Screening Tests of Platelet Function: Update on their appropriate uses for diagnostic testing

Paul Harrison
Oxford Haemophilia & Thrombosis Centre
Churchill Hospital
Oxford, UK

10th February 2011
Namur Thrombosis and Hemostasis Center Inauguration
15:00-18:30
### Workshop Presenter Disclosure Information (2011)

**Speaker:** Paul Harrison, PhD, FRCPath

<table>
<thead>
<tr>
<th>Company Name</th>
<th>Relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sysmex UK</td>
<td>Consultant &amp; Winner of Sysmex Outstanding Science Award 2009</td>
</tr>
<tr>
<td>Instrumentation Laboratory (IL-UK)</td>
<td>Partner employed</td>
</tr>
<tr>
<td>Siemens Diagnostics</td>
<td>Research Grant</td>
</tr>
<tr>
<td>Eli Lilly</td>
<td>Research Grant</td>
</tr>
</tbody>
</table>
Table I. A suggested classification of the heritable platelet disorders.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>OMIM number*</th>
<th>Site of gene defect†</th>
<th>Estimated no. cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Disorders of platelet number</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe disorders of platelet function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wiskott–Aldrich syndrome</td>
<td>302000</td>
<td>WAS</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Glanzmann thrombasthenia</td>
<td>273800</td>
<td>ITGA2B, ITGB3</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Bernard–Soulier syndrome</td>
<td>231200</td>
<td>GP1BA, GPIBB, GP9</td>
<td>&lt;100</td>
</tr>
<tr>
<td><strong>Disorders of receptors and signal transduction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet cyto-oxygenase deficiency</td>
<td>605735</td>
<td>Unknown</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Thromboxane synthase deficiency</td>
<td>274180</td>
<td>Unknown</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Thromboxane A2 receptor defect</td>
<td>188070</td>
<td>TBXA2R</td>
<td>&lt;10</td>
</tr>
<tr>
<td>ADP receptor defect (P2Y12)</td>
<td>600515</td>
<td>P2RY12</td>
<td>&lt;10</td>
</tr>
<tr>
<td><strong>Disorders of the platelet granules</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic dense-granule disorder (δ-storage pool disease)</td>
<td>185050</td>
<td>Unknown</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Hermansky–Pudlik syndrome</td>
<td>203300</td>
<td>HPS1, AP3B1, HPS3, HPS4, HPS5, HPS6, DYNBP1, HPS8</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Chediak–Higashi syndrome</td>
<td>214500</td>
<td>LYST</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Grey platelet syndrome</td>
<td>139090</td>
<td>Unknown</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Paris–Trousseau/Jacobsen syndrome</td>
<td>188025 and</td>
<td>11q23 deletion (FLI1)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>147791</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic α- and dense-granule storage pool disease</td>
<td>185050</td>
<td>Unknown</td>
<td>&lt;100</td>
</tr>
<tr>
<td><strong>Disorders of phospholipid exposure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scott syndrome</td>
<td>262890</td>
<td>ABCA1</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Evaluation of Platelet Function

• **Clinical Evaluation**
  
  Site of Bleeding  
  Clinical History including drugs  
  Family History  
  Bleeding Score?

• **Laboratory Evaluation**
  
  Platelet Count  
  Blood Film  
  Bleeding Time  
  PFA-100  
  Impact - CPA  
  Platelet Aggregation  
    turbidometric or impedance  
  Stored and Released adenine nucleotides  
  Electron Microscopy  
  Flow cytometry  
  Molecular Biology  
  Research and New Methods
Laboratory techniques

Guidelines on platelet function testing

THE BRITISH SOCIETY FOR HAEMATOLOGY
BCSH HEMOSTASIS AND THROMBOSIS TASK FORCE

Following a questionnaire from the Haematology and
Thrombosis Task Force of the British Society for
Haematology in 1985, there was obviously consider-
able variability and confusion as to how haematolo-
gists in the United Kingdom investigated platelet
function. This document outlines a standardised
approach which could be followed by most routine
laboratories for the investigation of bleeding dis-
orders. Platelet release studies are included for
interest; these are not recommended for routine
laboratories. Platelet function studies used specifically
to investigate thrombotic disorders or an assumed
hypercoagulable state will not be discussed in these
guidelines. Tests which are primarily of a research
nature or are in use only at a highly specialised referral
unit are also not discussed. It is essential to have a
working knowledge of platelet physiology so that the
relevant platelet function tests can be performed in
an orderly sequence and interpreted correctly. For
this reason we have supplied some basic details on platelet
biochemistry and structure.

