LABORATORY APPROACH TO PLATELET DISORDERS

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Disclosures
for Catherine P. M. Hayward

• No financial or conflicts of interest to disclose
Objectives

Review laboratory testing for platelet disorders, including:

- Causes of platelet disorders and typical laboratory findings
- Focus: inherited conditions, some important acquired disorders where testing is key
- Pitfalls
Platelet Disorders

- Many inherited disorders alter platelet numbers and/or function
- Important causes of abnormal bleeding
- Estimated prevalence ~1-6/1000
  - some forms (e.g. dense granule deficiency) have similar prevalence to von Willebrand disease (VWD)
- Laboratory testing is complex
  - relies on “laboratory developed methods/strategies”
  - no simple kits or methods
Pathogenesis of Inherited Thrombocytopathies

- Complex! Platelets contain >1000 proteins
- Genome wide association studies
  - Many genes affect platelet function
- Disorders: often a defect or deficiency of a protein affecting platelet formation, function and/or numbers
  - Rare causes with striking aggregation defects are largely characterized
  - Causes of common disorders that impair platelet secretion and aggregation is only emerging
**Guidelines for Laboratory Testing**


3. **NASCOLA guideline:** Hayward CPM et al. Development of North American consensus guidelines for medical laboratories that perform and interpret platelet function testing using light transmission aggregometry. AJCP, 2010;134:955-63**


** addresses test interpretation
Platelet function analyzer (PFA)-100® closure time in the evaluation of platelet disorders and platelet function

C. P. M. HAYWARD,*† P. HARRISON, ‡ M. CATTANEO,§ T. L. ORTEL¶ and A. K. RAO**†† ON BEHALF OF THE PLATELET PHYSIOLOGY SUBCOMMITTEE OF THE SCIENTIFIC AND STANDARDIZATION COMMITTEE OF THE INTERNATIONAL SOCIETY ON THROMBOSIS AND HAEMOSTASIS

*Chair, Working Group on the PFA-100®, ISTH-SSC Platelet Physiology Subcommittee; †McMaster University and the Hamilton Regional Laboratory Medicine Program, Hamilton, ON, Canada; ‡Oxford Haemophilia Centre and Thrombosis Unit, Churchill Hospital, Oxford, UK; §Unità di Ematologia e Trombosi, Ospedale San Paolo, DMCO, Università di Milano, Milan, Italy; ¶Duke University Medical Center, Durham, NC, USA; **Chairperson, ISTH-SSC Platelet Physiology Subcommittee, 2001–2004; and ††Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, USA
Platelet function analyzer (PFA)-100 closure time guideline

- Key recommendations related to inherited platelet disorders
  - Test: considered optional
    - Insufficient sensitivity and specificity to be used for screening to determine which individuals need further testing for platelet disorders
      - *Is this followed in all practice settings?*
    - Abnormal findings can reflect other conditions
      - Anemia, von Willebrand disease, etc.
  - Uncertain role in predicting clinical outcomes and/or therapy monitoring for other disorders
Light transmittance platelet aggregometry

- Important assay for diagnosing platelet disorders
- Detects many but not all platelet function disorders
Aggregometry: Realities of Practice

• Need better method standardization
• Laboratories have greater difficulty with aggregometry interpretation than with interpreting other platelet tests (e.g., electron microscopy tests for platelet dense granule deficiency)
• Performance is particularly problematic for interpreting common aggregation findings and abnormalities

Hayward, Moffat, Plumhoff, Timleck, Hoffman, Spitzer, Van Cott, Meijer. STH 2012;38(6):622-31
Sensitivity and Specificity of LTA as an Initial Test for Bleeding Disorders

- Hamilton Regional Laboratory Medicine Program (HRLMP) Study
- Approach
  - Estimated sensitivities and specificities of lab tests for bleeding disorder diagnosis, using a prospective cohort study design (Hayward, Moffat, Liu. STH 2012;38(7):742-52)
  - Standardized panel of initial investigations
  - Light transmission aggregometry (LTA) included in the panel to assess for platelet disorders at the same time as testing for VWD and coagulation defects
## Sensitivities and Specificities

