Thrombophilia: Laboratory Diagnostic Approach

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Questions

• Why order a thrombophilia risk factor laboratory evaluation?
• What are the most prevalent inherited and acquired risk factors for VTE?
• Which patients should be considered for thrombophilia testing?
• What preanalytic variables and conditions should be considered for a thrombophilia investigation?
• How does thrombophilia testing affect VTE treatment?
Why order a thrombophilia risk factor laboratory evaluation?

• To provide an explanation for VTE
• To identify risk factors that can be modified
• To assess risk for recurrent VTE
• To predict risk of VTE in asymptomatic kin
• To identify risk factors for pregnancy complications
Pathology Consultation on the Laboratory Evaluation of Thrombophilia

When, How, and Why

Riley B. Ballard, MD, MPH, and Marisa B. Marques, MD, for the Education Committee of the Academy of Clinical Laboratory Physicians and Scientists

Am J Clin Pathol 2012;137:553-560
Congenital Thrombophilias

Patients with laboratory-confirmed thrombophilia are at greater risk for VTE, but most will never have an event.

- More than 60% of patients with idiopathic (spontaneous or unprovoked) VTE have inherited thrombophilia;
- abnormal APCR, a negative workup, and P20210 were found in 33%, 32%, and 18% of patients, respectively.

Natural anticoagulant deficiencies:
- Antithrombin (AT)
- Protein C (PC)
- Protein S (PS)
Which patients should be considered for thrombophilia testing?

**Laboratory Investigation Guidelines**

- **Evidence-based guidelines** recommend laboratory evaluation for thrombophilia *only for selected patients*.
- **Testing should encompass all risk factors** because combined defects are common.
- If high-priority tests are negative, low priority studies (e.g., dysfibrinogenemia testing or factor IX and XI activity) *should be considered*.
- It is generally recommended only to perform thrombophilia testing *where the management of the patient will be altered* by the results.
Laboratory Investigation Guidelines, cont.

- *Unique circumstances can dictate the workup:*

  unexplained intra-abdominal VTE (portal, splenic, mesenteric, or hepatic) suggests the need for *JAK2 mutation testing* and *flow cytometric* to evaluate for *MPNs* and *PNH*, respectively
Laboratory Investigation Guidelines, cont

• The patient’s **personal** and **family history** of thrombosis are almost always more important than the thrombophilia test results.
• A **negative result** should not be allowed to provide false reassurance for the patient and clinician.
• It is now apparent that testing for heritable thrombophilia **does not usually predict likelihood of recurrence** in unselected VTE patients and does not reduce **recurrence rates**.
• There is a **low risk** of thrombosis in affected **asymptomatic relatives** and the results of thrombophilia tests are frequently misinterpreted.
Outline of the clinical circumstances that suggest a thrombophilia laboratory evaluation and the high priority screening tests.
Outline of the clinical circumstances that suggest a thrombophilia laboratory evaluation and the high priority screening tests.

Only selected patients from the above clinical circumstances should undergo a laboratory evaluation for thrombophilia.

Selected patients should be considered for thrombophilia testing if at least one of the following is present:
- "Spontaneous" thrombosis (unprovoked) in patients <50 years of age
- Recurrent venous thrombosis (at any age)
- First-degree relatives with VTE
- Thrombosis in unusual sites, such as portal, mesenteric, splenic, hepatic, renal, or cerebral veins (without a risk factor)

Testing should be considered in the following situations:
- Women who intend to become pregnant and/or use oral contraceptives and who have symptomatic first-degree relatives with AT, PC or PS deficiency, or FVL

Testing is recommended only in limited situations:
- Woman with a history of pregnancy loss that is either recurrent or late in pregnancy (second or third trimester)
- Woman with a thrombotic event during pregnancy, HRT, or while taking oral contraceptives
- Patients clinically suspected of having APS

Physical exam and complete medical history to evaluate for presence of acquired risk factors for thrombosis (see text)
Outline of the clinical circumstances that suggest a thrombophilia laboratory evaluation and the high priority screening tests.