The bleeding time

BACKGROUND
When investigating patients suspected of having a
bleeding disorder, it is essential to obtain a detailed
clinical history before embarking on tests of haemo-
static function.

The peripheral platelet count, blood film examina-
tion, and the skin bleeding time are the first line basic
laboratory tests of platelet function. If these tests are
within normal limits it is unlikely that a clinically
important platelet defect is responsible for excessive
clinical bleeding.

A drug history is particularly important, and as far
as possible the use of drugs should be avoided when
platelet function is assessed. This applies particularly
to patients with congenital platelet disorders. In
acquired bleeding states the drugs that patients are
receiving may themselves be directly responsible for
the haemostatic defect. When this is suspected platelet
function should be assessed when the patient is both
off and on the drug. Recent aspirin ingestion is of
particular importance as a single dose may exert its
effect for up to 10 days. Other drugs which affect
the bleeding time include non-steroidal anti-inflammatory
agents, ticlopidine, heparin, penicillin (in high doses)
and the antibiotics carbenicillin and ticarcillin
(table 1).

The bleeding time is arguably the most useful test of
platelet function in that it provides clinically relevant
information about the contribution of platelets to
primary haemostasis.

How long it takes for bleeding to stop after a skin
incision is largely influenced by rapid accumulation of
metabolically active platelets at the site of the wound
and the formation of a haemostatically effective
platelet plug. The bleeding time reflects this process
and if it is performed in a standardised manner is
sensitive to changes in platelet function and platelet
numbers.

Several attempts have been made to improve the
sensitivity and reproducibility of the bleeding time
since its introduction in 1910 by Duke. In the original
method the ear lobe was punctured by a needle. Later,
between 1935–41, Ivy described a method which
consisted of three puncture wounds in the forearm.
Some improvement in sensitivity was achieved by the
application of a sphygmomanometer cuff, inflated to
Diagnostic Approach?

1) Clinical Evaluation

2) Primary Screening Tests
   - Full Blood Count, Blood Film, Bleeding Time, PFA-100, Impact, Clotting screen, VWF panel

3) Secondary Screening Tests
   - LTA, WBA, Stored and Released Nucleotides

4) Specialised Tests
   - Flow Cytometry, Electron Microscopy, Molecular Biology

5) Research & New Tests
   - e.g. Real Time Thrombus Formation
Bleeding Time

Normal Range 2 - 10 minutes

Bleeding Time is prolonged in classical platelet disorders, thrombocytopenia, severe anaemia, acquired disorders, and by some antiplatelet drugs.

Great Variability in results

Unreliable test, invasive, insensitive, unpopular with patients

Not suitable for repeat or consecutive analysis

Now Less popular - recent UKNEQAS survey Revealed many centres still use the BT
PFA-100® Platelet Function Analyzer

- Built-In Printer
- LCD screen
- Soft keys
- Trigger solution container
- Test Cartridge
- Cassette
- Carousel
In Vivo Haemostasis

Endothelium
Collagen
vWF
Platelet
Erythrocyte

PFA-100® Test Principle

PFA-100®

- 40 mbar
ADP / Epinephrine

Aperture
147 µm
Membrane
Collagen

Capillary
200 µm

FLOW
Shear Rate
5000 - 6000 s⁻¹

Haemostasis

PFA

In Vivo
Occlusion Process

T-15 SEC.  X370
T-45 SEC.  X370
T-80 SEC.  X370
T-110 SEC. X370
Platelet function analyzer (PFA)-100® closure time in the evaluation of platelet disorders and platelet function

C. P. M. HAYWARD, M. P. HARRISON, M. CATTANEO, T. L. ORTEL and A. K. RAO on behalf of the ISTH-SSC Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.

To cite this article: Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK on behalf of the ISTH-SSC Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Platelet function analyzer (PFA)-100® closure time in the evaluation of platelet disorders and platelet function. J Thromb Haemost 2006; 4: 312-9.

