal light transmission aggregometry tests with a fresh sample, in parallel with a normal control sample (2C).**
3 **Only experienced individuals should interpret tracings and results (1C).**
4 **Assess performance of new batches of agonists by comparison with a previous batch (1A).**

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 specialized 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 RT, 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 BSS 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 is ~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

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

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; Cattaneo, 2009; Hayward *et al*, 2009a). 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 (Moffat *et al*, 2005; Jennings *et al*, 2008). 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-Aggregometer (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 standardized 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 high performance liquid chromatography (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 specialized 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/10^9 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/10^9 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 standardized assays (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988; Zhou & Schmaier, 2005). Enzyme-linked immunosorbent assays (ELISA) for platelet serotonin content are also available. Mepacrine uptake and release by the dense granules can be measured by flow cytometry (Gordon *et al*, 1995; Wall *et al*, 1995).

Recommendations

1. **When there is a high clinical suspicion of a platelet function defect, adenine nucleotides should be measured even if the aggregation is normal (1B).**
2. **If aggregation results suggest storage pool disease or a release defect, measure stored and released nucleotides (1B).**

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 (Ingerman-Wojenski *et al*, 1983; Mackie *et al*, 1984; 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 μmol/l) give no impedance response. Reversible aggregation and biphasic responses to ADP cannot be demonstrated by whole blood impedance, (Ingerman-Wojenski *et al*, 1983; Mackie *et al*, 1984) while epinephrine responses tend to be absent or very weak, (Mackie *et al*, 1984; Swart *et al*, 1984). A study by the UK United Kingdom National External Quality Assessment Service 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, standardized 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.

Other tests

Platelet alpha granule proteins [e.g. Platelet Factor 4 (PF4) and Beta-Thromboglobulin (βTG)] can be measured by ELISA, radioimmunoassay 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*, 2009b).

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.b-ss.org/1.html and sinaicentral.mssm.edu). Clinical diagnostic services for GT and BSS by direct sequencing of polymerase chain reaction-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 $P2Y_{12}$ 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. As there is limited repertoire of reported mutations, it is usually difficult to assign pathogenicity to observed nucleotide variations without

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Table III. Minimal Diagnostic Criteria for various platelet defects. The pattern of expected platelet function test results are listed for the most common platelet disorders.

Disorder	Platelet Count/Morphology	PFA	LTA Pattern	Nucleotides	Flow Cytometry	Other
Type 1, 2A and 3 VWD	Normal	CADP/CEPI both equally prolonged. Very prolonged in types 2A and 3.	Defective response to high dose ristocetin (correctable by VWF source).	Normal	Normal	VWF panel will confirm subtype
Platelet type or type 2B VWD	Normal	Both abnormal	Platelets aggregate on addition of plasma or cryoprecipitate Gain of function with low dose ristocetin	Normal	Increased VWF binding to platelets	Abnormal VWF panel in Type 2B VWD/ Loss of high molecular weight VWF in platelet type VWD
GT	Normal	CADP/CEPI both very prolonged	Profound impairment to all agonists except high dose ristocetin	Normal	Significantly reduced copy number of αIIbβ3 (variants, heterozygotes or defective functioning can be investigated)	
BSS	Mild to moderate macrothrombocytopenia	CADP/CEPI both very prolonged	Defective aggregation to high dose ristocetin (not correctable by addition of VWF source)	Normal to high levels	Significantly reduced copy number of GpIb (heterozygotes can also be measured)	
Dense Granule Defects	Low to normal count Reduced electron dense granules by whole mount electron microscopy	CADP normal CEPI sometimes prolonged	Decreased secondary aggregation to ADP and epinephrine	Increased ATP:ADP ratio with reduced ADP level. Reduced ATP release by luminoaggregometry	Reduced mepacrine uptake and release	Reduced serotonin release Hermansky Pudlak and Chediak-Higashi syndromes are autosomal recessive and associated with oculocutaneous albinism
Secretion defect	Normal	CADP normal CEPI sometimes prolonged	Decreased secondary aggregation to ADP and epinephrine	Normal but with defective release. reduced ATP release by luminoaggregometry	Normal mepacrine uptake but defective release	
Aspirin-like defect	Normal	CADP normal CEPI normally prolonged (NB can by bypassed by high VWF levels)	Absent arachidonic acid response but normal to U46619. Decreased secondary aggregation to ADP and epinephrine	Normal		Retest or defer for 10 d if patient taking aspirin or NSAIDs
Thromboxane receptor defect	Normal	CADP normal CEPI sometimes prolonged	Absent Arachidonic Acid and U46619 response	Normal		
Giant platelet syndrome	Macrothrombocytopenia	Sometimes normal	Normal response to ristocetin	Normal to high	Normal-high receptor copy numbers per platelet	

Table III. (Continued).

Disorder	Platelet Count/Morphology	PFA	LTA Pattern	Nucleotides	Flow Cytometry	Other
Collagen receptor defects	Normal	Both abnormal	Decreased Collagen aggregation. Decreased CRP response if GpVI defect	Normal	Low GpIa/IIa or GpVI levels by flow cytometry	
$P2Y_{12}$ defect	Normal	Normal	Decreased ADP aggregation. Reversible response at high doses. Reduced secondary responses	Normal	Low $P2Y_{12}$ copy number using radioligand binding assay	Retest or defer if patient taking clopidogrel or other anti-$P2Y_{12}$ drugs
$P2Y_1$ defect	Unknown	Unknown	Decreased response to ADP – no shape change and curves not reversible	Normal		
Scott syndrome	Normal	Normal	Normal	Normal	Reduced expression of phosphatidyl serine on activated platelets by flow cytometry using Annexin-V	Reduced PCI and ETP

VWD, von Willebrand disease; VWF, von Willebrand factor; GT, Glanzmann Thrombasthenia; BSS, Bernard Soulier Syndrome; CADP, collagen and ADP; CEPI, collagen and epinephrine; NSAIDs, non-steroidal anti-inflammatory drugs; CRP, Collagen-Related Peptide; Gp, glycoprotein; PCI – Prothrombin Consumption Index; ETP – Endogenous Thrombin Potential.
The results for a potential $P2Y_1$ defect are hypothetical as none described yet.

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).

Diagnostic features

Typical clinical and laboratory findings of platelet function tests in many different platelet defects are detailed in Table III. For more diagnosis and treatment of all platelet disorders including the inherited thrombocytopenias see Bolton-Maggs *et al* (2006).

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, finger-pricks 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 first year of life. The available literature suggests that in infancy the platelets are generally hyporeactive (except to ristocetin and variably collagen) but beyond infancy, reactiveness 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 utilized 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).

Disclaimer

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