Basic coagulation applications and case studies

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Agenda

- Overview about 3 major phases of Hemostasis
- Laboratory Testing for von Willebrand Disease
- Routine PT, PTT, FIB, TT, Rept and DDIM tests
- Case study
3 major phases of Hemostasis

- **Blood vessel wall**
  Vasoconstriction
  Endothelium cells surface interactions
  - Make and express tissue factor with injury
  - Major synthetic and storage site for Von Willebrand factor.
    a. vWF is a large multimeric protein that acts as the glue
       Binding platelets to the subendothelium at an injury site. **glycoprotein Ib on platelet surface membrane binds to vWF**
    b. vWF acts as carrier protein for factor VIII. Another name: **Factor VIII related antigen (VIII R: Ag)**

- **Platelets**
  Form platelets plug at the injury

- **Coagulation component**
  Coagulation factors (proteins)
  - Names and numbers
  - Active and inactive forms
Platelet Structure: Unactivated/Activated

- **$\alpha$ granules** (raw materials): PF4, $\beta$-TG, Fibrinogen, VWF, Factor V, and PAI-1
- **Dense granules** (energy and glue): ATP, ADP, Serotonin, $Ca^{2+}$, $Mg^{2+}$, P

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Bernard-Soulier syndrome

Bernard-Soulier syndrome results from a deficiency of platelet GPIb, the receptor for von Willebrand factor. It is a rare but severe bleeding disorder. Platelets do not aggregate to ristocetin. The platelet count is low, but, characteristically, giant platelets.

Glanzmann thrombasthenia

Glanzmann thrombasthenia results from a deficiency of the GP IIb/IIIa complex. Platelets do not aggregate to any agents except ristocetin.
Platelet activation
## Coagulation factors and names

<table>
<thead>
<tr>
<th>Factor</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>II</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>III</td>
<td>Tissue factor or thromboplastin</td>
</tr>
<tr>
<td>IV</td>
<td>Calcium</td>
</tr>
<tr>
<td>V</td>
<td>Proaccelerin (Labile factor)</td>
</tr>
<tr>
<td>VII</td>
<td>Proconvertin (Stable factor)</td>
</tr>
<tr>
<td>VIII</td>
<td>Antihaemophilic factor A, Antihaemophilic globulin</td>
</tr>
<tr>
<td>IX</td>
<td>Antihaemophilic factor B, Plasma thromboplastin component, Christmas factor</td>
</tr>
<tr>
<td>X</td>
<td>Stuart-Prower factor</td>
</tr>
<tr>
<td>XI</td>
<td>Plasma thromboplastin antecedent, Haemophilia C, Rosenthal syndrome</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman factor</td>
</tr>
<tr>
<td>XIII</td>
<td>Fibrin stabilising factor, Laki-Lorand factor</td>
</tr>
</tbody>
</table>

**Note:**

The basis of the factor XIII screen is that normally a clot is stable for 24 hours in 5M urea, while in factor XIII deficiency the clot is unstable over a time period of minutes. Clinically affected individuals have <1% factor XIII activity.

Routine screen tests can not detect FXIII deficiency.
Coagulation cascade

intracellular pathway

XII → XIIa → Ca²⁺ → XI → XIa → Ca²⁺ → IX → IXa → Ca²⁺ → VIIIa → PL → Ca²⁺ → X → Xa → Va → PL → Ca²⁺ → prothrombin → thrombin

extracellular pathway

VIIa/TF → TF → VII → VIIIa → PL → Ca²⁺ → fibrinogen → fibrin monomer → fibrin clot → XIII → thrombin → XIIIa → stabilized fibrin clot

PL = platelet phospholipids
TF = tissue factor

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Fibrinogen

Extrinsic Pathway

TF

Intrinsic pathway

XIIa

XIIa

IXa

VIIIa

XIa

XIIa

XIIIa

Common pathway

Xa

VIIa

Fibrinogen

Thrombin

Fibrin

Soft clot

Hard clot

Fibrin
Prothrombin Time

- Platelet Poor Plasma
- Citrated 9:1; 3.2%
- Thromboplastin
  - Phospholipid
  - Source of tissue factor (e.g. rabbit brain, human placenta)
  - Ca+2
- Time to clot detection (seconds)
- Sensitive to factor VII, but also V and X
- Standardization
  - INR

\[
\text{tissue thromboplastin (contains CaCl}_2\text{)} + \text{Citrated Plasma} \downarrow \text{Fibrin clot}
\]

