Laboratory Diagnosis and Therapeutic Monitoring in Hemophilia: Problems, Pitfalls, and Testing Pearls

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Learning Objectives

• Review current issues, challenges, and problems related to the laboratory assessment of hemophilia A and B
• Discuss potential solutions, such as need to test with both chromogenic and clot-based assays to correctly diagnose non-severe hemophilia A, and the need to accurately measure factor activity levels
• Review challenges facing clinical laboratories when monitoring some of these recombinant replacement products utilizing existing factor assays used in clinical laboratories
• Discuss advantages and disadvantages of proposed solutions as well as current recommendations (i.e. MASAC)
• Recognize the need for accuracy of factor assays in pharmacokinetic monitoring of factor levels
Laboratory Diagnosis of Hemophilia A and B

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Outline

• Laboratory evaluation of hemophilia A and B
• Impact of assay methodology in the evaluation of hemophilia A and B
• Methodological differences between one-stage and chromogenic factor activity assays
• Discrepant hemophilia A and B
  – Definition
  – Incidence
  – Molecular basis
Hemophilia A and B

- **Hemophilia A**: genetic-based deficiency of factor VIII (FVIII)
- **Hemophilia B**: genetic-based deficiency of factor IX (FIX)

**Hemophilia Disease Severity:**
- <1% (<0.01 IU/mL) factor activity = severe hemophilia
- 1%-5% (0.01-0.05 IU/mL) factor activity = moderate hemophilia
- 6%-40% (0.06-0.4 IU/mL) activity = mild hemophilia

- **An accurate measure of factor activity is necessary to:**
  - Make a diagnosis of hemophilia
  - Classify the severity of the disease
  - Monitor therapy
Factor Activity Assays

**Methods to measure factor activity:**
- One-stage clot assay (OSA)
  - Based on the activated partial thromboplastin time (APTT)
- Two-stage clot assay
  - Complex, cannot be automated, no kit available, rarely performed
- Chromogenic substrate assay (CSA)
  - Based on the two-stage clot assay
  - Limited availability in clinical laboratories, considered more expensive, often performed as a batched analysis

**One-stage assay (OSA)**
- Method used by the majority of clinical laboratories for all factor activity assays, many different instrument and reagent combinations available

**Chromogenic activity assay (CSA)**
- FVIII activity: multiple FDA-approved kits, currently offered by only a few laboratories
- FIX activity: no FDA-approved kit currently, limited number of manufacturers, offered by few to no laboratories
Factor Activity Assays: OSA vs CSA

Does assay methodology matter?

- **Discrepant non-severe hemophilia**
  - More than twofold difference in results between OSA and CSA assays; OSA may be higher or CSA may be higher\(^1\)
    - May impact diagnosis
    - May impact classification of disease severity
  - Reported to occur in \textbf{30\%} with non-severe hemophilia A,\(^2\) just recently described in hemophilia B\(^3\)

- **Discrepancies in recovery of some FVIII and FIX replacement therapies**
  - Reported with some new modified recombinant factor replacement products\(^4,5\)

Factor Activity Assays: OSA vs CSA

Are these discrepancies real?

• In non-severe hemophilia A, the OSA/CSA discrepancy:
  – Is consistent between family members¹
  – Is consistent in all individuals bearing the same mutation
  – Has a molecular basis that depends on the underlying defect and its impact on the different activity assays

• In post-infusion factor replacement monitoring, the discrepancy in OSA/CSA results depends on the modification of the recombinant factor and its impact on the assays

FVIII Activity Assays: OSA vs CSA

What is the difference?

One-Stage Activity

Test Plasma + FVIII-Def Plasma
Phospholipids + Surface Activator + Calcium

Chromogenic Activity

Test Plasma
FX and FIXa (excess) +/- Thrombin, Phospholipids + Ca²⁺

Stage One

FXa + FVIIIa
FX
FXa

Stage Two

Chromogenic Substrate, Thrombin Inhibitor

Clot

Formation Measured in Seconds

OSA and CSA Standard Curves

OSA and CSA assays are each read against a standard curve of known FVIII concentration that is referenced against an international standard.

Standard curves from STA-R Evolution® and BCS® XP, Colorado Coagulation.
Factor Activity Assays: OSA vs CSA
What is the difference?

