Laboratory Testing Issues for Protein C, Protein S and Antithrombin Assays

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Consultant: IL and Novo Nordisk
Research: Stago

Off Label Usage
None
Testing Recommendations for AT, PC and PS Assays

Outline

- Concept of Laboratory Phenotype
  - Genotype
  - Clinical phenotype (disease)

- General Recommendations
- Protein C Testing
- Protein S Testing
- Antithrombin Testing
- Algorithms for Testing
Role of Laboratory Phenotype in Coagulation

Genotype

Protein Phenotype

Clinical Phenotype

Laboratory Phenotype
Confusion of Laboratory Phenotype Reflecting Genotype & Clinical Phenotype

- Usually Laboratory Phenotype mirrors Genotype
  - In most cases, the activity method accurately reflect the genotype
  - HOWEVER, unique Type II molecules may not reflect the true genotype

- In a high percentage, Laboratory Phenotype does NOT reflect the Clinical Phenotype
  - Incidence of deficiency in the general population without thrombosis is high (PC, PS, APC-R)
  - Exception may be Antithrombin
General Recommendations for PC, PS and AT Testing
Components for the Evaluation of AT, PC and PS Assays

Post-Analytical
Result reporting
Intervals

Analytical
Assay method

Laboratory Phenotype

Pre-Analytical
Specimen Handling

Patient
Variability

Ref
General Recommendations for AT, PC and PS Assays

- Pre-Analytical Variables and Post-Analytical Processes must be controlled or may cause an erroneous diagnosis
  - Correct timing with respect to patient history
  - Presence of anticoagulant
  - Testing during pathology
  - Correct Reference Interval
- Not all methods measure material the same way
- Repeat abnormal results
Effect of Anticoagulant Drugs
## Effect Intravenous Anticoagulants on PC, PS and AT Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>UFH</th>
<th>LMWH</th>
<th>Fonda</th>
<th>IV DTI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PC-</strong> Clot Based</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Chromo</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Antigen</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>PS-</strong> Activity</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Free</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Total</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>AT-</strong> FXa</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Thr</td>
<td>↑</td>
<td>N</td>
<td>N</td>
<td>↑</td>
</tr>
<tr>
<td>Antigen</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
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</table>
**Effect Oral Anticoagulants on PC, PS and AT Assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Warfarin</th>
<th>Dabigatran</th>
<th>Rivaroxaban</th>
<th>Apixaban</th>
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<tbody>
<tr>
<td>PC- Clot</td>
<td></td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>Chromo</td>
<td>↑↑</td>
<td></td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Antigen</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>PS- Activity</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>Free</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Total</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>AT- FXa</td>
<td>N</td>
<td>N</td>
<td>↑↑</td>
<td>N</td>
</tr>
<tr>
<td>Thr</td>
<td>N</td>
<td></td>
<td>↑↑</td>
<td>N</td>
</tr>
<tr>
<td>Antigen</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>
Interpretation of coagulation data is a COMPARATIVE DECISION-MAKING PROCESS!

- Compare patient result to reference interval
- Reference interval set up for:
  - “Normal” or healthy
  - Physiologic conditions- age and/or gender
  - Therapeutic interventions
Reference Range Issues
Required Reference Ranges

- **Antithrombin:**
  - Adult Range
  - Newborn Range

- **Protein C:**
  - Adult Range
  - Pediatric Range
  - Newborn Range

- **Protein S:**
  - Adult Range
  - Gender Specific Range
  - Newborn Range
  - Pregnancy Range (?)
  - Hormone Range (?)
Protein C Assays

Major Issues Associated with Testing
Protein C Clotting & Chromogenic Assays

- Clotting assay measures activation, activity & cofactor/PL interactions
  - Based on PTT or RVVT
  - Clotting time proportional to PC conc.
  - Assay reagents vary significantly - may affect results

- Chromogenic assay measures activation & APC activity only
  - Cofactor and PL not necessary for assay
  - Less interfering effects
  - Miss defects in cofactor and PL binding
Clinical Aspects of Protein C
Quantitative and Qualitative Defects

- **Quantitative Defects (Type I):**
  - Major gene defect
  - Decrease of both activity and antigen

- **Qualitative Defects (Type II):**
  - Due to point mutation
  - Decrease of activity with normal antigen

- **Sub-Types:**
  - Type IIa- defect in activation site or active site
  - Type IIb- defect in cofactor and/or PL binding
Type II Protein C Deficiency
Genetic Defects in Function

- 10-15% of Protein C deficiencies are Type II deficiencies.
- Over 50 different Type II deficiencies described.
- Dysfunctions measurable in clotting assays but about 25% have normal chromogenic activity and antigen levels.
### Diagnosis of PC Deficiency
**Genetic and Acquired Defects**

