Thrombophilia Testing: Workshop

Rajiv K. Pruthi, MBBS
Special Coagulation Laboratory & Comprehensive Hemophilia Center
Division of Hematology/Internal Medicine
Dept of Laboratory Medicine & Pathology
Mayo Clinic

pruthi.rajiv@mayo.edu
Disclosure (s)

• Relevant financial relationship(s)
  • None
• Off-label usage
  • None
Learning Objectives

• Explain the concept of thrombophilia
• Recognize the congenital and acquired thrombophilias
• State the practical application of thrombophilia in patient management
• Understand limitations of selected assays
• Realize the value of algorithmic approach to testing
Multifactorial Disease

Thrombosis

Acquired + inherited

Acquired

Inherited

Acquired + acquired

Inherited + inherited
## Thrombophilia markers: What laboratory assays are indicated?

<table>
<thead>
<tr>
<th>Good evidence</th>
<th>Weak Evidence</th>
<th>Lack of evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC-R/Factor V Leiden</td>
<td>High TAFI</td>
<td>Plasminogen deficiency</td>
</tr>
<tr>
<td>Prothrombin G20210A</td>
<td>Elevated fibrinogen, factor IX and factor XI</td>
<td>High PAI-1 levels</td>
</tr>
<tr>
<td>AT/PC/PS deficiency</td>
<td>EPCR polymorphism</td>
<td>Factor XIII Leu34Val</td>
</tr>
<tr>
<td>Non-O blood group</td>
<td></td>
<td>Lp(a)</td>
</tr>
<tr>
<td>High factor VIII</td>
<td></td>
<td>MTHFR (677 and 1298)</td>
</tr>
<tr>
<td>Dysfibrinogenemia</td>
<td></td>
<td>Thrombomodulin/ACE/PZ polymorphism</td>
</tr>
<tr>
<td>Hyperhomocysteinemia</td>
<td></td>
<td>ADAMTS 13 polymorphism</td>
</tr>
</tbody>
</table>
Acquired Clinical Risk Factors for VTE Nested Case-Control Study (625 Case-Control Pairs)

- Surgery
- Trauma
- Inpatient
- Malignancy with chemotherapy
- Malignancy without chemotherapy
- Central venous catheter or pacemaker
- Neurologic disease
- Superficial vein thrombosis
- Varicose veins/age 45 yr
- Varicose veins/age 60 yr
- Varicose veins/age 70 yr
- CHF, VTE incidental on autopsy
- CHF, antemortem VTE/causal for death
- Liver disease

Odds ratio

• Pre-analytical:
  • Patient selection

• Analytical (laboratory aspects):
  • Types and sequence of testing
  • Influence of anticoagulants

• Post analytical:
  • Application to patient care
Patient selection: A suggested approach

Arterial thrombosis

Antiphospholipid Antibodies
Dysfibrinogenemia etc

Venous thrombosis

Temporary risk factor

Testing not indicated

Testing not indicated
Thrombophilia Profile

Testing begins with:
- Prothrombin Time (PT), Plasma
- Activated Partial Thromboplastin Time (APTT), Plasma
- Dilute Russells Viper Venom Time (DRVVT), Plasma
- Thrombin Time (Bovine), Plasma
- Fibrinogen, Plasma
- D-Dimer, Plasma
- Soluble Fibrin Monomer
- Antithrombin Activity, Plasma
- Protein C Activity, Plasma
- Protein S Antigen, Free, Plasma
- Prothrombin G20210A A Mutation, Blood
- Activated Protein C Resistance V (APCRV), Plasma
- Special Coagulation Interpretation

All initial testing within reference ranges for age and gender:
- No evidence of thrombotic diathesis
- No further testing is performed

**Antithrombin Activity:**
- <80%
- No evidence of an acquired deficiency

**PT:** ≥14.0 seconds
- PT Mix 1:1
  - ≥14.0
  - <14.0
- Evidence of inhibition*  
- Evidence of coagulation factor deficiency**
- No evidence of heparin in sample

**APTT:** >36 seconds
- APTT Mix 1:1
  - ≤36
  - >36
- Evidence of inhibition*  
- Evidence of coagulation factor deficiency**
- Platelet neutralization procedure (PNP)