<table>
<thead>
<tr>
<th>Thrombophilia: High Priority Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>The best time to evaluate a patient for an inherited thrombophilia is in the outpatient setting after the acute thrombotic episode has resolved and the patient is no longer acutely ill. If thrombophilia testing must be done during a thrombotic event or anticoagulation, a number of assays may be affected depending on the patient’s condition and the drug used.</td>
</tr>
<tr>
<td>1. Global coagulation tests: PT, PTT, TT, fibrinogen</td>
</tr>
<tr>
<td>2. APCR to screen for FVL</td>
</tr>
<tr>
<td>3. Prothrombin G20210A mutation (P20210)</td>
</tr>
<tr>
<td>4. AT activity</td>
</tr>
<tr>
<td>5. PC activity</td>
</tr>
<tr>
<td>6. PS activity: see Figure 2 for a diagnostic algorithm</td>
</tr>
<tr>
<td>7. Factor VIII activity</td>
</tr>
<tr>
<td>8. Antiphospholipid antibodies - LA (see Figure 3 for a diagnostic algorithm)</td>
</tr>
<tr>
<td>IgM and IgG aCL antibodies, and β₂GP1 antibodies</td>
</tr>
<tr>
<td>9. Homocysteine level</td>
</tr>
</tbody>
</table>
Prevalence of thrombophilia risk factors in VTE patients: most abnormal low results for PC, PS, AT are not inherited

Table 17.1  *Defects in thrombophilia: prevalence in the general population, in unselected and selected patients with thrombosis*

<table>
<thead>
<tr>
<th>Type of defect</th>
<th>Prevalence (%)</th>
<th>In general population</th>
<th>In unselected patients</th>
<th>In thrombophilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin deficiency</td>
<td>0.02</td>
<td>1</td>
<td>1–7</td>
<td></td>
</tr>
<tr>
<td>Protein C deficiency</td>
<td>0.2–0.4</td>
<td>3</td>
<td>1–9</td>
<td></td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>~0.1?</td>
<td>1–3</td>
<td>1–13</td>
<td></td>
</tr>
<tr>
<td>Factor V R506Q&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3–7</td>
<td>12–20</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Prothrombin 20210G→A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3</td>
<td>6.2</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>
## FREQUENCY OF INHERITED THROMBOPHILIAS AMONG HEALTHY SUBJECTS AND UNSELECTED AND SELECTED PATIENTS WITH VENOUS THROMBOSIS

<table>
<thead>
<tr>
<th>Inherited thrombophilia</th>
<th>Healthy subjects</th>
<th>Unselected patients</th>
<th>Selected patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td><strong>Protein C deficiency</strong></td>
<td>0.2 – 0.4</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td><strong>Protein S deficiency</strong></td>
<td>—</td>
<td>2.3</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>Antithrombin deficiency</strong></td>
<td>0.02</td>
<td>1.9</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>Factor V Leiden</strong></td>
<td>4.8</td>
<td>18.8</td>
<td>40</td>
</tr>
<tr>
<td><strong>G20210A prothrombin</strong></td>
<td>2.7</td>
<td>7.1</td>
<td>16</td>
</tr>
</tbody>
</table>
### Frequencies of Common Mutation/Polymorphism, Iranian Patients

<table>
<thead>
<tr>
<th>Total requested</th>
<th>Test</th>
<th>Normal (N/%)</th>
<th>Heterozygote (N/ %)</th>
<th>Homozygote (N/ %)</th>
<th>Male (N/%)</th>
<th>Female (N/ %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>F.V.L</td>
<td>287 (%91)</td>
<td>27 (%8.69)</td>
<td>1 (%0.31)</td>
<td>119 (%37.7)</td>
<td>196 (%62.23)</td>
</tr>
<tr>
<td>190</td>
<td>FII</td>
<td>177 (%93.15)</td>
<td>13 (%6.84)</td>
<td>0</td>
<td>44 (%23.15)</td>
<td>146 (%76.84)</td>
</tr>
<tr>
<td>154</td>
<td>MTHFR A1298C</td>
<td>47 (%30.52)</td>
<td>81 (%52.6)</td>
<td>26 (%16.88)</td>
<td>31 (%20.13)</td>
<td>123 (%79.87)</td>
</tr>
<tr>
<td>154</td>
<td>MTHFR C677T</td>
<td>58 (%37.67)</td>
<td>71 (%46.1)</td>
<td>25 (%16.23)</td>
<td>31 (%20.13)</td>
<td>123 (%79.87)</td>
</tr>
<tr>
<td>109</td>
<td>PAI 4G/5G</td>
<td>28 (%25.68)</td>
<td>61 (%55.08)</td>
<td>20 (%18.34)</td>
<td>20 (%18.35)</td>
<td>89 (%81.65)</td>
</tr>
</tbody>
</table>
Hereditary hypercoagulability factors tested by ARMS - PCR

