Pearls and Pitfalls in the Hemostasis Laboratory

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Esoterix Coagulation
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Outline

• Pearls and Pitfalls:
  – In the evaluation of a bleeding patient or patient with a history of bleeding
  – In the evaluation of a prolonged APTT and PT
  – In the performance and interpretation of normal plasma mixing studies
  – As they relate to pre-analytical variables
  – In the evaluation and interpretation of lupus anticoagulants and antiphospholipid antibodies
  – In regard to new oral anticoagulant therapies

Disorder of Primary Hemostasis
Platelet Defect or Von Willebrand Disease

• “Mucosal Bleeding”
  – Bleeding at time of insult “immediate”
  – Menorrhagia/Postpartum hemorrhage
  – Petechiae
  – Epistaxis
  – Gingival bleeding
  – Easy bruising
  – Post surgical or trauma-related bleeding
Disorder of Secondary Hemostasis

Factor Deficiency

- Defective Secondary Hemostasis
  - Deep ecchymoses
  - Joint hemorrhage
  - Hematomas (especially intramuscular)
  - Bleeding noted hours to days after injury
  - Poor wound healing

Hemostatic Screen

Evaluation of Patient with Bleeding

- Platelet Count
- Some form of platelet function test, especially if bleeding is “mucosal” in nature
- Activated Partial Thromboplastin Time (APTT)
- Prothrombin Time (PT)
- Thrombin Clotting Time (TT) and/or
- Fibrinogen Activity

OTC Drugs/Products that Affect Hemostasis

- Non steroidal anti-inflammatory agents (NSAIDS)
- Salicylates (Aspirin)
- Herbal Products: “Few G’s” – Feverfew, garlic, ginger, ginkgo and ginseng
  - Anise
  - Dong Quai
  - Vitamin E
  - Fish Oils
Prescribed Drugs that Affect Hemostasis

- Selective serotonin reuptake inhibitors (SSRIs)
- Antibiotics
  - Moxalactam
  - Tobramycin
- Anticonvulsants
- Chemotherapeutic agents

Underlying conditions associated with bleeding

- Myeloproliferative/myelodysplastic disorders
- Uremia
- Amyloidosis
- Liver disease
- Autoimmune disorders
- Thrombocytopenia

Hemostatic Screen

**Primary Hemostasis**

- **Platelet count**
  - $<10/\mu\text{L}$ = spontaneous hemorrhage
- **Some form of platelet function test**
  - Bleeding Time - Obsolete
  - Platelet Function Analyzer (PFA-100 Siemens)
  - Platelet Aggregation Studies
- **Von Willebrand factor antigen/activity**
Hemostatic Screen

Primary Hemostasis

- **Platelet Function Testing**
  - Platelets don’t travel – evaluation must be performed essentially on site
    - **Platelet Function Analyzer**
      - May not detect mild deficiencies
    - **Platelet Aggregometry**
      - Poorly standardized, pre-analytical factors, difficult to interpret
      - Performed in relatively few locations

Von Willebrand Disease

- **Deficiency or dysfunction of VW factor**
- **VW factor serves two functions:**
  - Anchors platelets to sites of injury and supports platelet aggregation (formation of the platelet plug)
  - Stabilizes factor VIII in the circulation

\[
\text{Von Willebrand Disease} \\
\text{Laboratory Diagnosis}
\]

- **Do not screen using Global Assays or BT**
  - APTT is prolonged only in a minority
  - Bleeding time is obsolete
  - Also: PT, TT and fibrinogen are normal
- **Screening requires a panel of assays:**
  - FVIII, VWF:Ag, VWF:RCo
  - Using fewer assays may lead to missed diagnosis
- **If one or more screening tests are abnormal,** supplemental tests can be performed to characterize the type of VWD
Isolated Elevated APTT

- Deficiency or Specific Factor Inhibitor
  - FXII, Prekallikrein (PK), High molecular weight kininogen (HMWK) - Not associated with bleeding
  - Factor XI
  - Factor IX
  - Factor VIII
  - Combination of several low normal factors
- Lupus Anticoagulant
  - Rarely associated with bleeding
    - Thrombocytopenia or Antibodies to FII (elevated PT)
- Heparin Therapy or Contamination
- Spurious Elevation
Evaluation of prolonged APTT