**DRVVT:** ≥1.2 seconds
- DRVVT Mix 1:1
  - ≥1.2
  - <1.2
- Evidence of inhibition*  
- Evidence of coagulation factor deficiency**
- No evidence of heparin or dys/hypofibrinogenemia

**Thrombin Time (Bovine):**
- 15-23 sec
- >23 sec

**APCRV:**
- <2.3
- OR
- Prolonged baseline APTT
- Evidence of inhibition*  
- Evidence of coagulation factor deficiency**

**Protein C Activity:** <70%
- Protein C Antigen

**Protein S Antigen, Free:**
- Males <65%
- Females <50 years: <50%
- ≥50 years: <65%
- Protein S Antigen, Total

**Fibrinogen:**
- ≥14.0
- <14.0
- ≤36
- >36
- ≥1.2
- <1.2
- DRVVT Confirmation
- Anticoagulant effect **
- Possible dys/hypofibrinogenemia

**Factor V Leiden (R506Q) Mutation:**
- 14-23 sec
- >23 sec

**Reptilase Time:**
- Factor V Leiden (R506Q) Mutation
- Repitlase Time

**Antithrombin Antigen, Plasma:**
- Does not shorten
- Shortens by 4-5 seconds
- Possible factor inhibitor*
- Evidence of lupus-like anticoagulant
- No diagnostic of lupus-like anticoagulant

*Additional assays may be performed if further clarification or confirmation is necessary. These may include:
- Coagulation Factor Assays
- Staclot Lupus Anticoagulant
- Protein S Activity
- **Unfractionated/low-molecular weight heparin or direct thrombin inhibitor (e.g., dabigatran, argatroban)

An interpretive report is provided that includes all profile tests (always performed) and any reflex tests performed (if appropriate).
Advantages/Limitations of profile approach

• Tests for all known markers in one venepuncture
• Costly
• Indicated once in a lifetime
  • Hereditary, repeat order may not be intercepted prior to collection (electronic order entry systems needs optimizing)
• Profile approach may not be applicable to individuals of different ethnicity
Activated Protein C Resistance and Factor V Leiden

• Most common congenital hereditary thrombophilia among whites

• Protein phenotype
  • Normally: Activated protein C (APC) inactivates activated factor V (fVa)
  • APC resistance: Mutated factor V resists inactivation by APC

• Genetic basis
  • Factor V Leiden (R506Q) mutation

• Testing strategy
  • Initial APC-R assay, FV Leiden only if indicated
APC Resistance assay: Normal

Baseline aPTT (30 sec)

aPTT after addition of APC (inactivates factor V, prolongs aPTT) (90 sec)

APCR ratio \( \frac{\text{APC aPTT (90)}}{\text{aPTT (30)}} = 3.0 \)
APC Resistance assay: Abnormal (FV Leiden)

Baseline aPTT (30 sec)

aPTT after addition of APC (inactivates factor V, prolongs aPTT, but not as much as normal) (60 sec)

APCR ratio \[
\frac{\text{APC aPTT (60)}}{\text{aPTT (30)}} = 2.0
\]
Testing Strategy for APC-R and FV Leiden

Screening With the Activated Protein C Resistance Assay Yields Significant Savings in a Patient Population With Low Prevalence of Factor V Leiden

Laura J. Taylor, MT(ASCP), Robert A. Oster, PhD, George A. Fritsma, MS, MT(ASCP), Patricia H. Tichenor, MT(ASCP), Cari E. Reed, MT(ASCP), Barbara M. Eiland, MT(ASCP), Christine L. Hudson, MT(ASCP), and Marisa B. Marques, MD

Key Words: Factor V Leiden; Activated protein C resistance; Cost savings

DOI: 10.1309/4370VLV9PBDEWF6
Mayo Clinic, Rochester experience vs Optum labs

- Optum labs data warehouse
  - >100 million enrollees
  - Medical claims data for laboratory testing etc
  - Inpatient and outpatient
## Mayo APCR/FV Leiden vs Optum Labs database 2013 data analysis