Summary. Background: Closure time (CT), measured by platelet function analyzer (PFA-100®) device, is now available to the clinical laboratory as a possible alternative or supplement to the bleeding time test. Aim: On behalf of the Platelet Physiology Subcommittee of the ISTH-SSC, this document reviews recent literature and provides consensus recommendations on the use of the PFA-100 CT in the evaluation of platelet function within the clinical laboratory. Method: The Medline database was searched to review the published information on the PFA-100 CT in the evaluation of platelet disorders and platelet function. This information, and expert opinion, was used to prepare a report and generate consensus recommendations. Results: Although the PFA-100 CT is abnormal in some forms of platelet disorders, the test does not have sufficient sensitivity or specificity to be used as a screening tool for platelet disorders. A role of the PFA-100 CT in therapeutic monitoring of platelet function remains to be established. Conclusions: The PFA-100 closure time should be considered optional in the evaluation of platelet disorders and function, and its use in therapeutic monitoring of platelet function is currently best restricted to research studies and prospective clinical trials.

Introduction

Closure time (CT), measured by platelet function analyzer (PFA-100®) device, is now available to the clinical laboratory as a possible alternative or supplement to the bleeding time test.

© 2006 International Society on Thrombosis and Haemostasis

Optional Screening Test for Platelet Disorders
What can the PFA-100 detect or do?


- Improvement on the BT
- Good sensitivity for VWD (III, IIA & IIB), GT and BS
- Variable sensitivity for Type I VWD (related to VWF level in plasma and platelets) – overall – 80-90%
- CEPI more sensitive than CADP for SPD and RD but overall sensitivity can be 50%
- CEPI but not CADP detects ASA – can be bypassed as non specific
- Cannot detect Clopidogrel and related compounds in current spec
- CEPI can predict surgical/clinical bleeding in some studies
IMPACT (immediate microscopic platelet adhesion cone and plate technology)

Based upon cone and plate(let) viscometer developed by Varon

A 130ul of whole blood (citrated) is placed in a polystyrene plate

Shear rate of 1800/s is applied for 2 minutes

Platelets adhere and aggregate on the plate surface

Washing and staining

The plate is placed under a microscope connected to an image analysis system and a computer

Successive images of different fields of the plate are taken and the percentage of the well covered by the stained objects and the average size of these objects are quantified.
NORMAL

SC=15% AS=50µm²

Glanzmann Thrombasthenia

SC=0% AS=0µm²

VWD Type III

SC=5% AS=24µm²

PRP

SC=0% AS=0µm²
Light Transmission Aggregometry (LTA)

- Invented by Born in 1960’s
- Regarded as the ‘Gold Standard’
- Useful for diagnosis of a wide range of platelet defects
- Labour Intensive - reagent and sample preparation
- Non Physiological - PRP and Low Shear
- Poorly standardised – range of agonists and concentrations.
  Recent Surveys - (UKNEQAS, CAP and ISTH Platelet Physiology SSC)
- Mainly used within specialized laboratories
- Modern Aggregometers – Computerized Multichannel instruments
- Increasing number of agonists available e.g. TRAP peptides, CRP etc
- Increasingly recognised that PRP dilution with PPP introduces artifacts
Platelet Aggregation

- Blood is centrifuged gently (170 g-200g) to obtain PRP
- PRP is stirred in a cuvette at 37°C between a light source and photocell
- Addition of agonist results in platelet aggregation - absorbance decreases and transmission increases as the platelets clump and form aggregates
- The addition of different agonists at a range of concentrations allows detection of certain defects
- Agonists all bind to their own specific receptor resulting in activation
  - Shape Change
  - Granule Release
  - Aggregation
- Above activation induced changes depends upon the normal platelet function
- Useful data: lag phase, aggregation rate (slope) and amplitude (maximal or after a set period of time e.g. 10 minutes
- Also threshold response or EC50 (dose that causes 50% of Max)
Indices of aggregation

Different indices reflect different underlying molecular processes
Platelet Agonists used for Turbidometric Aggregation

Standard/Minimal Panel of agonists

• ADP (range 0.5 - 10 µM)
• Collagen (range 1 - 5 µg/ml),
• Epinephrine (range 0.5 - 10 µM)
• Arachidonic Acid (Single dose 0.5 -1.6 mM)
• Ristocetin – Low Dose (< 0.6 mg/ml) and High Dose (0.8-1.5 mg/ml)