### Bleeding Disorder Panels

Hayward, Moffat, Liu. STH 2012;38(7):742-52

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT, PT/INR, Fibrinogen, Thrombin Time</td>
<td>3.0%</td>
<td>98%</td>
</tr>
<tr>
<td>APTT, PT/INR, Fibrinogen, Thrombin Time &amp; VWD Screen</td>
<td>7.8%</td>
<td>95%</td>
</tr>
<tr>
<td>APTT, PT/INR, Fibrinogen, Thrombin Time, VWD Screen &amp; LTA</td>
<td>29%</td>
<td>93%</td>
</tr>
</tbody>
</table>

**Practice points:**

1. Tests for primary hemostatic defects (i.e., LTA, VWD screens) are needed for the panel to have reasonable detection of common bleeding problems (factor deficiencies are important but less common causes of bleeding)
2. Some disorders are diagnosed without these tests (e.g. aspirin-induced bleeding, ITP, etc.) or by other tests (e.g., disorders of fibrinolysis)
3. Doing VWD screens and LTA at the same time reduced false negatives
USEFULNESS OF TESTS FOR PLATELET DISORDERS

Data from Prospective Studies
Hayward et al, JTH 2009;7;676-84; Castilloux et al, TH 2011;106(4):675-82;
Pai et al AJCP 2011;136(3):350-8

• Aggregometry – LTA abnormalities with ≥ 2 agonists (based on well established reference intervals)
  • 2009: Odds Ratio 32 for a bleeding disorder (95% CI: 4-245)
  • 2011: Odds Ratio 23 for a bleeding disorder (95% CI: 5-105)

• How does this compare to other tests?
  • Dense granule ATP release by Lumi-Aggregometry
    • Will review in detail later

  • Dense granule deficiency (DGD) testing by electron microscopy
    • completely predictive of a platelet disorder (95% CI: 1.12-infinity)
      Many true negatives, rare false positives

  • Bleeding time (BT) – NOT recommended
    • Odds Ratio 3.7 for a platelet function disorder (95% CI: 1.4-9.6)
Principle of aggregation tests

Test evaluates platelet aggregate formation, in response to multiple agonists, using either:

- **Platelet rich plasma (PRP)** - optical or light transmission (LTA) method (“Born” aggregometry)
  - When platelets aggregate, the percentage of light transmission through the sample increases

- **Whole blood** - electrical impedance methods
  - When platelet stick to the electrode, it changes the impedance
  - Not identical to LTA
    - e.g., with collagen, both platelets and WBC stick to the electrode
  - Has not been validated as much as LTA for diagnostic purposes
Aggregometry (citrated PRP) with measurement of ATP release

Evaluate results for:

1) Maximal aggregation (MA)
2) ATP release (nM), using a luciferin/luciferase method (with an ATP standard). Note: ADP and ATP are both stored in dense granules.

Strong agonist: immediate ATP release
Weak agonist: ATP release with secondary aggregation

% Maximal aggregation

Time (min:sec)

Aggregation
6 μM epinephrine
5 μg/mL collagen
ATP release
ATP release is delayed and less complete with epinephrine

d-Luciferin + ATP
Firefly luciferase
Oxyluciferin + AMP + light
Whole blood aggregometry with measurement of ATP release

**NOTE:**
Ristocetin causes agglutination but this is followed by dense granule release and aggregation.
How should labs interpret the aggregation findings?
### Evaluation of LTA – Maximal Aggregation (MA) Findings Are Important