<table>
<thead>
<tr>
<th>Test description</th>
<th>Mayo Sp Coag Lab</th>
<th>Optum Labs</th>
</tr>
</thead>
<tbody>
<tr>
<td>APCR-R</td>
<td>1256</td>
<td>5,395</td>
</tr>
<tr>
<td>FV Leiden</td>
<td>268</td>
<td>80,129 (78,525)</td>
</tr>
<tr>
<td>Ratio: APCR:FVL</td>
<td>~1: 0.2</td>
<td>~1:15</td>
</tr>
<tr>
<td>Cost per evaluated individual</td>
<td>$36.38 (savings: $47.39)</td>
<td>$83.77</td>
</tr>
</tbody>
</table>

© 2011 Mayo Foundation for Medical Education and Research. All Rights Reserved.
### ECAT 2016: APCR Normal control plasma sample

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Assigned value</th>
<th>CV(%)</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group</td>
<td>178</td>
<td>(Varied with kit)</td>
<td>33.1</td>
<td>0.76-5.90</td>
</tr>
</tbody>
</table>

#### Classification

<table>
<thead>
<tr>
<th>Classification</th>
<th>Normal</th>
<th>Borderline</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>175</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

#### Secondary classification

<table>
<thead>
<tr>
<th>Secondary classification</th>
<th>Homozygous FVL</th>
<th>Heterozygous FVL</th>
<th>Non-conclusive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

![Histogram of Ratio](chart.png)

- **Count**: The number of plasma samples falling into different ratio ranges.
- **Ratio**: The range of values for the ratio.
- **N**: The total number of samples analyzed.
ECAT 2016: APCR Heterozygous FVL plasma sample

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Assigned value</th>
<th>CV(%)</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group</td>
<td>178</td>
<td>Varied with kit</td>
<td>14.8</td>
<td>0.62-2.38</td>
</tr>
</tbody>
</table>

Classification

<table>
<thead>
<tr>
<th>Classification</th>
<th>Normal</th>
<th>Borderline</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1</td>
<td>1</td>
<td>195</td>
</tr>
</tbody>
</table>

Secondary classification

<table>
<thead>
<tr>
<th>Secondary classification</th>
<th>Homozygous FVL</th>
<th>Heterozygous FVL</th>
<th>Non-conclusive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>9</td>
<td>117</td>
<td>24</td>
</tr>
</tbody>
</table>

ECAT 2016: APCR Heterozygous FVL plasma sample

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.2</td>
<td></td>
</tr>
<tr>
<td>&lt;0.5</td>
<td></td>
</tr>
<tr>
<td>&lt;0.9</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>1.7</td>
<td>100</td>
</tr>
<tr>
<td>2.1</td>
<td>50</td>
</tr>
<tr>
<td>2.5</td>
<td>15</td>
</tr>
<tr>
<td>&gt;2.8</td>
<td>5</td>
</tr>
</tbody>
</table>
Conclusion: APCR and FVLeiden assay

• Initial APCR assay with reflex factor V Leiden is most cost effective approach
• Laboratories need to establish their reference intervals
• False positive APCR:
  • EDTA plasma
Antithrombin assays

• Acquired causes for AT deficiency
  • Liver disease, DIC, L-asparaginase therapy, neonates etc

• Congenital AT deficiency
  • Type I: decreased antigen and activity
  • Type II: low or normal antigen and decreased activity
    • Subtype II RS: reactive site domain
    • Subtype II HBS: heparin-binding domain
    • Subtype II PE: pleiotropic effect mutations (both)
Types of antithrombin assays

- Antigenic:
  - Antigen assays (if performed alone) will miss type II variants
  - C.V: 40 to 50% [CG2-A Survey. CAP 2000]
- Using antigenic assays alone is not advisable
- Initial functional assay with reflex to antigenic assay to sub-classify defect
Functional AT assays

- Functional (activity) amidolytic assays
  - C.V: 9-14% [CG2-A Survey. CAP 2000]

- Principles of assays

- Patient plasma(AT) + heparin + excess thrombin
  - Residual thrombin cleaves PNA (405nm)
  - Human vs bovine thrombin

- Factor Xa based assays
  - Less interference from heparin cofactor II
<table>
<thead>
<tr>
<th>Subtype</th>
<th>Progressive assay</th>
<th>Heparin cofactor activity assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II RS</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Type II HBS*</td>
<td>Normal</td>
<td>Decreased</td>
</tr>
<tr>
<td>Type II Pleotropic</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