Mixing study using 1:1 normal plasma
- Correct into normal range – deficiency
  - Consider time and temp dependent inhibitor
- Incomplete correction – consider inhibitor

Specific factor inhibitor
- Two major types:
  1. Develop in deficient patients in response to transfused factor
     - Factor VIII inhibitor, Factor IX inhibitor
  2. Develop in “normal” individual due to variety of mechanisms such as autoimmune, drug-induced

Non-specific inhibitor
- Lupus anticoagulant
- Heparin or heparin-like anticoagulant
- Direct thrombin inhibitors, FXa inhibitors

Mixing Studies:
Technical Considerations

• Incubated Mixing Study
  - Should be performed when correction is obtained with immediate mix in a bleeding patient or if FVIII inhibitor is suspected
  - Few inhibitors require 37°C incubation over time
  - FVIII inhibitors are classically time dependent
  - LA: 10-15% can demonstrate time dependence
    - Possible artifact due to pH effect
  - Method is not standardized
    - Variety of different methods performed
  - More applicable to APTT than PT

Incubated Mixing Study: Method

![Incubation Diagram](image)
### Mixing Study Results

<table>
<thead>
<tr>
<th></th>
<th>Lupus Anticoagulant</th>
<th>FVIII Inhibitor</th>
<th>Factor Def</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># 1</td>
<td># 2</td>
<td># 3</td>
</tr>
<tr>
<td>APTT normal</td>
<td>53.5</td>
<td>75.8</td>
<td>46.6</td>
</tr>
<tr>
<td>24-36 sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APTT 1:1 Normal Plasma</td>
<td>40.8</td>
<td>56.8</td>
<td>46.6</td>
</tr>
<tr>
<td>Control</td>
<td>53.5</td>
<td>75.8</td>
<td>46.6</td>
</tr>
<tr>
<td>APTT 1:1 Incub Control</td>
<td>42.0</td>
<td>58.2</td>
<td>48.6</td>
</tr>
<tr>
<td>Plasma (1 hr incub)</td>
<td>41.0</td>
<td>58.0</td>
<td>47.0</td>
</tr>
</tbody>
</table>

### Factor Assays

#### Demonstration Of An Inhibitor
- Should be performed at multiple dilutions
- Non-parallelism of factor assay curves - typical of non-specific inhibitor (LA)

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Factor deficiency</th>
<th>Lupus anticoagulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>20%</td>
<td>60%</td>
</tr>
<tr>
<td>1:20</td>
<td>22%</td>
<td>100%</td>
</tr>
<tr>
<td>1:40</td>
<td>19%</td>
<td>150%</td>
</tr>
</tbody>
</table>

### Specific Factor Inhibitor
- High affinity IgG antibody directed against a single coagulation factor
  - **Neutralizing**: inhibits the activity of the relevant clotting factor
  - Detected with plasma mixing study
  - **Non-neutralizing**: non-inhibitory, targeted against a non-functional epitope (FII and VWF)
    - Clinically relevant if it causes increased clearance of factor
    - Not detected with plasma mixing study
- Can occur against any factor but, some are more common than others
  - Intrinsic factor inhibitors most common
**Isolated Elevated APTT**

- Clinically significant hereditary factor deficiencies
  - Factor VIII – Hemophilia A (x-linked)
  - Factor IX – Hemophilia B (x-linked)
  - Factor XI – Hemophilia C (autosomal)
- No increased risk for bleeding
  - Deficiency XII, PK, HMWK
  - Spurious elevation – poor collection, improper storage, elevated hematocrit, PEG compounds
  - Lupus anticoagulants (unless ↑PT or ↓platelet count)
  - Heparin contamination

**Effects of an Elevated Hematocrit and a Short Draw**

- Hct > 55% or short draw leads to reduced plasma volume for the amount of citrate present in the tube = spuriously ↑PTT


**Sample Handling**

1. Transport/Storage of *Whole Blood* at 2-4°C is Not Recommended
   - Loss of FVIII/VWF *
   - Time and temperature dependent
   - As much as 50% from baseline
   - Activation of FVII
2. Factor VIII is a labile factor:

<table>
<thead>
<tr>
<th>FVIII</th>
<th>FVIII</th>
<th>Aliquotted plasma 1 - 4°C</th>
<th>FVIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>51%</td>
<td>47%</td>
<td>39%</td>
<td>33%</td>
</tr>
<tr>
<td>110%</td>
<td>102%</td>
<td>71%</td>
<td>65%</td>
</tr>
<tr>
<td>0 hr</td>
<td>8 hr</td>
<td>24 hr</td>
<td>48 hr</td>
</tr>
</tbody>
</table>

*Favalaro E, et al. AJCP 2004;122:686*
Normal APTT Does Not Rule Out Intrinsic Factor Deficiency!