Almost NEVER see an abnormal tracing without abnormal MA

<table>
<thead>
<tr>
<th></th>
<th>RI %MA</th>
<th>Glanzmann Thrombasthenia</th>
<th>Secretion Defect Case 1</th>
<th>Secretion Defect Case 2</th>
<th>Thromboxane Generation Defect (e.g., Aspirin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP 5 µM</td>
<td>&gt;43</td>
<td>0</td>
<td>56</td>
<td>44</td>
<td>71</td>
</tr>
<tr>
<td>Collagen 5 µg/mL</td>
<td>&gt;85</td>
<td>0</td>
<td>83</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>Collagen 1.25 µg/mL</td>
<td>&gt;51</td>
<td>0</td>
<td>43</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Epinephrine 6 µM</td>
<td>&gt;8</td>
<td>0</td>
<td>6</td>
<td>15</td>
<td>36</td>
</tr>
<tr>
<td>Arachidonic Acid 1.6 mM</td>
<td>&gt;77</td>
<td>0</td>
<td>84</td>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td>Thromboxane analogue 1 µM*</td>
<td>&gt;70</td>
<td>0</td>
<td>21</td>
<td>21</td>
<td>94</td>
</tr>
<tr>
<td>Ristocetin 0.5 mg/Ml</td>
<td>&lt;7</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Ristocetin 1.25 mg/mL</td>
<td>&gt;75</td>
<td>47</td>
<td>76</td>
<td>84</td>
<td>90</td>
</tr>
</tbody>
</table>

*reduced MA with thromboxane analogue U46619 is almost always accompanied by significant deaggregation
All RI: 95% confidence intervals (takes into account that 1/5 individuals don’t have a secondary wave with epinephrine)
ADP and epinephrine responses – don’t follow a normal distribution
Evaluation of LTA findings

Several prospective studies:

• Reduced maximal aggregation (MA) with ≥2 agonists is associated with a bleeding disorder

• Reduced MA with a single agonist (except collagen and ristocetin) often represents a false positive
  Exceptions: ristocetin, collagen – reduced aggregation can have biological reasons

Hayward et al, JTH 2009;7;676-684
Castilloux et al, TH 2011;106(4):675-82
Guidance document for evaluating light transmittance aggregation (LTA) results


First questions to ask:
  Was LTA done in accordance with guidelines?
  Was the sample tested at usual platelet count?

Next question: are any maximal aggregation findings outside the Reference Interval (RI)?

  • No – test is nondiagnostic
  • Yes, but with only one agonist
    • With ristocetin? is it VWD? Bernard Soulier syndrome? Congenital or acquired?
    • With collagen? Collagen receptor defect?
    • Other - False positive is likely
  • Yes, and with two or more agonists
    • What is the pattern?
      • Aspirin? Hallmark - Impaired MA with AA, normal MA with thromboxane analogue
      • Glanzmann thrombasthenia? Impaired aggregation with all agonists, aggregation present with ristocetin
      • P2Y12 (ADP) receptor type abnormality? Rare…
      • Other? Typical of “SECRETION DEFECTS” – most common
<table>
<thead>
<tr>
<th>LTA Finding (from AJCP 2010;134(6):955-63)</th>
<th>Recommended Interpretation</th>
<th>Follow-up Investigation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or marked ↓ with AA, normal with U46619, ↓ with low dose collagen.</td>
<td>Aspirin-like defect (drug induced or inherited).</td>
<td>Repeat testing when subject not on NSAIDs.</td>
</tr>
<tr>
<td>Aggregation is present with only ristocetin.</td>
<td>Possible Glanzmann thrombasthenia (inherited or acquired).</td>
<td>Glycoprotein analysis of fibrinogen receptor αIIbβ3.</td>
</tr>
<tr>
<td>Aggregation absent with high conc. of ristocetin and patient has thrombocytopenia with very large platelets.</td>
<td>Possible Bernard Soulier Syndrome (inherited or acquired). VWD should be excluded.</td>
<td>Glycoprotein analysis to assess glycoprotein IbIXV.</td>
</tr>
<tr>
<td>Aggregation ↓ with high conc. of ristocetin, no thrombocytopenia.</td>
<td>Possible von Willebrand disease.</td>
<td>VWF levels.</td>
</tr>
<tr>
<td>Aggregation abnormally ↑ with low conc. of ristocetin.</td>
<td>Possible type 2B or platelet-type VWD.</td>
<td>VWF levels. Consider genetic testing.</td>
</tr>
<tr>
<td>Aggregation abnormally reduced with multiple agonist. Markedly ↓ with ADP with significant deaggregation.</td>
<td>Possible platelet ADP receptor defect (P2Y12). Drug induced defect should be excluded.</td>
<td>Repeat aggregation testing.</td>
</tr>
<tr>
<td>Other abnormalities with two or more agonists.</td>
<td>Suggest a platelet function disorder is present. Confirm on repeat testing.</td>
<td>Platelet ATP release and/or EM for dense granule deficiency (DGD).</td>
</tr>
<tr>
<td>Abnormalities with only one agonist (excluding collagen or ristocetin).</td>
<td>Nondiagnostic and could represent a false positive.</td>
<td>Repeat aggregation, ATP release and/or EM for DGD.</td>
</tr>
</tbody>
</table>
Examples of abnormal ristocetin induced platelet aggregation (RIPA)