One-Stage Activity
Test Plasma + FVIII-Def Plasma
Phospholipids + Surface Activator + Calcium

Chromogenic Activity
Test Plasma
FX and FIXa (excess) +/- Thrombin, Phospholipids + Ca^{2+}

Stage One

Stage Two
Chromogenic Substrate, Thrombin Inhibitor

FXIa → FIX → FIXa + FVIIIa → FX → FXa → FVa, FII → Thrombin → Fibrinogen → Fibrin → Clot
Formation Measured in Seconds

Factor Activity Assays: OSA vs CSA

What is the difference? (cont)

One-Stage Activity

Test Plasma + FVIII-Def Plasma
Phospholipids + Surface Activator + Calcium

- Reaction occurs quickly
- Factors are present at physiologic concentrations

Formation Measured in Seconds

Factor Activity Assays: OSA vs CSA
What is the difference? (cont)

- Stage one is incubated for 2-10 minutes
- Factors are present in excess

Discrepant Non-Severe Hemophilia A

- Due to missense mutations, often novel
  - **OSA:CSA > 1.5**: mutations localized to A1-A2-A3 interface of FVIII molecule
    - Destabilizes FVIIIa – this is better detected in the incubated CSA assay
  - **OSA:CSA < 0.5**: mutations localized close to or within the thrombin cleavage sites or FIX binding sites
    - Thrombin and FIX at physiologic concentrations in OSA; therefore, OSA is more sensitive to these defects, and incubation may overcome some binding defects
- Characteristically, have high antigen levels (CRM+)
Discrepant Non-Severe Hemophilia A (cont)

- OSA/CSA discrepancy may lead to missed diagnosis or misclassification of hemophilia A (HA)
  - 11% reported to have normal FVIII OSA result\(^1\)
  - Those classified with mild HA may have moderate HA with a significant bleeding tendency\(^2\)

- In general, the lower result better correlates with clinical bleeding tendency and results of thrombin generation studies\(^2-4\)

Discrepant Non-Severe Hemophilia B

- Recently described in a small cohort of patients\(^1\)
  - CSA > OSA
  - Due to a mutation at the N-terminal site of the activation peptide at Arg191
  - Phenotype appears to correlate to CSA result
  - CRM+ deficiency (antigen > activity)

- Discrepancy between OSA results using different activated partial thromboplastin time (APTT) reagents described in a cohort of mild hemophilia B patients\(^2\)
  - Higher result with Actin FS vs STA-PTT or STA-C.K. PREST

Laboratory Screening for Hemophilia

- Do not rely on an APTT to screen for hemophilia A and B
  - Depending on the APTT reagent, OSA FVIII activity may have to fall below 25% and FIX below 15% before APTT prolongs
Reagent Responsiveness to Factor Deficiency  
*Varies by laboratory, reagent, and factor-deficient plasma used*

**Level of factor deficiency needed before the APTT prolongs**

A normal APTT does not rule out mild deficiency of FVIII, FIX, or FXI

Colorado Coagulation data, unpublished.
Laboratory Diagnosis of Hemophilia A and B: Conclusion

• In the initial evaluation of non-severe hemophilia A:
  – Evaluate both one-stage clot-based FVIII and chromogenic FVIII activity assays
  – Confirm diagnosis on a new plasma sample
  – Consider molecular testing to identify underlying mutation

• Recommendations on the initial evaluation of non-severe hemophilia B forthcoming

• Do not rely on an abnormal APTT to screen for non-severe hemophilia A and B
Pitfalls Associated with Monitoring FVIII and FIX Replacement Therapy

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Outline

• Provide an overview of the modified recombinant factor VIII (rFVIII) and recombinant factor IX (rFIX) replacement products

• Provide examples of reagent-dependent recovery of modified rFVIII and rFIX replacement products

• Review the challenges facing clinical laboratories involved in monitoring modified rFVIII and rFIX replacement products
# Modified rFVIII Products

<table>
<thead>
<tr>
<th>Name</th>
<th>Modification for Half-Life Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax 855 (FL)(^a)</td>
<td>20 kDa branched PEG</td>
</tr>
<tr>
<td>rFVIII-Fc (BDD)(^a)</td>
<td>Fusion to Fc domain of IgG1</td>
</tr>
<tr>
<td>N8-GP (BDtrunc PEGylated)</td>
<td>40-kDa glycoPEGylation</td>
</tr>
<tr>
<td>CSL627 (BDD)(^a)</td>
<td>Single-chain</td>
</tr>
<tr>
<td>BAY 94-9027 (BDD)</td>
<td>Site-specific 60-kDa PEG</td>
</tr>
</tbody>
</table>

\(^a\)FDA-approved.