Optional/Other agonists

• Thrombin - α/GPRP or χ
• TRAP peptides (PAR-1 – SFLLRN or PAR-4- AYPGKF)
• Calcium ionophore
• U46619 – thromboxane analogue
• CRP – Collagen Related Peptide
• Serotonin, PAF and others
# Typical Patterns of Aggregation Responses to Various Inherited and Acquired Platelet Defects

<table>
<thead>
<tr>
<th>Disorder</th>
<th>ADP Primary</th>
<th>ADP Secondary</th>
<th>AA</th>
<th>Epi</th>
<th>Collagen</th>
<th>Ristocetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWD</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>Glanzmann thrombasthenia</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>N</td>
</tr>
<tr>
<td>Bernard Soulier syndrome</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>SPD</td>
<td>N</td>
<td>A</td>
<td>N</td>
<td>N, A</td>
<td>N, A</td>
<td>N</td>
</tr>
<tr>
<td>Secretion Defects</td>
<td>N</td>
<td>A</td>
<td>N, A</td>
<td>N, A</td>
<td>N, A</td>
<td>N</td>
</tr>
<tr>
<td>Scott syndrome</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Aspirin</td>
<td>N</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>N, A</td>
<td>N</td>
</tr>
<tr>
<td>P2Y12 inhibitors</td>
<td>A</td>
<td>A</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>GpIIb/IIIa antagonists</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>N</td>
</tr>
</tbody>
</table>

N = Normal, A = Abnormal
Reference curves for aggregation and ATP secretion to aid diagnosis of platelet-based bleeding disorders: Effect of inhibition of ADP and thromboxane A₃ pathways

BAN B. DAWOOD, JONATHAN WILDE, & STEVE P. WATSON

1Centers for Cardiovascular Sciences, Division of Medical Sciences, Institute of Biomedical Research, Walsden Drive, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK and 2Adult Haemophilia Centre, Queen Elizabeth Hospital, Birmingham B15 2TH, UK

(Received 13 September 2006; accepted 18 September 2006)

Abstract
Platelet aggregation is widely used in clinical laboratories to evaluate patients with bleeding disorders of suspected platelet etiology. Simultaneous monitoring of ATP release as a measure of dense granule secretion provides additional information to diagnose. There is, however, no standard way of performing or interpreting these tests. The present study has evaluated aggregation and ATP secretion to eight platelet agonists in healthy donors and has evaluated the reproducibility of response for a number of variables, including platelet number and time after donation. The effect of inhibition of the two major platelet feedback mediators, ADP and thromboxane A₃ (TXA₂), was investigated using the P2Y₁ and P2Y₁₁₁ receptor antagonists, MK2179 and AR-C6993, and the cyclooxygenase inhibitor, indomethacin. The results demonstrate that, if used within certain boundaries, the investigation of platelet aggregation and secretion is a powerful way to distinguish between differing pathways of platelet activation. The present data set can be an invaluable resource to the clinical laboratory to aid evaluation of patients with suspected platelet-based bleeding disorders.

Keywords: Platelet testing, platelet aggregation, dense granule secretion, ADP receptor, thromboxane A₃

Introduction
Platelets are critical elements in the maintenance of normal haemostasis. Abnormalities in platelet number or platelet function may result in excessive bleeding. Platelet function testing is routinely used in the clinic to diagnose patients with suspected platelet-based bleeding disorders. One of the most widely used tests for assessing platelet reactivity is platelet aggregation in platelet-rich plasma (PRP). However, there is no accepted way to perform or interpret these studies [1], with practices varying between laboratories as recently documented by a review of 46 North American Clinical Laboratories [2]. Variables include factors such as sample collection, platelet preparation, platelet number, agonist selection and agonist concentrations. Furthermore, the full value of aggregation testing is seldom achieved because of the use of a limited number of agonists and concentrations. Indeed, platelet aggregation methodology remained largely unchanged since the late 1980s despite important advances in our understanding of platelet activation. For example, the guidelines in the UK for investigation of patients with suspected platelet-based bleeding disorders, including information on platelet aggregation studies, were drawn up by British Society for Haematology Task Force in 1988 [3]. A more recent report on behalf of the Rare Haemostatic Disorders Working Party of the UK Haemophilia Centre Doctors Organisation (UKHCDO) summarizes heritable platelet disorders and gives guidelines on their analysis and clinical management [4]. This report emphasizes the importance of aggregation testing in clinical diagnosis but does not give experiential details on how to perform these studies. Further, development of expertise in platelet function testing is hampered by the small number of clinical patients who require testing and...
Figure 3.1. Aggregation and release tracings from a normal donor reflecting responses to typical agonists used in the laboratory. Deflection in baseline tracing indicates point at which agonist is added. Aggregation response is lower tracing; release response is upper tracing. Note classic biphasic response of epinephrine. ADP second wave is masked by strong response to exogenous agonist. Lumi-responses to both mAb7 and γ-thrombin are off the scale.
Figure 3.3. Aggregation and release tracings from a patient with storage pool disease (SPD). Note the relatively high response to ADP in the absence of release; this is due to the high concentration of ADP used that can mask release defects and SPD. The collagen response is high as well, indicating the potent aggregating power of this amount of collagen. Characteristic of SPD is the absent or significantly diminished release response.
Multiplate analyzer