Images from Hayward Moffat Chapter in Platelets (Editor: A. Michelson)

- Test of patient’s platelets in their plasma → ristocetin induced agglutination → aggregation

- Gain of function (type 2B or platelet type) defects:
  - Increased MA with ~ 0.5 mg/mL ristocetin

- Loss of function defects:
  - Delayed and reduced MA with 1-1.25 mg/ml ristocetin when VWF:RCo is low
Absent RIPA from Bernard Soulier Syndrome

Caution: sometimes the platelet count is very low and only the response to ristocetin can be evaluated
Example of an Aspirin-like defect

Based on RI:
Reduced MA with AA (arachidonic acid), normal MA with thromboxane analogue
Loss of secondary aggregation with epinephrine (not shown)
Reduced MA with low concentrations of collagen and ADP (some deaggregation)
Sometimes also reduced MA with ristocetin
LTA findings
“native” (undiluted) vs. platelet count adjusted PRP samples for healthy controls

Modified from Castilloux et al, TH 2011;106(4):675-82

important messages: need to know how test is done to interpret the findings
more variability with weak agonists for adjusted samples and absent secondary aggregation with epinephrine is only abnormal for native samples. On the other hand, there is more variability with ristocetin for native samples.
Which is the best sample type for LTA?

used healthy controls or patients with “no bleeding disorder” as comparison

clinically relevant question: does this person have a bleeding disorder, or not

Castilloux et al, TH 2011;106(4):675-82

DBD, diagnosed bleeding disorders
IPSD, inherited platelet secretion defects
NBD, no bleeding disorder
# How Good are Labs at Interpreting Aggregation Findings?

*Findings for real cases (NASCOLA Study)*

<table>
<thead>
<tr>
<th>% Correct for type of case see STH 2012:38;622-31</th>
<th>2008</th>
<th>2009</th>
<th>2010-1</th>
<th>2010-2</th>
<th>2011-1</th>
<th>2011-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non diagnostic (within RI)</td>
<td>94%</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td>83%</td>
</tr>
<tr>
<td>Possible secretion defect/multiple agonist abnormalities</td>
<td>93%</td>
<td>36%</td>
<td>75%</td>
<td>45%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Dense granule deficiency</td>
<td>33%* (~1/3 said NSAID)</td>
<td>-</td>
<td>92%</td>
<td></td>
<td>83%</td>
<td></td>
</tr>
<tr>
<td>Non-diagnostic findings: Single agonist abnormality (below RI)</td>
<td>19%</td>
<td>26%</td>
<td>47%</td>
<td>90%</td>
<td>67%</td>
<td></td>
</tr>
<tr>
<td>Non-diagnostic: No 2° wave with epinephrine (within RI)</td>
<td>16%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSAID-like abnormality (congenital or acquired)</td>
<td></td>
<td>86%</td>
<td>72%</td>
<td></td>
<td>80%</td>
<td></td>
</tr>
<tr>
<td>von Willebrand disease (type 2B)</td>
<td></td>
<td></td>
<td>95%</td>
<td></td>
<td>83%</td>
<td></td>
</tr>
<tr>
<td>Bernard Soulier syndrome</td>
<td>85%</td>
<td>93%</td>
<td></td>
<td>96%</td>
<td>35%</td>
<td></td>
</tr>
<tr>
<td>Glanzmann thrombasthenia</td>
<td>100%</td>
<td></td>
<td></td>
<td>92%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH

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2015 ISTH Guidance Document

Stepwise testing, after exclusion of VWD

- **First step:**
  - Platelet count, platelet size, morphology by light microscopy
  - Light transmission platelet aggregometry (LTA) with platelet rich plasma
    - Limited agonist panel – extend if abnormalities are found
    - Test for dense granule release defects (or at second step)