• Mild deficiencies of factors VIII, IX and XI cannot be excluded with a normal APTT
  – APTT is not sensitive to factor levels above 10% to 30%
  – Not as much a problem with the PT
• Different APTT reagents vary in their responsiveness to factor deficiency
  – Responsiveness = factor level required to cause clotting time to fall out of the normal range

APTT/PT Responsiveness Determination

Use normal plasma, determine factor activity
Dilute into factor deficient plasma
Run aPTT and PT at each dilution
Determine factor activity at each dilution
Dilution at which aPTT or PT becomes abnormal determines responsiveness

APTT/PT Responsiveness

Varies by laboratory, reagent and factor deficient plasma used
Coagulation Cascade: *In vitro* model

**Isolated Elevated PT**

- Deficiency of FVII - Acquired vs Congenital
  - FVII has the shortest T½ of all factors and is vitamin K dependent, first factor to decrease in response to:
    - Early vitamin K deficiency
    - Early warfarin therapy
    - Early liver disease
  - Inhibitor of FVII - very rare
- Mild deficiency of common pathway factors
  - FX, FV or FII
- Deficiency of fibrinogen

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Coagulation Cascade: *In vitro* model
Elevated TCT

- Hypofibrinogenemia
- Dysfibrinogenemia
- Drugs: Heparin, Direct Thrombin Inhibitor
- Mechanical interference with fibrin polymerization
  - Fibrin Spit Products
  - Monoclonal Proteins

Coagulation Cascade: In vitro model

Deficiency Vitamin K Factors

- Factors II, VII, IX and X
- Congenital - Rare
- Acquired - Poor diet (prolonged fasting), malabsorption, antibiotics (cephalosporins), alcoholism, hyperemesis gravidarum
- Warfarin therapy
- Superwarfarin poisoning
- Treatment: 10 mg Vitamin K if stable
  FFP if bleeding (8 – 10 ml/kg) or PCC
Coagulation Cascade: *In vitro* model

Fibrin Clot Formation & Plasminogen System

Life-long Bleeding in a Patient With Normal APTT and PT

- Intrinsic factor deficiency
  - Factor VIII
  - Factor IX
  - Factor XI
- von Willebrand disease
- Hypofibrinogenemia or dysfibrinogenemia
- FXIII deficiency
- Alpha-2-antiplasmin deficiency
- PAI-1 deficiency
- Abnormal platelet number or function
- Vascular disorders
FXIII Deficiency

- Rare: Hereditary (life-long) or acquired
- **Only > 2 - 4 % required for normal hemostasis**
  - Probably NOT TRUE!!!!
- Umbilical stump, intracranial bleeding, habitual abortion, menorrhagia, soft tissue hematomas
- Screening assay: Urea solubility, not useful
- Diagnosis: Factor XIII activity
- Other screening tests normal

Factor XIII Inhibitor

- Autoimmune, frequently elderly population
- New onset bleeding, no family history
  - Severe bleeding diathesis, large ecchymoses
  - 26% mortality
- Factor XIII undetectable, mixing studies with normal plasma → no correction
  - Must suspect and order FXIII specifically
- Rx - plasma products, immunosuppression

Hemostatic Screen

- Platelet Count
  - Some form of platelet function test
- APTT/PT
- Thrombin Clotting Time (TT)
- Fibrinogen Activity

*This battery of assays does not always exclude a cause of bleeding*
Platelet and Vascular Disorders

• Thrombocytopenia
• Congenital or acquired platelet dysfunction
  – Glanzmann’s thrombasthenia; storage pool defect
  – Drug-induced, uremia, post CABG
• Vascular disorders
  – Vasculitis, senile purpura, hereditary hemorrhagic telangiectasia, hemangioma, scurvy
  – Structural Disorders: Ehlers-Danlos syndrome, osteogenesis imperfecta, Marfan’s syndrome
  – Surgical or traumatic vascular defect, non-accidental trauma, factitious purpura