- Compact (10" x 15" x 4")
- 5 independent channels
- Integrated computer
- Windows XP based software
- Automatic analysis and documentation
- Electronic pipetting available
firm adhesion and aggregation of platelets on the sensor surface enhances the electrical resistance between the 2 sensor wires
**Multiplate parameters**

- **aggregation Area under the curve = AUC**
- **test 1+2**
- **velocity**
- **time [min]**

- **most important parameter**
- **expressed in AU*min or U (10 AU*min = 1 U)**
blood sample for Multiplate analysis

- citrated blood is the typical sample anticoagulant used for platelet function analysis
- citrate complexes approx. 98% of the free calcium in the sample
- however calcium is an important second messenger of platelet activation
- therefore citrate has the potential to disturb platelet function tests
- for Multiplate analysis a tight attachment of platelets onto the impedance sensor is mandatory, which enhances the effect of calcium depletion on this method
- therefore the analysis of blood anticoagulated by hirudin or heparin is recommended
- hirudin blood collection tubes are commercially available from the manufacturer of Multiplate

Since hirudin and other direct acting thrombin inhibitors do not reduce the concentration of divalent cations in plasma, these can be considered to provide a more physiological environment than the use of sodium citrate. On this basis, we would agree with the recommendation by Dynabyte that the use of hirudin or another direct acting thrombin inhibitor is preferable for measurements of platelet aggregation performed by MEA (multiple electrode aggregometry = Multiplate). A. Johnson, Thromb Haemost. 2008 Jun;99(6):1127-9.
Multiplate tests 1/2

- Collagen
- Aspirin®
- Arachidonic Acid
- TXA2
- ADP
- PGE1
- COLtest
- ASPItest
- TRAPtest
- ADPtest
- ADPtest HS

Platelet activation:
- GpIIb/IIIa receptor exposure
- Degranulation

Platelet inhibitors:
- Aspirin®
- NSAR
- TXA2
- ArA
- TRAP

GpIIb/IIIa antagonists:
- Reopro® (abciximab)
- Aggrastat® (Tirofiban)
- Integrillin® (Eptifibatid)
Control (DMSO)

1 µM R138727

10 µM R138727
absent aggregation in all tests due to Glanzmann Thrombasthenia
Detection of P2Y12 Defect in a patient with a lifelong bleeding history?

Absence of Secondary Aggregation to all doses of ADP by LTA
Detection of P2Y12 Defect in a patient with a lifelong bleeding history?

Patient Control

ADP AUC = 24
ADP AUC = 53
ADPHS AUC = 9
ADPHS AUC = 31
ASP Test AUC = 64
ASP Test AUC = 53
Multiplate is a platelet function analyzer using whole blood

- platelet function is recorded with a single-use test cell with a double sensor unit
- the device has 5 channels for parallel determinations
- good sensitivity for anti-platelet drugs Aspirin®, Plavix® and ReoPro®
- Does not measure released nucleotides

Other applications include:
- the peri-operative assessment of platelet function
- evaluation of platelet disorders
- Clinical experience limited but promising

Experience growing

More studies required

Conclusion
Why do we need new guidelines?