- **Second step:**
  - LTA with more agonists, secretion if it wasn’t evaluated
  - Flow cytometry
  - Electron microscopy
  - Biochemical assays for granule contents

- **Third step:**
  - Genetic tests
Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH

• RAND method used
  • formal consensus among experts about appropriateness of health care interventions, when scientific evidence is absent, scarce and/or heterogeneous
  • Experts scored statements:
    • “inappropriate” (1-3), “uncertain” (4-6) or “appropriate” (7-9)
    • highest and lowest scores discarded before determining consensus score
  • Public presentations for feedback
  • Formal literature review: 14/1830 potentially relevant studies considered relevant for review, leading to modification of some consensus statements
“Appropriate” recommendations about LTA related to bleeding disorders (abbreviated)

- Is clinically useful for the study of subjects with bleeding disorders

- Preanalytical (abbreviated) recommendations:
  - Collect after short rest
  - Collect after refraining from smoking at least 30 minutes
  - Collect after refraining from caffeine intake for at least 2 hours
  - Record all drugs taken
  - Stop reversible platelet function inhibitors at least 3 days before, and irreversible inhibitors at least 10 days before. Consider drug effects if inhibitors can’t be stopped
  - Discard first 3-4 ml blood (or use for other tests)
  - Underfilled tubes can only be used to evaluate for severe disorders
“Appropriate” recommendations about LTA related to bleeding disorders (abbreviated)

- Rest blood samples 15 mins before centrifuging and for 15 mins after obtaining PRP
- PRP: 200 g X 10 min, at ambient temperature, without a brake. Allow to sediment if platelets are very large
- Discard if grossly hemolyze
- Comment if lipemic
- Check PRP platelet count – caution: LTA may not be accurate if <150 X10⁹/L but still acceptable to evaluate severe disorders (e.g., Bernard Soulier syndrome)
- Do not adjust PRP to standard count (acknowledgment that this is valid though)
“Appropriate” recommendations about LTA related to bleeding disorders (abbreviated)

- Test in parallel with control sample
- Don’t add more than 10% volume for agonist addition
- Complete test within 4 hours
- Use as agonists:
  - 2 \( \mu M \) ADP
  - 5 \( \mu M \) epinephrine
  - Low concentration of collagen (2 \( \mu g/ml \) Horm collagen) that aggregates normal platelets
  - 10 \( \mu M \) PAR1-AP
  - 1 \( \mu M \) thromboxane analogue U46619
  - 1mM arachidonic acid
  - 1.2 mg/ml ristocetin, and if it aggregates, 0.5-0.7 mg/ml
    - Test 2 mg/ml if reduced
“Appropriate” recommendations about LTA related to bleeding disorders (abbreviated)

• Evaluate:
  • Presence of shape range
  • Length of lag phase
  • Slope of aggregation
  • Maximal, final and dis- aggregation, if there is secondary aggregation with epinephrine
  • Perform a visual inspection of tracing
**Expert Panel for this Working Party:**

The guideline was developed using:

• Consensus of expert opinion
  • 10 experts from various specialities and the current and past Chairman of the ISTH SSC on Platelet Physiology

• Review of the literature
  • 14 relevant publications were identified

• Feedback from two public presentations
LABORATORY DIAGNOSTIC WORK-UP

FIRST STEP TESTS

Blood smear
LTA (screening)
Granules release
Flow cytometry (FC) (screening)

Platelet size

normal

altered

S N L

Scott

BSS GPS GT-variant PT-VWD PTS VCF ARC Stormorken GATA1

GPS QPD WAS(±) ARC Stormorken GATA1 Filaminopathy Medich White MYH9-RD

EPI (*) → QPD/α₂ receptor(±)/GT/WAS(±)/FPD AML MDS/LADIII/δ-SPD/PSD/cPLA₂/ARC(±)/White(±)

ADP → P2Y₁₂ defect/GPS/PT-VWD/WAS(±)/FPD AML MDS/LADIII/δ-SPD/PSD/cPLA₂/ARC(±)

COLLAGEN → α₂β₁/GPVI/SPD/P2Y₁₂/GT/Stormorken/GPS/WAS/LADIII/δ-SPD/PSD/cPLA₂/ARC(±)/FPD AML MDS/GATA1/Filaminopathy(±) White(±)