Quick test: Dr Quick first invented this test
(Dr. Armand Quick published his method in 1935.)
Anticoagulant: 3.2% sodium citrate

The formula to calculate the appropriate sodium citrate volume is:

\[(100 - \text{HCT}) / (595 - \text{HCT}) \times \text{total anticoagulated blood (5, 3 ,2 ml)}\]

Given a normal HCT ~ 45, \((100 - 45) / (595 - 45) = 1 : 10\)
Anticoagulant : whole blood would be 1 : 9 ratio

According to the latest CLSI (formerly NCCLS) guideline on coagulation testing, it is important to adjust the sodium citrate volume when a patient’s hematocrit is greater than 55%.

High HCT, less plasma, less anticoagulant needed
Monitoring Coumadin Therapy: INR (International Normalized Ratio)

\[
\text{INR} = \left\{ \frac{\text{Patient PT (sec)}}{\text{Mean Normal PT (sec)}} \right\}^{\text{ISI}}
\]

Where ISI = International Sensitivity Index (fudge factor to make different reagent and instrument systems agree)
Therapeutic range for coumadin is INR 2-3 for most indications (e.g., treatment of venous thromboembolism)
Activated partial thromboplastin time (aPTT)

- Platelet Poor Plasma
- Citrated 9:1 3.2%
- Activator (e.g. silica, kaolin)
- Partial Thromboplastin
  - No source of tissue factor
  - Phospholipid
- Ca2+
- Time to Clot Detection (seconds)
- aPTT can be shortened due to elevated FVIII as an acute phase reactant

In 1953, in stead of using a complete tissue extract, such as PT reagent, only a partial extract was used for assay which is called PTT (Partial thromboplastin time)

- Cephalin (platelet substitute) from rabbit cerebral tissue + particulate activator silica
  + Citrated Plasma
  + CaCl₂
  ↓
  Fibrin clot
In vitro clotting pathways and clotting tests

Hmw kininogen, prekalikrein
Factor XII

Tissue Factor
Factor VIIa

PTT

XIIa

IXa  VIIIa

Xa

Va

IIa

Fibrin

PT
Principles of Methodology

Principle of Clotting Methods:

The STA-R® uses a mechanical clot detection system. A constant pendular swing of the ball in each cuvette is created by an electromagnetic field applied on each side of the cuvette. As the plasma starts to clot the viscosity of the plasma increases, decreasing the swing of the ball. The instrument uses the variation in the amplitude of the swing to determine the clotting time.
The reduction in light transmission caused by particle formation.

LED Light Source → 671/405 Wavelength → Cuvette → Photodetector

Clot Curve Formation
- Baseline
- Acceleration
- Deceleration
- Endpoint
Fibrinogen

- The time required for clot formation is inversely proportional to plasma fibrinogen concentration.
- A standard curve with 4 points of clotting times versus fibrinogen concentration is plotted on a linear graph. The fibrinogen concentration is read from the standard curve. Clotting time is inversely converted to concentration.
- Fibrinogen is produced in the liver and has a plasma half-life of 3 days.
- Fibrinogen is an acute reactant.
### Calibration Points

<table>
<thead>
<tr>
<th>Calibrators mg/dl</th>
<th>Meas. Sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>33.3</td>
</tr>
<tr>
<td>300</td>
<td>16.7</td>
</tr>
<tr>
<td>600</td>
<td>8.3</td>
</tr>
<tr>
<td>900</td>
<td>5.6</td>
</tr>
</tbody>
</table>

### Equation

\[ \log(c) = (-1.0015 \times \log(t)) + 3.7812 \]
Fibrinogen molecular structure

The major domains (E and D) and the coiled-coil segments (C) connecting them in fibrinogen and fibrin monomer. Catalyzed conversion of fibrinogen to fibrin results in release of fibrinopeptides A (FpA, sequence A1-16) and B (FpB, sequence B1-14) from the amino termini of the A- and B-chains of fibrinogen, and exposure of two knobs, the EA and EB association sites, respectively.
Abnormal Fibrinogen

Afibrinogenemia
Hypofibrinogenemia
Dysfibrinogenemia
Primary and secondary fibrinolysis (DIC)
Liver disease
A/Hypo/Dysfibrinogenemia

- Congenital
  - Can’t convert fibrinogen to fibrin
    - Abnormal fibrinopeptide release
    - Fibrin polymerization defect
    - Abnormal stabilization
    - Resistance to fibrinolysis
  - Afibrinogenemia
    - Mutation in any of the three chains

- Presentation varies from no complications to hemorrhagic and thrombotic complications
Thrombin cleaves both fibrinopeptides A and B.