• Last guideline published in 1988, few other guidelines

• Many new tests available now e.g. flow cytometry and PFA-100 etc

• BT shown to be a poor screening test

• Our knowledge of platelet biology and disorders has increased

• Newer agonists available e.g. TRAP peptides, CRP

• Recent Surveys - CAP, ISTH and UKNEQAS have all demonstrated that platelet function testing and LTA are all poorly standardised
New Guidelines

1) CLSI
   LTA, WBA & PFA-100 – now published

2) ISTH platelet physiology SSC
   LTA - completed - to be published

3) New BCSH guidelines
   Platelet Function Testing - In Prep
Screening Tests – BSCH Recommendations

• A full blood count is recommended on all samples

• Samples with abnormalities in platelet distribution and count should be checked on a blood film

• The BT is not recommended anymore

• The PFA-100 provides an optional screening test but test results should be interpreted with caution in conjunction with the clinical background as the test is not diagnostic or sensitive for mild platelet disorders and abnormal results can be frequent

• Consider performing Prothrombin Consumption Index
BSCH LTA Recommendations

• LTA should be performed in parallel with normal samples as an internal QC of all reagent preparations.

• Prepare PRP at 170-200 g for 10 minutes at RT – swingout rotor (no brake)

• Autologous PPP – 1500 g for > 15 mins at RT

• Keep PRP in capped tubes at RT, record platelet count, do not adjust platelet count

• Perform at 37°C, at 1000 rpm (with a stir bar) and monitor aggregation tracings for at least 5 - 10 minutes.

• Use baseline panel of agonists and depending upon pattern of results obtained with each agonist perform additional testing?

• In house normal ranges should be established for each concentration of agonist.
BSCH LTA Recommendations (2)

• Tracings and results should be interpreted by an experienced individual

• New batches of agonists should be verified for performance by comparison with a previous batch.

• Reproducibility between instruments (where applicable) and channels should be validated

• Regularly check instrument and individual channel linearity with diluted PRP and PPP mixtures

• Results > 100% suggest a problem with calibration or contaminating platelets in PPP.

• Stored/Released Nucleotides should be also be assessed by lumiaggregometry or Bioluminescence assay of platelet lysates
Platelet Nucleotide Measurement:

BSCH Recommendations

Both stored and released nucleotides should be measured in addition to aggregometry.

Lumiaggregometry cannot distinguish between a storage and a release defect.
Blood samples for LTA should be collected from subjects who:

- refrain from smoking for at least 30 minutes
- abstain from caffeine for at least 2 hours
- rest for a short period

- A record of all drugs that the subject has taken in the week prior to testing should be collected

- Treatment with drugs known to reversibly inhibit platelet function (e.g. NSAIDs) should be stopped at least 3 days before sampling

- Treatment with drugs known to irreversibly inhibit platelet function (e.g. aspirin, thienopyridines) should be stopped at least 10 days before sampling

- When treatment with drugs that inhibit platelet function cannot be stopped before sampling, drug-induced effects on platelet function should be considered when interpreting the LTA results

- It is uncertain whether blood samples for LTA should be collected from fasting patients, and whether treatment with any drug should be stopped before sampling
Blood samples for LTA should be drawn:

- with minimal or no venostasis
- using a needle of at least 21 gauge
- into plastic (polypropylene) or siliconized glass tubes
- into 109 or 129 mM sodium citrate, buffered anticoagulant
- The first 3-4 ml of blood drawn should be discarded or used for tests other than LTA
- When difficulties are encountered in obtaining sufficient blood for LTA, underfilled tubes may only be used to exclude severe platelet function disorders, such as Glanzmann Thrombasthenia or Bernard-Soulier Syndrome
Blood samples should be allowed to “rest” at room temperature for 15 min before centrifugation

Preparation of PRP for LTA:

- should be prepared by centrifuging blood samples at 200 x g for 10 min, at ambient temperature (approximately 21°C), without using a brake

- should be prepared by blood sedimentation for samples with very large platelets (it is uncertain whether it is advisable to keep the tubes at 45°)

Preparation of PPP for LTA: PPP should be prepared by centrifuging whole blood, or the tubes of blood from which PRP was removed, at ambient temperature, at 1500 x g for 15 min

Grossly hemolyzed samples should be discarded
If the sample tested is lipemic, the final report should indicate this

It is necessary to check the platelet count of the PRP sample tested
The results of LTA studies could be inaccurate when the platelet count in the PRP samples is lower than 150 x 10^9/L, therefore, caution should be taken when interpreting abnormal results in samples with low platelet counts.