DNA samples were obtained from 196 patients and relatives of patients with thromboembolic diseases.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Samples (n)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type homozygous</td>
<td>Risk allele heterozygous</td>
<td>Risk allele homozygous</td>
</tr>
<tr>
<td>ACE (intron 16 I/D)</td>
<td>42</td>
<td>102</td>
<td>52</td>
</tr>
<tr>
<td>Factor V (1691G/A)</td>
<td>144</td>
<td>47</td>
<td>4</td>
</tr>
<tr>
<td>Factor V HR2 (4070A/G)</td>
<td>48</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Factor VII (Gln353Arg)</td>
<td>108</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>Factor XIII (Val34Leu)</td>
<td>76</td>
<td>34</td>
<td>3</td>
</tr>
<tr>
<td>β-fibrinogen (−455G/A)</td>
<td>51</td>
<td>34</td>
<td>3</td>
</tr>
<tr>
<td>Glycoprotein Ia (807C/T)</td>
<td>42</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>MTHFR (677C/T)</td>
<td>79</td>
<td>98</td>
<td>18</td>
</tr>
<tr>
<td>PAI-1 (−675 I/D, 5G/4G)</td>
<td>47</td>
<td>88</td>
<td>55</td>
</tr>
<tr>
<td>Prothrombin (20210G/A)</td>
<td>123</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Thrombomodulin (127G/A)</td>
<td>59</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>TFPI (536C/T)</td>
<td>59</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>tPA (intron h D/I)</td>
<td>38</td>
<td>93</td>
<td>64</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>E2/E2</td>
<td>E2/E3</td>
<td>E2/E4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Reference DNA samples were tested for hereditary hypercoagulability by ARMS. The number of samples tested per allelic constellation are indicated.
Variables and/or Interferences That Affect Results of Thrombophilia Evaluation

- **Natural anticoagulants**
- Antithrombin deficiency (plasma activity) decreased by
  - Unfractionated or low molecular weight heparin
  - Ongoing or recent thrombosis
  - Hepatic dysfunction (acute or chronic)
  - Nephrotic syndrome
  - DIC
  - Pregnancy and puerperium (avoid testing until 3 mo postpartum)
- Estrogen therapy (COC or HRT; delay testing until 3 mo after discontinued)
- L-asparaginase therapy
Variables and/or Interferences That Affect Results of Thrombophilia Evaluation

- **Natural anticoagulants**
- **Protein C deficiency** (plasma activity) decreased by
- Warfarin (delay testing until at least 3-4 wk after discontinued)
- Ongoing or recent thrombosis
- Delay testing 2-3 months after acute event
- Preferable to discontinue oral anticoagulant therapy at least 2 weeks to 1 month before testing
- **Hepatic dysfunction** (acute or chronic)
- **DIC**
- **Vitamin K deficiency**
- **L-asparaginase therapy**
- Elevated FVIII levels (acute phase reactant) may interfere in some functional assays and result in falsely decreased values
- **Heparin and direct thrombin inhibitors** may interfere in some functional assays, resulting in falsely elevated values
Variables and/or Interferences That Affect Results of Thrombophilia Evaluation

- **Natural anticoagulants**
- Protein S deficiency (plasma activity) decreased by
  - Warfarin (delay testing until at least 3-4 wk after discontinued)
  - Acute phase reaction
  - Ongoing or recent thrombosis
  - Hepatic dysfunction (acute or chronic)
  - DIC
  - Pregnancy and puerperium (avoid testing until 3 mo postpartum)
  - Estrogen therapy (COC or HRT; delay testing until 3 mo after discontinued)
  - Vitamin K deficiency
  - Nephrotic syndrome
  - L-asparaginase therapy
Problems with tests for antithrombin, protein C, and protein S

- **Physiologic states associated with variable levels of AT, PC, and PS:**
  - **Pregnancy**: ↓AT, ↑PC, ↓↓ PS
  - **Age**: adult PC levels not reached until age 18
  - **Gender**: females have lower PS levels than males
Problems with tests for antithrombin, protein C, and protein S

<table>
<thead>
<tr>
<th>Pathologic states associated with variable levels of AT, PC, and PS:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute thrombosis</td>
</tr>
<tr>
<td>Liver disease</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
</tr>
<tr>
<td>Warfarin</td>
</tr>
<tr>
<td>Heparin</td>
</tr>
</tbody>
</table>
How “good” are thrombophilia test results?