The thrombin time measures the final step of the clotting pathway, the conversion of fibrinogen to fibrin. Therefore, it is normal even with severe deficiencies of the other intrinsic or extrinsic factors leading up to the conversion of fibrinogen to fibrin.
Abnormal Thrombin Time

- Afibrinogenemia
- Hypofibrinogenemia
- Dysfibrinogenemia
- Heparin (very sensitive!!!!)
- Fibrin Degradation Products
- High [Immunoglobulins] interfere with fibrin polymerization
- Anti-bovine thrombin antibodies if bovine thrombin used
Reptilase Time

- Reptilase is an enzyme similar to thrombin that is found in the venom of Bothrops snakes.
- Batroxobin cleaves Fibrinopeptide A off fibrinogen to form Fibrin I.
- The Reptilase Time is **not** sensitive to heparin and can be used to evaluate a prolonged Thrombin Time.
D-dimer

D-dimer is a specific degradation fragment of cross-linked fibrin.

High plasma D-dimer is an indicator of intravascular fibrin formation and plasmin-mediated fibrinolysis.

Measurement of plasma D-dimer concentration (ELISA method with 99% NPV) is useful to aid in the diagnosis of systemic thrombosis, including pulmonary thromboembolism (PTE) and disseminated intravascular coagulation (DIC).
D-dimer latex Agglutination

Citrated plasma + Latex microparticles coated with monoclonal antibodies specific for D-Dimer ↓ agglutination (Big clumps that are visible to the naked eye)

Clotting increases turbidity and thus increases absorbance. (immunotubidimetrically)

D-Dimer level determined from calibration curve
<table>
<thead>
<tr>
<th>Calibrators ng/ml</th>
<th>Meas. DOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.00</td>
<td>-0.008</td>
</tr>
<tr>
<td>500.00</td>
<td>0.011</td>
</tr>
<tr>
<td>1000.00</td>
<td>0.036</td>
</tr>
<tr>
<td>2000.00</td>
<td>0.095</td>
</tr>
<tr>
<td>3000.00</td>
<td>0.144</td>
</tr>
<tr>
<td>4000.00</td>
<td>0.178</td>
</tr>
</tbody>
</table>

D-DI - 3rd Order Polynomial

\[ c = (585196.0 \cdot d3) - (127010.0 \cdot d2) + (26447.0 \cdot d) + 223.0 \]
Case 1: no clot everywhere

- 66Y female from E2
- 4 specimens in three hours (from 3:15 am – 6:20am) all gave PT>300sec, INR>60, and PTT>300.0

Questions

- Can we trust the specimen?
- Is this real? Bubbles? Instrument problem?
- What should we do?
Case 1: no clot everywhere solution

1. Ask for a phlebotomist draw. (the last one was from phlebotomist draw)
2. Check all 4 specimens of clot, hemolysis and tube type.
3. Rerun all the specimens. Add DDIM, FIB, TT and HEPAR.
<table>
<thead>
<tr>
<th>T</th>
<th>Identity</th>
<th>Rack</th>
<th>Po</th>
<th>Sts</th>
<th>D-D1 1/5 ng/ml</th>
<th>PT Sec.</th>
<th>APTT Sec.</th>
<th>FIB mg/dl</th>
<th>UFH 8 UI/ml</th>
<th>TT 10ML Sec.</th>
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<tbody>
<tr>
<td>✔</td>
<td>E195147407</td>
<td>239865</td>
<td>2</td>
<td></td>
<td>V&gt;VMax</td>
<td>204.8</td>
<td>V&gt;VMax</td>
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<td></td>
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<tr>
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<td></td>
<td>V&gt;VMax</td>
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<td>V&gt;VMax</td>
<td>0.00</td>
<td>V&gt;VMax</td>
</tr>
</tbody>
</table>
Pathophysiology