PRP with low platelet counts may be tested to exclude severe platelet function disorders (BSS, type 2B and platelet type von Willebrand disease).

Platelet count of PRP samples should NOT be adjusted to a standardized value with autologous PPP (uncertain for PRP samples with platelet counts > 600 x 10^9/L).

LTA studies must include a known normal subject, run in parallel with the subject(s) under study.

After centrifugation, PRP samples should be allowed to sit at room temperature for 15 min before testing.

PRP should be used to set 0% light transmission in the aggregometer.

Autologous PPP should be used to set 100% light transmission in the aggregometer.

LTA studies should be performed at 37°C.

During LTA testing, PRP samples should be constantly stirred at 1,000 rpm using a disposable stirrer, unless otherwise specified by the manufacturer of the aggregometer.
Before adding an agonist, baseline tracings for LTA should be observed for oscillations and stability for at least 1 minute.

The volume of agonist added for LTA should be consistent, and never more than 10% of the total sample volume.

Platelet aggregation should be monitored for:

- a minimum of 3 minutes after adding an agonist
- a minimum of 5 minutes after adding an agonist that does not cause maximal aggregation by 3 minutes with most control samples
- a minimum of 10 minutes after adding an agonist that does not cause maximal aggregation by 5 minutes with most control samples

LTA studies should be completed within a maximum of 4 hours after blood sampling.
The following platelet agonist should be used for diagnostic LTA studies:

**ADP**: 2 µM (higher concentrations if abnormal results with 2 µM)

**Epinephrine**: 5 µM (higher concentrations if abnormal results with 5 µM)

**Collagen**: 2 µg/mL (Horm collagen) (higher concentrations if abnormal results with 2 µg/mL)

**Thrombin Receptor Activating Peptide (TRAP)**: 10 µM (higher concentrations if abnormal results with 10 µM)

The **thromboxane A2 mimetic U46619**: 1 µM (higher concentrations if abnormal results with 1 µM)

**Arachidonic acid**: 1 mM (higher concentrations if abnormal results with 1 mM)

**Ristocetin**: 1.2 mg/mL

- In case platelet agglutination induced by Ristocetin 1.2 mg/mL is normal, testing should be repeated using **Ristocetin 0.5-0.7 mg/mL**

- In case platelet agglutination induced by Ristocetin 1.2 mg/mL is absent, testing should be repeated using **Ristocetin 2 mg/mL**.
The platelet aggregation tracing should be evaluated based on:

- presence of shape change
- length of the lag phase
- slope of aggregation
- maximal amplitude or % aggregation
- amplitude or % aggregation at the end of the observation
- disaggregation
- visual examination of the aggregation tracings

The presence of a "secondary wave" induced by epinephrine should be evaluated.

Studies completed more than 4 hours after blood collection should be reported with a comment of this.

Clinical laboratories must establish an appropriate reference interval and validate test performance with each lot of reagents.
New ISTH SSC WP on *Diagnosis of Congenital Platelet Function Disorders*

- What patients should be screened for platelet function disorders?
  - Type of bleeding manifestations
  - Usefulness of bleeding scores?
  - Criteria orienting towards an inherited defect
  - Presence of associated alterations in other cells/organs
  - Any role for global tests of primary hemostasis?
  - Do we need to rule out other bleeding disorders (e.g., VWD) before studying platelet function?
  - Drug history

- What first-line screening tests should we use?
  - Should platelet secretion be measured in parallel with platelet aggregation in all patients?

- What second-line, confirmatory tests should we use to test the diagnostic hypothesis that was raised based on the results of the first-line screening tests?

- Proposal of a diagnostic algorithm
Conclusions

• LTA remains the “gold standard” for diagnosing platelet disorders

• Since the late 1980’s, many alternative tests have been developed

• Some are now widely used in clinical practice
  e.g. Flow cytometry, PFA-100 and Multiplate

• Quality assurance increasingly recognised

• New platelet function testing and LTA guidelines becoming available

• This should lead to improvements in inter-laboratory practice and the diagnosis of platelet disorders
Acknowledgements

CLSI

ISTH platelet physiology SSC

BCSH

David Keeling
Kampta Sukhu
James Beavis
Abbie Edwards

Oxford Haemophilia & Thrombosis Centre