Laboratory investigation of platelet function disorders

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Objectives

1. To develop an approach to investigation of platelet disorders
2. To understand the utility and limitations of commonly used platelet function assays
3. To consider the future of diagnostic testing for platelet disorders
In vivo activation

Primary Hemostasis

SECRETION
- Release of
  - glycoproteins
  - vWF
  - fibrinogen
  - coagulation factors
- Release of
  - ADP
  - serotonin
  - calcium
  - etc

PROCOAGULTANT
- Flip Flop
- PS Exposure
- Microvesicle generation

ACTIVATION

AGGREGATION
- GPIb-IX receptor-complex
- von Willebrand - Factor
- collagen fibers

ADHESION

endothelial cell

(Harrison, Blood Rev 2005; 19:111)
Prevalence of platelet function disorders:

- There are no population-based studies.
- Prevalence studies hampered by:
  - Access to testing
  - Comprehensiveness of testing: heterogeneity of disorders
  - Quality of testing: standardization and interpretation

**Registry Data**

<table>
<thead>
<tr>
<th>Registry Type</th>
<th>Year</th>
<th>PD</th>
<th>VWD</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK registry (2012)</td>
<td></td>
<td>1575</td>
<td>9377</td>
</tr>
<tr>
<td>Canadian registry (2014)</td>
<td></td>
<td>802</td>
<td>3963</td>
</tr>
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</table>
Prevalence of platelet function disorders:

Quiroga et al. Haematologica 2007;92:357

280 bleeders and 299 controls
Clinical histories and bleeding scores
Bleeding times
VWD testing, clotting factor testing
Platelet aggregation and secretion studies
Clot lysis assays

RESULTS in 280 patients
VWD 17.9%
PFD 23.2%
Factor deficiencies 3.9%
BT prolonged 18.6%
No laboratory abnormality 36.4%

RESULTS in 299 controls
2.5% had decreased VWF
7% had decreased platelet aggregation or secretion
Pre-pre-analytical variables:
Does the patient require testing?

- Why are you testing?

- Patient and family history
  - Clinical associations

- Medication history
  - Can the patient stop their medication?
  - Should the patient stop their medication?

<table>
<thead>
<tr>
<th>Healthy Individuals (n &gt; 700)</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epistaxis</td>
<td>5-11</td>
</tr>
<tr>
<td>Menorrhagia</td>
<td>17-44</td>
</tr>
<tr>
<td>Dental bleeding</td>
<td>5-11</td>
</tr>
<tr>
<td>Hematomas</td>
<td>12</td>
</tr>
<tr>
<td>Gum bleeding</td>
<td>7-37</td>
</tr>
<tr>
<td>Post-surgical bleeding</td>
<td>1-6</td>
</tr>
<tr>
<td>Post-partum bleeding</td>
<td>3-23</td>
</tr>
<tr>
<td>Joint bleeding</td>
<td>6</td>
</tr>
</tbody>
</table>

(Rydz & James, JTH 2012)
Bleeding assessment tools

Does the patient require testing?

• Standardized questionnaires to:
  1. Improve diagnostic accuracy
  2. Predict bleeding risk
  3. Describe symptom severity

– Which BAT to use?
  • Global bleeding symptom BATs
  • Focused BATs (eg, menorrhagia, pediatric)

– Can we use them as a screening tool to know who we should be testing?
  • Maybe. Very good NPV.

– Can they direct us to the specific diagnosis?
  • No. Poor specificity.

Rodeghiero et al, JTH 2010
Rydz & James, JTH 2012
Lowe et al, JTH 2013
Laboratory testing for PFD


- Global survey of 202 laboratories in 37 countries
- 14,000 patients investigated yearly: PFD identified in 40%

Initial laboratory testing
Global tests of hemostasis

Bleeding Time

- Invasive
- Poorly reproducible
- Not specific
- Poor sensitivity for common PFD

Platelet Function Analyzer

- Simple
- Reproducible
- Not specific
- Even less sensitive to common PFD
CBC and blood film

- Platelet count
- MPV
- Blood film
In vivo activation

(www.youtube.com/watch?v=0pnpoEy0eYE)

(Rand, Israels, McNicol, 2010)
Light transmission aggregometry (LTA)

(Jackson, Blood 2007;109:5087)
Platelet aggregometry

- Lack of standardization
  - Surveys of existing practices: 2005-2012

Issues that required attention:
1. Patient screening
2. Collection and sample preparation
3. Agonist panel and concentrations
4. Laboratory specific reference intervals and controls
5. Interpretation of results
6. Quality assurance/EPT
Standardization recommendations and guidelines:

- **Clinical and Laboratory Standards Institute**: Christie et al. Platelet function testing by aggregometry: approved guideline. [www.clsi.org](http://www.clsi.org) 2008

- **NASCOLA**: Hayward et al. Development of NA consensus guidelines for medical laboratories that perform and interpret platelet function testing using light transmission aggregometry. *AJCP 2010*

- **British Committee for Standards in Haematology**: Harrison et al. Guidelines for the laboratory investigation of heritable disorders of platelet function. *BJH 2011*

- **ISTH Platelet Physiology Subcommittee**: Cattaneo et al. Recommendations for the standardization of light transmission aggregometry. *JTH 2013*
Pre-analytical variables