Definition
Antiphospholipid Antibodies

• A heterogeneous population of immunoglobulins directed against phospholipid binding proteins that fall into one or two groups:
  – Lupus Anticoagulants: identified using plasma-based clotting assays
  – Solid Phase Antibodies: identified using enzyme immunoassays or ELISA, e.g. anticardiolipin antibodies, B2GP1 antibodies, anti-prothrombin antibodies, anti-phosphatidylserine antibodies

Concordance between these 2 groups is only 60%

Classification Criteria for Definite APS*
Sydney Criteria - 2004 (revised Sapporo)

• Clinical
  – Venous thrombosis
  – Arterial thrombosis
  – Small vessel thrombosis
  – Pregnancy morbidity
    • Fetal death ≤ 10 weeks
    • Severe preeclampsia/UGR
    • 3 or more embryonic losses

• Laboratory
  – aCL positive, IgG or IgM at moderate to high levels (> 40)
  – aB2GPl positive, IgG or IgM; > 99%
  – Lupus Anticoagulant positive: based on ISTH criteria

* At least 1 clinical and 1 laboratory finding with demonstration of persistence. A repeat positive test in 12 weeks is required for the diagnosis.
Sydney Criteria for APS Diagnosis

Limitations

• Originally developed for research purposes – to help define a homogenous population for research studies

• Clinically, there are patients who have APS who do not meet these criteria
  – In an individual patient, may want to use less stringent criteria
  – “Seronegative” APS

Clinical Significance

Antiphospholipid Antibodies

• A common cause of acquired thrombophilia
  – Obesity is now the most common cause

• Common, occur in 1 – 2% population

• Associated with the development of thrombosis and obstetric complications

• In children aPL are most commonly transient (6 – 8 weeks), typically appear follow an infection and are clinically insignificant

Clinical Significance

Antiphospholipid Antibodies

Arterial thrombosis

• Cerebral
  – 30% of strokes under 50 years of age

• Coronary
  – 20% of AMI under 45 years of age *
  – In a study of 1000, 2.8% with APS initially presented with AMI **

Venous thrombosis

• 15% of all lower extremity deep venous thrombosis***

• Other sites: Renal, hepatic, axillary, subclavian, cerebral sinus

Clinical Significance
Antiphospholipid Antibodies

Obstetric complications
- Late fetal loss (>10 weeks gestation) and possibly 5 - 15% of all recurrent spontaneous abortions
- Severe preeclampsia, intrauterine growth restriction (IUGR)

Catastrophic antiphospholipid syndrome
- Rare, often fatal accelerated form of APS
- Disseminated small and large vessel clots with multi-organ failure
- Refractory to anticoagulant therapy alone
  - Mortality 50%, even with therapy
    - Plasma exchange (mandatory if microangiopathic features), steroids, gamma globulin
  - Laboratory: usually high titer aPL

Laboratory Diagnosis
Assays for antiphospholipid antibodies
- aPL are a heterogeneous population of antibodies
  - Epitope specificity varies widely between patients
- No single test is 100% sensitive and specific
- Panel of assays necessary
  - LA may react + in some test systems, not in others
  - Some patients may be positive with some ELISA assays and not others

Laboratory Testing
Antiphospholipid Antibodies
- In order to adequately screen for antiphospholipid antibodies, both clot-based assays (to screen for a lupus anticoagulant) and immunoassays must be tested
  - Concordance between clot based and immunoassays is about 60%
  - Screening/diagnostic testing using an inadequate panel of assays may lead to a false negative diagnosis
  - Using too many assays may lead to a false positive diagnosis
Laboratory Testing

Immunoassays for antiphospholipid antibodies

- aCL and aB2GP1: Isotype Significance (IgG; IgM & IgA)
  - IgG aPL are more strongly associated with APS than IgM aPL
  - IgM may be falsely elevated in the presence of rheumatoid factor or with recent infection
  - Significance of IgA is questionable
    - May be worthwhile in cases where APS is strongly suspected but IgG and IgM testing is negative
    - Likely has greater significance with aB2Gp1 than aCL