<table>
<thead>
<tr>
<th></th>
<th>PTT Assay</th>
<th>Chromogenic Assay</th>
<th>PC Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>96%</td>
<td>98%</td>
<td>95%</td>
</tr>
<tr>
<td>OAT</td>
<td>39%</td>
<td>76%</td>
<td>81%</td>
</tr>
<tr>
<td>Type I</td>
<td>42%</td>
<td>46%</td>
<td>40%</td>
</tr>
<tr>
<td>Type IIa</td>
<td>58%</td>
<td>53%</td>
<td>98%</td>
</tr>
<tr>
<td>Type IIb</td>
<td>48%</td>
<td>89%</td>
<td>94%</td>
</tr>
</tbody>
</table>
## Diagnosis of PC Deficiency

### Type IIb Defect

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Chromo. Assay</th>
<th>PTT Assay #1</th>
<th>PTT Assay #2</th>
<th>RVVT Assay</th>
<th>PC Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type IIb</td>
<td>98 IU/mL</td>
<td>50 IU/mL</td>
<td>91 IU/mL</td>
<td>69 IU/mL</td>
<td>96 IU/mL</td>
</tr>
</tbody>
</table>

From: Cooper, et al IJLH, 2011
Protein S Assays
Clinical and Laboratory Aspects of Protein S Assays

- Complex interactions and functions of Protein S
- Deficiency states are also complex
  - Type I, Type II and Type III
- Therefore assay methods can be complex as well

3 Types of Assays
- Protein S Activity Assay: Clot Based (PTT or RVVT)
- Free Protein S Antigen Assay: Monoclonal Antibody
- Total Protein S Antigen Assay: Polyclonal Antibody
Spurious Protein S Activity Levels in Non-PS Deficient Patients
Protein S Activity Assays
Assay Inconsistencies

- False low PS activity compared to Free PS Ag
  - Assay #1: 11% of samples
  - Assay #2: 14% of samples
  - Assay #3: 16% of samples
  - Assay #4: 19% of samples

- Cause of Discrepancy between Activity & Antigen
  - Elevated FVIII
  - Inflammatory interactions

- On repeat, >95% low PS Act compare to Free PS

Comparison of Free PS Levels with PS Activity

![Graph showing comparison of Free PS Levels with PS Activity]
Issues for Free PS Antigen and Total PS Antigen Assays

**Free PS Antigen**
- Free PS Antigen usually correlates with PS activity
- Possibly miss Dysfunctional PS (Type II) molecules
- Estimated 1-3% of Protein S deficiencies

**Total PS Antigen**
- Poor correlation with PS Act or Free PS Ag assays
- Miss numerous PS deficiencies
- Increased cost with no additional clinical or mechanistic information
Antithrombin Assays
Clinical Aspects of Antithrombin
Quantitative and Qualitative Defects

- **Quantitative Defects (Type I):**
  - Major gene defect
  - Decrease of both activity and antigen

- **Qualitative Defects (Type II):** (May be Difficult to Diagnose)
  - 3 different sub-types due to point mutations
  - Decrease of activity with normal antigen

- **Sub-Types:**
  - Type IIa or Type II RS- defect in reactive site
  - Type IIb or Type II HBS- defect in heparin binding site
  - Type IIc- Pleiotropic effect
Laboratory Assays for Antithrombin

- Both Activity and Antigenic AT assays available
- Activity assays are chromogenic methodology based using one of two enzymes
  - Factor Xa
  - Thrombin (human or bovine)
- Majority use heparin
- Assay Variables: incubation time, enzyme conc., buffers
Diagnosis of Antithrombin Deficiency
Type I and Type II Defects

Type I
- Decreased activity & antigen (Ratio 0.7-1.3)
- Detects Type I with all assays
  - Both thrombin based and factor Xa based
  - Possible interference from Heparin Cofactor II with human thrombin

Type II
- Decreased activity & normal antigen (Ratio 0.3-0.7)
- Values different to thrombin or FXa.
  - This can lead to the wrong diagnosis of “normal”
Interference of Heparin Cofactor II in the Diagnosis of Type I AT Deficiency

Enzyme and Antigen Levels

<table>
<thead>
<tr>
<th></th>
<th>Human FIIa</th>
<th>Bovine FIIa</th>
<th>Human FXa</th>
<th>AT Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>93-135%</td>
<td>85-125%</td>
<td>80-124%</td>
<td>87-130%</td>
</tr>
<tr>
<td>Act/Ag Ratio</td>
<td>0.92-1.42</td>
<td>0.82-1.24</td>
<td>0.78-1.22</td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>47-75%</td>
<td>42-62%</td>
<td>35-58%</td>
<td>45-64%</td>
</tr>
<tr>
<td>Act/Ag Ratio</td>
<td>0.47-0.65</td>
<td>0.34-0.59</td>
<td>0.38-0.60</td>
<td></td>
</tr>
</tbody>
</table>
# Diagnosis of Antithrombin Deficiency