- Instructions for patients on day of collection
- Pre-collection medication questionnaire
- Sample collection
- Sample transport/handling
- PRP/PPP preparation and quality assessment
- Standardization of the platelet count
  - ISTH SSC Survey 2009 results showed that 95% of clinical laboratories adjusted the platelet counts in PRP.
  - Recent studies show that there is no advantage to adjusting PRP platelet counts and addition of autologous PPP may inhibit aggregation.
  - Published guidelines provide contrary advice on adjustment of platelet counts.
Analytical variables

- Standard settings for temp, rpm, etc
- Baseline tracings should be observed for oscillations and stability before the addition of agonists
- LTA should be completed within 4 hours of collection of the sample.
- Comparison with normal controls
- A basic testing panel with single concentrations of standard agonists
  - Published guidelines give a range of recommended agonist concentrations
  - Laboratory-determined reference intervals for % maximal aggregation for each agonist and concentration

### Agonist

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>5 µM</td>
</tr>
<tr>
<td>ADP</td>
<td>2.5 µM</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.5 µg/mL</td>
</tr>
<tr>
<td>Arach. Acid</td>
<td>1 mM</td>
</tr>
<tr>
<td>U46619</td>
<td>1 µM</td>
</tr>
<tr>
<td>Ristocetin</td>
<td>1.2 mg/mL</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/mL</td>
</tr>
</tbody>
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(Zhou & Shmaier, AJCP 2005)
Extended agonist panels are not clearly better than streamlined panels of LTA agonists

- Testing algorithms
- Interpretation

Hayward et al, JTH 2009
Dawood et al, Blood 2012
Diagnosis Value of LTA

- Goal: to distinguish PFD from no PFD
- Studies evaluating diagnostic utility of LTA
  - Consensus on definition of patients vs. controls
  - Additional laboratory information in defining the patients vs controls
  - Agonist panels and definition of abnormal results
- LTA alone will not identify all patients with PFD
- The most useful additional test is a measure of granule secretion

**NOT for the evaluation of antiplatelet therapy**

Hayward et al, JTH 2009
Quiroga et al, Br J Haematol 2009
Dawood et al, Blood 2012
Investigating platelet secretion defects

Frequently, but not always demonstrate abnormal LTA

The frequency of release abnormalities in the setting of normal LTA results varies across studies from 14.3% to 56%.

ATP release assay measures DG secretion

ATP + D-luciferin + O₂ → AMP + PPI + oxyluciferin + CO₂ + light
Investigating platelet secretion defects

- Not all guidelines address secretion assays
- Evidence for improved sensitivity for diagnosis PFD when LTA and secretion assays are combined.
- How to best use secretion assays:
  - Should all patients have secretion assays done in conjunction with LTA?
  - Should patients with LTA results suggestive of a secretion defect have secretion assays done?
  - Should patients with normal LTA but a high pre-test probability of PFD have secretion assays done?
Investigating platelet secretion defects

Release defect or DG deficiency?

DG quantification by EM whole mount, mepacrine labeling, biochemical measures of ATP/ADP, or $^{14}$C-serotonin uptake.
External proficiency testing

• An additional challenge
  – College of American Pathologists (CAP)
    o Platelet Function Analyzer
    o LTA
  – NASCOLA
    o Interpretation of aggregation tracings
      – Case-based
    o Dense granule enumeration
      – Electron micrographs

Hayward et al, Sem Thromb Hemost 2012
Flow cytometric analysis

RESTING

α-granules
δ-granules
GPIb-IX-V
CD62P
CD63

ACTIVATED

↑ CD62P
↑ αIIbβ3
↓ GPIb-IX-V
↑ CD63
↑ PS

Fibrinogen

- Membrane receptors
- Dense granule enumeration

- Platelet activation
  - Granule exocytosis
  - Fibrinogen binding
  - Procoagulant surface development
  - Microparticle release
Other platelet function tests

Roche Diagnostics

Haemonetics

PlaCor, Inc

Accumetrics
The role of genetic testing

• **THE BAD NEWS:** The combination of clinical assessment, and laboratory evaluation still leaves approximately 50% of patients with evidence of a platelet disorder without a specific diagnosis.

• **THE GOOD NEWS:** Characterization of molecular defects in recognized disorders and in previously unrecognized disorders is advancing, aided by powerful and efficient genomic analysis.
Clinical utility of genetic testing?

It depends on what you have:

1. Well characterized syndromic conditions with known genetic cause
2. Well characterized pedigree with definitive phenotype
3. Everybody else

Genetic variation and platelet phenotype

• SNP associations with platelet count, volume or reactivity
• Multiple SNPs associated with one trait
• Identified loci not previously known to be associated with platelet traits
Summary

1. Platelet disorders are common; diagnostic evaluation is a challenge.
2. Screening assays are neither sensitive nor specific; BAT may more helpful in determining who requires further investigation.
3. Aggregometry and secretion assays are the most useful functional assays for the clinical laboratory but will still leave a portion of patients without a definitive diagnosis.
4. Improved understanding phenotype-genotype relationships will move us towards clinically useful genetic diagnostics.