*lower risk of venous thrombosis
Most available assays will not detect: due to long incubation times.
UK NEQUAS: AT Cambridge

No of participants

AT activity ug/dL

- Factor Xa
- Bovine thrombin
- Human thrombin

Walker, ID & Jennings, I. Quality in Laboratory Hemost & Thromb. 2nd edition
Conclusions: antithrombin assays

- Bovine thrombin: miss approximately 0.5% of AT deficiency
- Human thrombin: miss approximately 2% of AT deficiency
- Xa based assays: miss approximately 1% of AT assays
Protein C deficiency: assays & variables

- Chromogenic assay: miss 1 to 2% of deficiencies
  - Will miss PL and PS cofactor binding variants
- Clotting based assay: miss ~1% of deficiencies
  - Multiple interferences
- Antigen assays: miss 14% of deficiencies
  - Miss type 2 deficiency
- Variables:
  - Vitamin K dependent
  - False increase with anti-Xa/Direct thrombin inhibitors
UK NEQUAS: PC assays on homozygous factor V Leiden plasma

![Bar chart showing distribution of Protein C levels with categories for Clotting based and Chromogenic assays.]

- Clotting based
- Chromogenic

Participants #

Protein C (u/dL)

30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105

Walker, ID & Jennings, I. Quality in Laboratory Hemost & Thromb. 2nd edition
Limitations of PS activity assays

- PS activity measurements in normals:
  - Levels reduced in 10 to 15% normal donors
  - Upon recheck levels returns to normal

- Subject to technical limitations
  - Measuring a cofactor function
  - Significantly affected by biological and analytical variables

**Reference ranges: lab established vs manufacturer provided information**

<table>
<thead>
<tr>
<th>Assay</th>
<th>%below established reference range</th>
<th>%below manufacturer’s reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free PS Ag</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Kit A PS activity</td>
<td>11%</td>
<td>13%</td>
</tr>
<tr>
<td>Kit B PS activity</td>
<td>18%</td>
<td>38%</td>
</tr>
<tr>
<td>Kit C PS activity</td>
<td>24%</td>
<td>Not available</td>
</tr>
<tr>
<td>Kit D PS activity</td>
<td>10%</td>
<td>20%</td>
</tr>
<tr>
<td>Total PS Ag</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

UK NEQUAS: normal protein S plasma

![Histogram of protein S activity with four kits A, B, C, D showing distribution of participants across different activity levels.](image-url)
Limitations of PS activity assays

- Measurement of a cofactor activity
- Influenced by different biological and preanalytical variables.
- Interferences:
  - Artifactual elevation of PS activity:
    - Lupus anticoagulants
  - Artifactual reduction of PS activity
    - Elevated factor VIII:C
    - Factor V Leiden mutation (selected assays)

## ECAT Proficiency testing: PS deficiency sample (2015-3)

<table>
<thead>
<tr>
<th>Total Group</th>
<th>Assigned value</th>
<th>Range of results</th>
</tr>
</thead>
<tbody>
<tr>
<td>155 Laboratories (5 different kits)</td>
<td>34%</td>
<td>21-111</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Classification</th>
<th>Normal</th>
<th>Borderline normal</th>
<th>Borderline abnormal</th>
<th>Abnormal</th>
<th>No classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>147</td>
<td>2</td>
</tr>
</tbody>
</table>

©2015 MFMER | slide-31
Lupus anticoagulant testing

- Complex
- Preanalytic variables:
  - Anticoagulants
  - Optimal sample processing
  - Calculation of ratios etc
  - Confirmation of positive test results 12 weeks later
Investigation of failed proficiency testing
• Exclude typographical/data entry errors.
• Inspect quality of sample (clotted; ?reconstituted).
• Review Instrument maintenance.
• Review QC and calibrations for the day testing was performed and for trending of assay.
• Review previous PT to see if consistent trend;
  • If so review calibration assignment (is assignment specific for the instrument and kit? Inaccurate?).
• Repeat sample (alternate and same instrument if possible)
Case examples
Case 1