Thrombosis—brief period of hypercoagulability

1) Coagulation cascade is initiated, causing widespread fibrin formation
2) Microthrombi are deposited throughout the microcirculatory system
3) Fibrin deposits result in tissue ischemia, hypoxia, necrosis
4) Leads to multi organ dysfunction

Fibrinolysis—period of hypocoagulability (the hemorrhagic phase)

1) Activates the complement system
2) Byproducts of fibrinolysis (fibrin/fibrin degradation products [FDP]) further enhance bleeding by interfering with platelet aggregation, fibrin polymerization, & thrombin activity
3) Leads to Hemorrhage

(Porth, 2004) & (Otto, 2001)
<table>
<thead>
<tr>
<th>Test</th>
<th>Abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count</td>
<td>Decreased</td>
</tr>
<tr>
<td>Fibrin degradation product (FDP)</td>
<td>Increased</td>
</tr>
<tr>
<td>Factor assay</td>
<td>Decreased</td>
</tr>
<tr>
<td>Prothrombin time (PT)</td>
<td>Prolonged</td>
</tr>
<tr>
<td>Activated PTT</td>
<td>Prolonged</td>
</tr>
<tr>
<td>Throbimn time</td>
<td>Prolonged</td>
</tr>
<tr>
<td>Reptilase time</td>
<td>Prolonged</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Decreased</td>
</tr>
<tr>
<td>D-dimer</td>
<td>Increased</td>
</tr>
</tbody>
</table>
Case 1: no clot everywhere
Happy ending

1. Doctor informed patient was in DIC.
2. Patient had transfusion.
3. 5 hours later, patient PT 20.3 sec, INR 1.8, PTT 46.8 sec, FIB 145 mg/dl TT 20.2 sec.

We are in the front line of saving patient’s life!
Extra steps could save a life!
Case 2: Put all the results together

<table>
<thead>
<tr>
<th>Patient</th>
<th>PTINR (1.1 - 1.3)</th>
<th>PTT (30.0-38.0 sec)</th>
<th>FIB (287-347 mg/dl)</th>
<th>TT (16.2-19.1 sec)</th>
<th>REPT (17.4-21.0 sec)</th>
<th>DDIM (&lt;451 ng/dl)</th>
<th>explanation</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4</td>
<td>117.0</td>
<td>237</td>
<td>&gt;120</td>
<td>19.6</td>
<td>325</td>
<td></td>
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<tr>
<td>2</td>
<td>3.1</td>
<td>68.3</td>
<td>33</td>
<td>33.2</td>
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<tr>
<td>3</td>
<td>1.4</td>
<td>27.5</td>
<td>82</td>
<td>32.4</td>
<td>&gt;100.0</td>
<td>&lt;220</td>
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</tr>
<tr>
<td>4</td>
<td>1.1</td>
<td>24.9</td>
<td>524</td>
<td>52.6</td>
<td>30.7</td>
<td>2749</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>26.4</td>
<td>53</td>
<td>25.3</td>
<td>28.5</td>
<td>1724</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.1</td>
<td>66.1</td>
<td>52</td>
<td>27.1</td>
<td>50.6</td>
<td>2290</td>
<td></td>
</tr>
</tbody>
</table>
## Case 2: Reptilase time and Thrombin time

<table>
<thead>
<tr>
<th>Patient</th>
<th>PTINR (1.1-1.3)</th>
<th>PTT (30.0-38.0 sec)</th>
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<th>DDIM (&lt;451 ng/dl)</th>
<th>explanation</th>
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<tr>
<td>1</td>
<td>1.4</td>
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<td>&gt;120</td>
<td>19.6</td>
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<td>3.1</td>
<td>68.3</td>
<td>33</td>
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<td>&gt;20000</td>
<td>DIC</td>
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<td>1.4</td>
<td>27.5</td>
<td>82</td>
<td>32.4</td>
<td>&gt;100.0</td>
<td>&lt;220</td>
<td>Dysfibrinogenemia</td>
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<tr>
<td>4</td>
<td>1.1</td>
<td>24.9</td>
<td>524</td>
<td>52.6</td>
<td>30.7</td>
<td>2749</td>
<td>Myeloma</td>
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<tr>
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<td>26.4</td>
<td>53</td>
<td>25.3</td>
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<tr>
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<td>6.1</td>
<td>66.1</td>
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<td>50.6</td>
<td>2290</td>
<td>End stage liver disease</td>
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