AA → COX-1/TP defect/GT/P2Y₁₂ defect/δ-SPD/FPD AML MDS/ARC(±)/White(±)

RISTOCETIN → PT-VWD/BSS/GATA1

Total amount of blood required: ~21-28 ml
LABORATORY DIAGNOSTIC WORK-UP

SECOND STEP TESTS

LTA (extension)

Flow cytometry (extension)

Granules content

α-thr → GPS/GT/LADIII
TRAP-6 → GPS/GT/LADIII
U46619 → TP defect/GT/LADIII
CRP → GPVI/GT/LADIII
CVX → GPVI/GT/LADIII/Filaminopathy(±)
PAR4-ap → GPS/GT/LADIII
PMA → PKC defect/GT/LADIII
A23187 → Ca²⁺ defects/GT/LADIII
Inhibition by Iloprost or PGE₁ → Gs platelet defect

Mixing tests (LTA/Flow cytometry)

Platelet GP

Platelet procoagulant activity

impaired enhanced

GPla/Ila → α₂β₁
GPIV → GPIV
GPVI → GPVI

A) Patient’s plasma + control plts enhanced
B) Patient’s plasma + control plts normal

A → 2B-VWD
B → PT-VWD

Clot retraction

GT Stormorken WAS

TEM

Granule content or morphology
Structural abnormalities

α-δ SPD

Total amount of blood required: ~3-15 ml
LABORATORY DIAGNOSTIC WORK-UP

THIRD STEP TESTS

Biochemical studies
Surface glycoproteins (WB) / Spreading assay/Adhesion and thrombus formation under flow conditions/Protein phosphorylation (WB, FC)/Second messengers (Ca^{2+}, IP3, cAMP)/Receptor binding studies/ Western Blotting for MYH10

Signalling pathway defects
Filaminopathy (±)
WAS
Gs platelet defect
Receptor defects
FPD/AML/MDS
PTS
MYH9-RD

Molecular genetic diagnosis

ITGA2B, ITGB3
GP1BA, GP1BB, GP9
GP1BA
WAS
NBEAL2, GFI1B
Del11q23 (FLI1)
del22q11.2
CD36
GP6
TMEM16F
PLAU (duplication)
TBXAS2R
HPS1, AP32B1, HPS3,
HPS4, HPS5,
HPS6, DTNBP1,
BLOC1S3, PLDN
LYST
P2RY12
GNAS
VPS33B, VIPAS39
STIM1, ORAI1
FERMT3
RUNX1
GATA1
FLNA
TBXAS1
PLA2G4A
MYH9

Total amount of blood required: ~3-50 ml

Comments on latest guideline

• Use of many laboratory-developed tests
  • Research vs. clinical laboratory setting

• Applicability to clinical laboratory setting

• Are laboratories following this recent guideline?
  • See: Moffat et al, Poster Session II, 1045, Friday May 13
**Illustrative case to work through testing options**

64 year old female, preop urgent cardiac surgery. ISTH Bleeding score>20

Very abnormal bruising, very heavy menses until menopause,

Sister: has a “platelet disorder”. Platelet count is normal. VWD excluded.

<table>
<thead>
<tr>
<th>Aggregation Findings:</th>
<th>Reference interval: Maximal aggregation (MA)</th>
<th>Patient’s MA</th>
<th>Sister’s MA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonist</td>
<td></td>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>2.5 µM ADP</td>
<td>&gt;24</td>
<td>89</td>
<td>26</td>
</tr>
<tr>
<td>5.0 µM ADP</td>
<td>&gt;43</td>
<td>92</td>
<td>48</td>
</tr>
<tr>
<td>1.25 µg/ml Collagen</td>
<td>&gt;51</td>
<td>54</td>
<td>5</td>
</tr>
<tr>
<td>5 µg/mL Collagen</td>
<td>&gt;85</td>
<td>91</td>
<td>75</td>
</tr>
<tr>
<td>6 µM Epinephrine</td>
<td>&gt;9</td>
<td>93</td>
<td>13</td>
</tr>
<tr>
<td>1.6 mM Arachidonic Acid</td>
<td>&gt;77</td>
<td>88</td>
<td>41</td>
</tr>
<tr>
<td>1 µM Thromboxane analogue U46619</td>
<td>&gt;70</td>
<td>90</td>
<td>39</td>
</tr>
<tr>
<td>0.5 mg/ml Ristocetin</td>
<td>&lt;7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>1.25 mg/ml Ristocetin</td>
<td>&gt;75</td>
<td>83</td>
<td>78</td>
</tr>
</tbody>
</table>