Type II (Reactive Site) Defects

<table>
<thead>
<tr>
<th>AT Defect</th>
<th>Name</th>
<th>Bovine Thrombin</th>
<th>Human FXa</th>
<th>AT Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Range</td>
<td></td>
<td>86-132%</td>
<td>83-135%</td>
<td>83-124%</td>
</tr>
<tr>
<td>AT (A416S)</td>
<td>Cambridge II</td>
<td>67%</td>
<td>93%</td>
<td>87%</td>
</tr>
<tr>
<td>AT (S426L)</td>
<td>Denver</td>
<td>63%</td>
<td>90%</td>
<td>88%</td>
</tr>
<tr>
<td>AT (G424D)</td>
<td>Stockholm</td>
<td>45%</td>
<td>88%</td>
<td>97%</td>
</tr>
</tbody>
</table>

## Diagnosis of Antithrombin Deficiency

### Heparin Binding Site Defect

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Normal</th>
<th>AT Type II (HBS)</th>
<th>AT Type II (RS)</th>
<th>AT Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AT Defect</strong></td>
<td><strong>30 sec</strong></td>
<td><strong>1 min</strong></td>
<td><strong>2 min</strong></td>
<td><strong>4 min</strong></td>
</tr>
<tr>
<td><strong>Normal</strong></td>
<td>85%</td>
<td>82%</td>
<td>83%</td>
<td>85%</td>
</tr>
<tr>
<td><strong>AT Type II (HBS)</strong></td>
<td>70%</td>
<td>85%</td>
<td>98%</td>
<td>115%</td>
</tr>
<tr>
<td><strong>AT Type II (RS)</strong></td>
<td>46%</td>
<td>49%</td>
<td>44%</td>
<td>48%</td>
</tr>
<tr>
<td><strong>AT Type I</strong></td>
<td>60%</td>
<td>53%</td>
<td>58%</td>
<td>51%</td>
</tr>
</tbody>
</table>
Summary of Recommendations
PC, PS and AT Testing: Recommendations

- All test kits measure the same analyte
- However not all test kits measure the analyte in the same manner and same extent
  - Hence different diagnosis
- Most cost-effective method:
  - Measure with single test
  - And if abnormal then reflex to confirmatory tests
- Question an assay result that is borderline normal or has significant variability on repeat
- Repeat questionable results
PC, PS and AT Testing: Recommendations

- Pre-Analytical Variables and Post-Analytical Processes:
  - If not controlled, may cause erroneous results
- Pre-Analytical
  - Acceptable specimen
  - Testing during pathology
  - Presence of anticoagulants
- Post-Analytical
  - Comparison to wrong Reference Interval
PC, PS and AT Testing: Test Recommendations

- AT Activity Assay can use FXa or thrombin (bovine)
  - If Abnormal confirm with antigen assay
- PS Free Assay should be initial test
  - If abnormal, then reflex test to PS activity
- PC chromogenic assay should be standard test
  - Unless Type II suspected, then PC Clotting Assay

**Remember:** Not all test kits measure the same parameters so alternatives may be necessary
Protein C Testing Algorithm

1. Assess for Causes of Acquired Deficiencies
   - Yes → Do NOT Test
   - No → Chromogenic PC Assay

2. Chromogenic PC Assay
   - Normal → No Further Testing
   - Abnormal → PC Antigen Assay

3. PC Antigen Assay
   - Abnormal → Confirm PC Deficiency
   - Abnormal → Evaluate for Acquired Causes & Repeat in 6-12 weeks
Protein S Testing Algorithm

1. Assess for Causes of Acquired Deficiencies
   - Yes: Do NOT Test
   - No: Free PS Ag Assay

2. Free PS Ag Assay
   - None
     - Do NOT Test
   - Normal: PS Activity Assay
   - Abnormal: Total PS Ag Assay

3. Total PS Ag Assay
   - Optional

4. PS Activity Assay
   - Abnormal: Evaluate for Acquired Causes & Repeat in 6-12 weeks
   - Abnormal: Confirm PS Deficiency

5. No Further Testing
Antithrombin Testing Algorithm

Assess for Causes of Acquired Deficiencies

- Yes: Do NOT Test
- No: AT Activity Assay

AT Activity Assay

- None: No Further Testing
- Normal: No Further Testing
- Abnormal: AT Antigen Assay

AT Antigen Assay

- Normal: Confirm AT Deficiency
- Abnormal: Evaluate for Acquired Causes & Repeat in 6-12 weeks

Optional Unique Assay or Genotyping

Possible Type II

Abnormal
Laboratory Testing for Protein C, Protein S and Antithrombin

Bottom Line

- Must know your patient
- Must have specimen & sample integrity
- Must know the assay limitations
- Must know Reference Interval
- Must confirm abnormal results
Questions??