• **Reference ranges**
  – 40 healthy adults:
    • 50% women, no OCP/HRT

• **Precision**
  – Quality control
    • Normal/abnormal controls within 2SD
    • Lot to lot comparisons

• **Accuracy/bias?**
  – No universal standards (Na, glu)-WHO plasma pools substitute
  – Proficiency testing: external proficiency testing
    • College of American Pathology
    • ECAT Foundation Leiden Netherlands
Lab Highlights

- **Collection tubes**, vacuum collection device
- 3.2% tri-sodium citrate (light blue-top) is the proper anticoagulant.
- **Needle size, Adults:** 20-21 gauge **pediatric** patient, a 21- to 23-gauge
- **Syringe draws are discouraged** because of the increased risk of hemolysis. Larger syringes, there is an increased chance that clotting may occur.
- If a syringe is used, a small volume syringe < 20mL is recommended.
- all tubes should be **gently inverted IMMEDIATELY at least five times to mix. DO NOT shake or mix vigorously.**
# Lab Highlights

**CLSI recommended order of draw**

<table>
<thead>
<tr>
<th>Tube</th>
<th>Top Color</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>YELLOW</td>
<td>(Culture tubes/vials)</td>
</tr>
<tr>
<td>2</td>
<td>RED</td>
<td>(Non-additive tubes/serum tubes)</td>
</tr>
<tr>
<td>3</td>
<td>LIGHT BLUE</td>
<td>(Citrate tubes-Coagulation tests)</td>
</tr>
<tr>
<td>4</td>
<td>SPECKLED</td>
<td>(Gel separator tubes)</td>
</tr>
<tr>
<td>5</td>
<td>GREEN</td>
<td>(Heparin tubes)</td>
</tr>
<tr>
<td>6</td>
<td>LAVENDER</td>
<td>(EDTA tubes)</td>
</tr>
<tr>
<td>7</td>
<td>OTHER</td>
<td>(Other additives)</td>
</tr>
</tbody>
</table>
Lab Highlights

- **Collection problems**
  - underfilling
  - overfilling
  - high hematocrit
  - clotted specimens
  - hemolysis, shortens the APTT
- **SPECIAL COAGULATION TESTS:** CLSI guidelines recommends drawing a discard tube before specimens collected for special coagulation tests.
TRANSPORT and STORAGE

• For best results, most sources recommend that specimens for Coagulation testing be delivered to the laboratory for testing within 1/2 hour of collection.

• Specimens for routine Coagulation testing should be transported either at room temperature* (18-24°C) or refrigerated (2-4°C). *Specimens for Prothrombin Time testing (PT) should be transported at room temperature. They should NOT be refrigerated.

• PT : within 24 hours of collection.

• APTT : within 4 hours of collection.

• ALL other COAGULATION tests : within 4 hours of collection.

• When samples cannot be assayed within the required time frame, the plasma must be separated from the red cells and frozen within one hour of collection.
Lab Highlights

- Special tests that require double spun to obtain platelet-free plasma
- Heparin Neutralization Procedure
- Lupus Panel (Dilute Russell’s Viper Venom Test)
- AT III (Anti-Thrombin III)
- APCR (Activated Protein C Resistance)
- Bethesda and Factor Assays (if saved for later testing)
- Heparin Anti-Xa Assay
- PROTEIN C
- PROTEIN S
Lab Highlights

• Clot-based activity assays: maximize sensitivity
  PC, PS, AT deficiencies
• PC in adults: about 3-5 µg/mL (70-140%).
• Term newborns have PC levels of ~25-45% of adult levels, which slowly rise to adult levels during adolescence
  ■ Protein C levels vary with age

• it is extremely important to exclude an acquired deficiency
Deficiencies of Natural Anticoagulants

- *Laboratory investigation of PS deficiency is more complex with 3 distinct assay types:*
  1. PS activity (clot-based) measures function as a cofactor for APC
  2. Free PS is an immunologic measure of the unbound fraction
  3. Total PS antigen quantifies free and bound PS.
    - *Antigenic tests for free protein S* are preferred for diagnosis
    - Free protein S values are higher in males than in females
Deficiencies of Natural Anticoagulants

- *Falsely low* values of PS are found in up to 10% to 15% of healthy people.
- *Free PS antigen is more reliable* and diagnoses 95% to 99% of PS deficiencies.
- *Anti-thrombin*: Healthy newborns have about half the normal adult concentration and gradually reach the adult level by six months of age.
Lab. message

• Many pre-analytical variables lower PC, PS, AT
• **Assay imprecision**: PS>PC>AT
• Overlap between heterozygous deficiency and wild type: PS=PC>AT
• Reference intervals are guidelines-
• Wide indeterminate range: ~40-60% PC/PS, 60-80% AT
Variables and/or Interferences That Affect Results of Thrombophilia Evaluation

- **Other risk factors**
- Factor VIII activity (functional and antigenic assays) increased by
  - HRT (delay testing until 3 mo after discontinued)
  - Illness, stress, surgery
  - Ongoing or recent thrombosis
  - DIC
  - Pregnancy and puerperium (avoid testing until 3 mo postpartum)
Variables and/or Interferences That Affect Results of Thrombophilia Evaluation