Would you do anything more?
Platelet Secretion Also Assessed at Step One
Dense Granule Adenosine Triphosphate (ATP) Findings

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Reference Interval nmol/L ATP release</th>
<th>Patient results nmol/L ATP release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin 1 unit/mL</td>
<td>0.77–2.42</td>
<td>0.33</td>
</tr>
<tr>
<td>Collagen 5 µg/mL</td>
<td>0.66–2.22</td>
<td>0.33</td>
</tr>
<tr>
<td>Epinephrine 6 µM</td>
<td>0.31–1.84</td>
<td>0.00</td>
</tr>
<tr>
<td>Arachidonic Acid 1.6 mM</td>
<td>0.25–2.24</td>
<td>0.10</td>
</tr>
<tr>
<td>Thromboxane Analogue U46619 1 mM</td>
<td>0.18–1.26</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Possible diagnosis?
Next: “Whole Mount” Electron Microscopy (EM) To Assess Platelet Dense Granules

calcium and phosphorus in dense granules makes them look black (EM dense)

Healthy control

Case

Expect, on average, about 4.9-10 electron dense granules per platelet
## Dense granule numbers (multiple tests)

<table>
<thead>
<tr>
<th>Average of 4.9-10 dense granules per platelet</th>
<th>Patient</th>
<th>Sister</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 0.5</td>
<td>2.0 3.0 3.0</td>
<td></td>
</tr>
</tbody>
</table>

### Important Laboratory Practice Points:

I. Aggregation doesn’t detect all platelet disorders.

II. ~30% of patients with dense granule deficiency have normal aggregation findings.

III. When dense granule release is impaired, one cause is a loss of dense granule contents.
<table>
<thead>
<tr>
<th>Other Tests Can Be Useful</th>
<th>Case 1: Bruises, bleeding with surgeries requiring transfusions, heavy periods</th>
<th>Case 2: No challenges, very large bruises and heavy periods</th>
<th>Case 3: Heavy menses but no major challenges. Father &amp; other relatives with unusual bleeding (massive bruises, joint bleeds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examples of three women with menorrhagia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ADP 2.5 ( \mu M )</strong></td>
<td>↓</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>ADP 5.0 ( \mu M )</strong></td>
<td>↓</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Collagen 1.25 ( \mu g/mL )</strong></td>
<td>↓</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Collagen 5.0 ( \mu g/mL )</strong></td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Epinephrine 6 ( \mu M )</strong></td>
<td>Normal</td>
<td>Normal</td>
<td>↓ (not detected)</td>
</tr>
<tr>
<td><strong>Arachidonic acid 1.6 mM</strong></td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Thromboxane analogue U46619 1 ( \mu M )</strong></td>
<td>↓</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Ristocetin 0.5 mg/mL</strong></td>
<td>Normal (&lt;7% aggregation)</td>
<td>Normal (&lt;7% aggregation)</td>
<td>Normal (&lt;7% aggregation)</td>
</tr>
<tr>
<td><strong>Ristocetin 1.25 mg/mL</strong></td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Additional Tests and Diagnosis</strong></td>
<td>Platelet secretion, VWD screen, dense granule EM</td>
<td>Platelet secretion, VWD screen, dense granule EM</td>
<td>Genetic testing for <strong>PLAU</strong> duplication mutation of Quebec platelet disorder (QPD)</td>
</tr>
<tr>
<td></td>
<td>Platelet secretion defect and “low VWF”</td>
<td>Dense granule deficiency</td>
<td>QPD</td>
</tr>
</tbody>
</table>
Warnings