Laboratory Testing

Immunoassays for antiphospholipid antibodies

- Antibodies to cardiolipin (aCL): IgG & IgM
  - Expressed in international GPS or MPS units
  - Clinically significant - only those moderate to high titer; ~ > 40 and only those that are persistent
    - Normal up to about 20 GPS or MPS
  - Considered sensitive but not specific for APS
  - High degree of variability between commercial kits and therefore results between laboratories do not compare well

Laboratory Testing

Immunoassays for antiphospholipid antibodies

- B2Glycoprotein1- antibodies (aB2Gp1): IgG & IgM
  - Only those > 99% (3SD) normal range (2SD = 95%) clinically significant and only if persistent
    - 99% slightly higher than normal which is usually 95%
  - Positive result more specific for APS than aCL
    - Domain 1 contains a major epitope for APS
  - Variability between commercial kits and therefore results between laboratories do not compare well
Laboratory Testing

Immunoassays for antiphospholipid antibodies

- Antibodies to Prothrombin, Phosphatidylserine, Annexin V,
  - Clinical significance questionable

Lupus Anticoagulant

Antibody interferes with the phospholipid needed for clotting - causing prolongation
- Interferes with APTT > PT
  - PT reagents inherently contain more PL than APTT reagents
  - Non-specific inhibitor
- THE most common non-specific inhibitor
- Despite prolongation of clotting assays, typically not associated with bleeding

Watch for an elevated PT, antibody induced hypoprothrombinemia; thrombocytopenia

Laboratory Diagnosis

Lupus Anticoagulant* 1995

# 1) Prolongation of one or more phospholipid dependent clotting assay(s)
  - APTT, DRVVT, dPT
  - 2 LA responsive assays should be performed before LA ruled out
# 2) Demonstrate inhibitor – mixing studies or factor assays
# 3) Evidence of phospholipid dependence
  - PNP, DRVVT ratio, hexagonal phospholipid neutralization
# 4) Rule out other coagulopathies (FVIII inhibitor, anticoagulant drug effect)

Mixing Studies in the Dx of LA
Are they or are they not needed?

• CLSI H60 – New guideline “Laboratory testing for the Lupus Anticoagulant” still in draft form
  — Recommends that “mixing test” be performed subsequent to finding both screening and confirmatory LA results to be abnormal
  — Correction of a screening mixing test does not exclude the presence of LA

Assay Interferences

— Heparin
  — May lead to false positive LA diagnosis
• Warfarin
  — May lead to false positive LA diagnosis
• Direct Thrombin Inhibitors
  — Dabigatran may lead to false positive LA diagnosis
• Direct Xa Inhibitors
  — Rivaroxaban/apixaban may lead to false positive LA diagnosis
• Factor VIII or FV inhibitors
  — May lead to false positive LA diagnosis

Will not interfere with ELISA aPL assays

Recommended Screening Panel

• ELISA Assays
  — aCL - IgG and IgM
  — aB2GP1 - IgG and IgM
• Clot-based assays for LA
  — Two PL dependent assays that point to different sites in the coagulation cascade
    • Such as dRVVT and aPTT based
If this panel is negative and APS strongly suspected clinically: repeat this panel and add aCL and aB2GP1 IgA and possibly other aPL antibodies such as aPS and aPT.
LA and aPL – Clinical Significance

- Strong correlation between positivity in multiple assays with clinical manifestations of APS (thrombosis and miscarriage)*
- Correlation with APS

![All three positive, significant titer, same isotype = Triple positivity](image)

**Triple Positivity**

- These patients are much more likely to have clinical manifestations (APS)
- Risk of first thromboembolic event is significantly increased*
  - Risk is greater in males > females
  - Risk is greater with additional thrombotic risk factors
- Aspirin is not adequate thromboprophylaxis in this population

LA and aPL – Clinical Significance

- aPL positivity does not always indicate a dx of APS
  - aPL positivity in isolation is not sufficient to make a dx APS
  - The > # lab test performed, > likelihood of false positive by chance alone
  - Positive results reported in 1 – 6% of healthy population
  - aCL IgM often associated with infection
- APS is not always associated with aPL positivity – SNAPS (seronegative APS)
  - Patients with typical clinical manifestations APS but persistently negative for detectable aPL
  - Negative for LA, aCL, aB2Gp1, all isotypes at time of thrombosis and following resolution and after careful exclusion of other pro-thrombotic conditions