- **Other risk factors**
- Hyperhomocysteinemia (fasting plasma level) increased by
  - Vitamin B6, vitamin B12, or folate deficiency
  - Renal disease
  - Malignancies
  - Hypothyroidism
  - Drugs such as oxcarbazepine and topiramate
Variables and/or Interferences That Affect Results of Thrombophilia Evaluation

- **Antiphospholipid antibodies**
  - Positive rheumatoid factor and/or cryoglobulins can cause false-positive IgM

- **aCL (ELISA)**
  - Positive rheumatoid factor and/or cryoglobulins can cause false-positive IgM

- **LA (clot-based tests such as PTT and dRVVT)**
  - Unfractionated heparin prolongs PTT and will interfere with the mixing study
  - Factor VIII inhibitor may mimic LA by prolonging PTT and yielding a positive phospholipid neutralization step (false-positive confirmatory test)
  - Warfarin prolongs the dRVVT
Criteria for APS

- include a positive APA result on 2 or more occasions at least 12 weeks apart plus 1 of the following clinical criteria:
  1. arterial, venous, or small vessel thrombosis occurring in any tissue or organ
  2. unexplained fetal loss at or beyond the 10th week of gestation;
  3. birth of a morphologically normal neonate before the 34th week because of eclampsia, preeclampsia, or placental insufficiency; or
  4. 3 or more unexplained consecutive spontaneous abortions before the 10th week of gestation.

VTE is the most common manifestation of APS, and LA is the strongest risk factor for thrombosis and pregnancy morbidity.
Pregnancy loss is at least 3 times more likely in women with positive aCL or LA results
Antiphospholipid Antibodies and Antiphospholipid Syndrome

- There are 3 major types of antiphospholipid antibodies (APAs):
  - *Lupus Anticoagulants, LAs*
  - *anticardiolipin, aCL*
  - *anti–β2-glycoprotein-1, anti-β2GP1, antibodies.*

- While most experts believe that patients suspected of having APS must be evaluated for all APAs, some prefer to evaluate for LAs and aCLs or anti-β2GP1.
Antiphospholipid Antibodies

10% of healthy donors, 30-50% of SLE patients

- **LA antibodies** are directed against **plasma proteins** bound to anionic phospholipids

- **aCL antibodies** are directed against **phospholipids** bound to proteins
  - Can be IgA, M, or G (subclasses 1-4)
  - IgG (esp G2) associated with a greater risk of APS

- **Anti β₂GPI antibodies** are directed against a **plasma protein** that binds phospholipid with high affinity
Antiphospholipid Antibodies and Antiphospholipid Syndrome

• Because of heterogeneous antibody specificity, *no single test is 100% sensitive*

• Thus, 2 or more phospholipid-based screening tests must be used to detect LAs, often the combination of a low-concentration phospholipid PTT and dilute Russell viper venom time.
Antiphospholipid Antibodies

• **Lupus Anticoagulant (LA) Antibodies**
  • Prolonged coagulation in phospholipid-dependent in vitro tests (aPTT, PT, dRVVT)
  • Failure to correct with 50:50 mix
  • Correction of coagulation time by adding phospholipid

• **Anticardiolipin (aCL) Antibodies**
  • ELISA assay in the presence of bovine B2GPI

• **Anti-Beta 2 Glycoprotein I Antibodies (β₂GPI)**
  • ELISA assay using human B2GPI coated plates
  • *most specific*
Algorithm for the laboratory detection of lupus anticoagulant (LA)

1. Perform 2 low-phospholipid screening tests: PTT and dRVVT
   - Neither test is prolonged
     - No further testing; no LA present
   - PTT prolonged
     - Assay thrombin time
       - Normal
         - PTT and/or dRVVT mixing study
           - Mix corrects
             - No LA present; suspect factor deficiency(ies)
           - Mix does not correct
             - Confirmatory test (high phospholipid)
               - Positive
                 - Warfarin prolongs the dRVVT (low factors X and II)
               - Negative
                 - LA present
               - No LA present
     - PTT prolonged
       - Add heparin neutralizer
         - Repeat PTT
           - Normal
             - No LA present; no further testing
           - Warfarin prolongs the dRVVT (low factors X and II)
   - dRVVT prolonged
     - No further testing; no LA present
NHS FORTH VALLEY

GUIDELINE ON
THROMBOPHILIA INVESTIGATION AND TESTING

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Final Approval

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UNCONTROLLED WHEN PRINTED
GUIDELINES FOR THROMBOPHILIA TESTING
Thank you, any question?