BD, B-domain; BDD, B-domain-deleted; FL, full length; PEG, polyethylene glycol.
## Modified rFIX Products

<table>
<thead>
<tr>
<th>Name</th>
<th>Modification for Half-Life Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFIX-Fc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Fusion to Fc domain of IgG1</td>
</tr>
<tr>
<td>N9-GP</td>
<td>40-kDa glycoPEGylation</td>
</tr>
<tr>
<td>CSL654&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Fusion to albumin</td>
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</tbody>
</table>

<sup>a</sup>FDA-approved.
Modified rFIX Product Recovery in OSA: APTT-Reagent Dependent

APTT, activated partial thromboplastin time; OSA, one-stage assay.
Modified rFIX Product Recovery in CSA

CSA, chromogenic assay.

* Values below auto validation limit.
Modified rFVIII Recovery in OSA: APTT-Reagent Dependent

Silica-Activated PTT

Ellagic Acid-Activated PTT

Modified rFVIII Product Recovery in CSA

Challenge #1: Alignment of Clinical Factor Activity Assay with the Potency Assay

• Factor product potency assignment\(^1\)
  – FVIII products: predominantly CSA using WHO IS concentrate
  – FIX products: OSA using WHO IS concentrate

• Factor product monitoring in clinical laboratories usually performed with OSA assay using pooled normal plasma standard

• Factor activity assays used for post-infusion monitoring should align with:
  – Assay used by manufacturer to assign potency to factor replacement product
  – Clinical efficacy of the factor replacement product

IS, International Standards; WHO, World Health Organization.
Challenge #2: Variability of FVIII & FIX One-Stage Factor Activity Assay

- FVIII and FIX OSA consistently demonstrate high variability between laboratories in PT surveys (CAP, ECAT, NEQUAS)
  - A normal plasma sample in a recent CAP survey (CGE-A 2016) yielded results ranging from 0.72–1.61 IU/mL

Data derived from RCPAQAP Haematology, ECAT, and NEQAS EQA programs (2011-2012).
Challenge #3: Limited Availability of Data on Modified rFVIII & rFIX Recovery in OSA & CSA

- Behavior of modified recombinant factor replacement products using OSA and CSA reagents commonly employed in clinical laboratories ideally should be studied and available prior to approval of product.

- Currently such data (published largely through field studies) are available for only 2 of the 5 modified rFVIII and 1 of the 3 modified rFIX products\(^1-3\)

Challenge #4: Limited Availability of IVD-Approved CSA in the US

FVIII Chromogenic Activity Assay
- 5 assay kits marketed in US: Coatest® SP FVIII; Coamatic® FVIII; Biophen FVIII:C; Siemens FVIII:C; and Technochrom® FVIII:C
- 3 assay kits are IVD-approved; only 1 kit IVD-approved for use on automated coagulation analyzer

FIX Chromogenic Activity Assay
- 2 assay kits marketed in US (both are RUO labeled!)
- Currently, no validated instrument applications/protocols for commonly used coagulation analyzers available

IVD, in vitro diagnostic; RUO, research use only.
Conclusions

- Selected modified rFVIII and rFIX replacement products show significant reagent-dependent recovery in the OSA, whereas recovery in CSA appears to be more consistent (especially for modified rFIX products).

- Clinical factor activity assays used for post-infusion monitoring of modified rFVIII and rFIX replacement products should closely align with (1) potency assay used by pharmaceutical or (2) clinical efficacy of product.