• While some families have quite reproducible findings, pre-analytical errors are common with platelet function tests
• Need to confirm abnormalities, use specific tests
  • Examples:
    • Flow cytometry to confirm the diagnosis if aggregation findings suggests Bernard Soulier Syndrome or Glanzmann’s thrombasthenia
    • EM to diagnose dense granule deficiency if release is impaired with strong agonists
### Clinical Laboratory Performance on Interpreting Aggregation Findings

*Findings for real cases (NASCOLA/ECAT Data, STH 2012:38;622-31)*

<table>
<thead>
<tr>
<th>% Correct for type of case</th>
<th>2008</th>
<th>2009</th>
<th>2010-1</th>
<th>2010-2</th>
<th>2011-1</th>
<th>2011-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non diagnostic (within RI)</td>
<td>94%</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td>83%</td>
</tr>
<tr>
<td>Possible secretion defect/multiple agonist abnormalities</td>
<td>93%</td>
<td>36%</td>
<td>75%</td>
<td>45%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Dense granule deficiency</td>
<td>33%*  (~1/3 said NSAID)</td>
<td>-</td>
<td>92%</td>
<td>83%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diagnostic findings: Single agonist abnormality (below RI)</td>
<td>19%</td>
<td>26%</td>
<td>47%</td>
<td>90%</td>
<td>67%</td>
<td></td>
</tr>
<tr>
<td>Non-diagnostic: No 2° wave with epinephrine (within RI)</td>
<td>16%</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSAID-like abnormality (congenital or acquired)</td>
<td>86%</td>
<td>72%</td>
<td>-</td>
<td>80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>von Willebrand disease (type 2B)</td>
<td></td>
<td></td>
<td>95%</td>
<td>83%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bernard Soulier syndrome</td>
<td>85%</td>
<td>93%</td>
<td>-</td>
<td>96%</td>
<td>35%</td>
<td></td>
</tr>
<tr>
<td>Glanzmann thrombasthenia</td>
<td>100%</td>
<td></td>
<td>92%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case Type</td>
<td>Common errors or omissions by clinical labs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possible secretion defect/multiple agonist abnormalities</td>
<td>Reported diagnoses were too specific for the findings and some suggested possibilities were incorrect</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dense granule deficiency</td>
<td>Reported diagnoses were too specific for the findings or were incorrect (e.g. case in 2008 was often reported to be an aspirin-like defect)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diagnostic findings: Single agonist abnormality (below RI)</td>
<td>Reported that the findings were diagnostic of a platelet function disorder</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diagnostic findings: No secondary aggregation with epinephrine (result within RI)</td>
<td>Reported that the findings were diagnostic of a platelet function disorder</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Usefulness of assessing dense granule ATP release for diagnosing platelet function disorders?

- Lumi-aggregometry assessment of platelet dense granule adenosine triphosphate (ATP) release is widely used for diagnosing platelet function disorders.

- Recent ISTH guidelines recommend this testing be done as part of the diagnostic-work up of platelet function disorders.
Our earlier study on the diagnostic usefulness of dense granule ATP release as a secondary investigation for a bleeding disorder is often cited as the evidence for doing this testing.

*Pai M et al, AJCP 2011;136(3):350-8*

- Reduced ATP release with ≥2 agonists by lumi-aggregometry:
  - *Odds Ratio 17 (95%CI: 6-46) for a bleeding disorder*
- Subjects: 76 referred patients, 78 healthy controls
- Controls: showed high CV for individual agonists responses (up to 30% with weak agonists; significant within and between test variability)
- We reevaluated the diagnostic usefulness of this test recently
Re-evaluation of the diagnostic usefulness of ATP release

Badin et al, submitted (presented at THSNA two weeks ago)

• Looked at a larger number of subjects, and the consistency of test findings
• Evaluated two cohorts, 150 unique patient subjects
Latest study findings

1. Platelet dense granule ATP release findings are often inconsistent amongst patients tested for a bleeding disorder AND consistent abnormalities are not predictive of a bleeding disorder (or increased bleeding scores)
   not surprising given the reported high coefficient of variation for healthy control tests

2. Our study raises questions about whether it is appropriate to use (or recommend) a test with such variability for diagnostic purposes

   *other publications: tests with CV >20% should not be used for diagnostic purposes*
Acknowledgments:

Colleagues and Collaborators