• Reference Laboratory sample referred for assessment of Lupus Anticoagulant

• Local PT and APTT markedly prolonged and inhibited.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
<th>Ref range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (INR)</td>
<td>192.1 (19.2)</td>
<td>8.4-12</td>
</tr>
<tr>
<td>PT mix</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td>APTT</td>
<td>&gt;240</td>
<td>21-33</td>
</tr>
<tr>
<td>APTT Mix</td>
<td>&gt;240</td>
<td></td>
</tr>
<tr>
<td>DRVVT screen (secs)</td>
<td>&gt;6.7</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRVVT mix (secs)</td>
<td>&gt;6.7</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRVVT confirm (secs)</td>
<td></td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>ratio</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Case 1:

Excess heparin (>1.6 u/mL)
Case 2

- Reference Laboratory sample referred for assessment of inhibitors against
  - Factors XII, XI, IX and VIII
- No bleeding symptoms
- On no anticoagulants
- Local APTT markedly prolonged and inhibited
<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
<th>Ref range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (INR)</td>
<td>15.2 (1.4)</td>
<td>8.4-12</td>
</tr>
<tr>
<td>PT mix</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>APTT</td>
<td>60</td>
<td>21-33</td>
</tr>
<tr>
<td>APTT Mix</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>FVIII</td>
<td>&gt;60</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>FIX</td>
<td>&gt;70</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>FIX</td>
<td>&gt;35</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>Assay</td>
<td>Result</td>
<td>Ref range</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>PT (INR)</td>
<td>15.2 (1.4)</td>
<td>8.4-12</td>
</tr>
<tr>
<td>PT mix</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>APTT</td>
<td>60</td>
<td>21-33</td>
</tr>
<tr>
<td>APTT Mix</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Thrombin time</td>
<td>&gt;300</td>
<td>16-25</td>
</tr>
<tr>
<td>Reptilase time</td>
<td>16</td>
<td>16-22</td>
</tr>
<tr>
<td>DRVVT screen (secs) ratio</td>
<td>3.2</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>DRVVT mix (secs) ratio</td>
<td>1.9</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>DRVVT confirm (secs) ratio</td>
<td>1.6</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>Staclot APTT</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Staclot APTT Hex (Delta)</td>
<td>4</td>
<td>(0-13)</td>
</tr>
</tbody>
</table>

**Dabigatran (Mayo)**
Case 2:
Patient was on dabigatran (oral direct thrombin inhibitor)
Phases of anticoagulation:

- **Initial Rx**: Parenteral
  - 0-7 days
- **Acute VTE Rx**: Parenteral or VKA or other agent
  - No effect of thrombophilia
- **Long term**: VKA or other agent
  - 3 months
- **Extended**: VKA or other agent
  - > 3 months
  - Multifactorial effect

Secondary prophylaxis of VTE

Kearon C et al Chest 2012;141 (2)(Suppl)e419S-e494S
Case 3

• Reference laboratory sample submitted for Lupus anticoagulant profile (no clinical information available)

• Laboratory data:
  • PT: 14.8* (8.3 – 10.8)
  • PT Mix 13.0*
  • APTT 67* (21 – 33)
  • APTT Mix 34*
• PT: 14.8* (8.3 – 10.8)
• PT Mix 13.0*
• APTT 67* (21 – 33)
• APTT Mix 34*  
  Thrombin time 142
• PNP 72  
  Reptilase time 163
• PNP buffer 69
• DRVVT Screen ratio 1.4 (49.7)
• DRVVT Mix ratio 1.2 (46.9s)
• DRVVT Confirm ratio 1.1 (46.9)
EDTA specimen

• Pre-analytical variable
• Reference lab receives frozen specimen
• Not possible to tell citrate vs EDTA
• Calcium is critical for in vivo and in vitro coagulation reactions
• EDTA is a more potent chelating agent, reduces available calcium in an assay
• Strong calcium chelation also affects other clot based assays
In house study of EDTA vs citrate

• False reductions in:
  • FV, FVIII, protein C/S activity
• False positive activated protein C resistance
• False reduction in ADAMTS-13 activity/inhibitor assay
Conclusions: Algorithmic approach to thrombophilia testing

- Algorithmic approach begins with patient selection
  - Judicious ordering of Thrombophilia testing if it affects patient management
  - Ensure patient off anticoagulants
  - Provides the most cost effective approach to testing

- Performing laboratories awareness of:
  - Preanalytic interference
  - Anticoagulant interference

- Not all thrombophilias will be detected with current repertoire and assay methodology
Thanks for your attention