- Clinical factor activity assays used for post-infusion monitoring of modified factor replacement products should be (at a minimum) verified for the particular product prior to use.
Modified Recombinant Factor Products: Case Examples and Discussion on Solutions

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Associate Professor, Mayo Clinic College of Medicine, Co-Director, Special Coagulation Laboratory, Director Comprehensive Hemophilia Center, Mayo Clinic, Rochester, MN
Outline

• Presentation of 2 cases of patients on modified recombinant factor products
• Discussion on implications of inaccurate laboratory measurements
• Overview of potential solutions
Standard of Care for Severe Hemophilia

• Prophylactic intravenous infusion of factor concentrates (modified and unmodified)

• Unmodified products with standard half-life infusion frequency:
  – rFVIII: approximately 3 times/week
  – rFIX: approximately 2 times/week
    • Frequency of infusion varies with individualized in vivo half-life

• Aim: maintain trough factor level of >1% (1%–5%)

• Dose and frequency of infusions based on pharmacokinetic analysis
  – Infusion of factor concentrate followed by serial measurement of factor levels
Phase 3 Study of rFVIII/Fc Fusion Protein in Severe Hemophilia A

Phase 3 Study of rFIXFc Fusion Protein in Hemophilia B

Consequences/Risks of Inaccurate Measurements

• Increased risk of bleeding
  – With underdosing of factor product

• Increased cost and risk of thrombosis
  – With overdosing of factor product
Case History: Pharmacokinetic Study With New Modified rFVIII

- 20-year-old male with severe hemophilia A
- Changed from rFVIII to new modified rFVIII
- Pharmacokinetic study (30 units/kg) infusion
  - Target FVIII:C approximately 60%

<table>
<thead>
<tr>
<th></th>
<th>OSA</th>
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<tbody>
<tr>
<td>Pre-infusion FVIII:C</td>
<td>&lt;0.01 IU/mL (&lt;1%)</td>
</tr>
<tr>
<td>1 hour Post-infusion FVIII:C</td>
<td>0.3 IU/mL (30%)</td>
</tr>
</tbody>
</table>

OSA, one-stage clot assay.
Case History: Pharmacokinetic Study With New Modified rFVIII (cont)

• Was the patient underdosed? *or*
• Was the measured OSA FVIII:C suboptimal for calculated dose?
• Generally, the next step is to increase the dose and repeat pharmacokinetics
• Potential pitfalls:
  – Definite increase in cost
  – Potential increased risk of thrombosis
    • Central line–associated or other
Case History: Pharmacokinetic Study With New Modified rFVIII (cont)

- Results of CSA FVIII

<table>
<thead>
<tr>
<th></th>
<th>OSA</th>
<th>CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-infusion FVIII</td>
<td>&lt;0.01 IU/mL (&lt;1%)</td>
<td>&lt;0.04 IU/mL (&lt;4%)</td>
</tr>
<tr>
<td>Post-infusion FVIII</td>
<td>0.3 IU/mL (30%)</td>
<td>0.6 IU/mL (60%)</td>
</tr>
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</table>

- Per package insert:
  - Multiply OSA FVIII result by 2 (30 x 2 = 60%)
  - Suggested “correction factor” correlated with CSA FVIII

CSA, chromogenic activity assay; OSA, one-stage clot assay.
Case History: New Modified rFIXFc

- 12-year-old male with severe hemophilia B
- Initiated on rFIXFc by hemophilia treatment center
- Dose calculated based on results of pharmacokinetic testing:

<table>
<thead>
<tr>
<th></th>
<th>OSA</th>
</tr>
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<tbody>
<tr>
<td>Pre-infusion FIX (trough)</td>
<td>0.05 IU/mL (5%)</td>
</tr>
<tr>
<td>Post-infusion FIX (peak)</td>
<td>0.8 IU/mL (80%)</td>
</tr>
</tbody>
</table>
Case History: New Modified rFIXFc (cont)

- Patient was followed in local hemophilia treatment center
- Advised to recheck FIX kinetics
- No bleeding events since initiation of prophylactic rFIXFc infusions
Case History: New Modified rFIXFc (cont)

- Rechecked FIX kinetics (in different laboratory)

<table>
<thead>
<tr>
<th></th>
<th>OSA (original)</th>
<th>OSA (recheck)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-infusion FIX (trough)</td>
<td>0.05 IU/mL (5%)</td>
<td>&lt;0.01 IU/mL (&lt;1%)</td>
</tr>
<tr>
<td>Post-infusion FIX (peak)</td>
<td>0.8 IU/mL (80%)</td>
<td>0.4 IU/mL (40%)</td>
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</table>
Case History: New Modified rFIXFc (cont)

- Results showed apparent under-recovery or suboptimal FIX dosing
- Local hemophilia treatment center planned to increase the dose of rFIXFc
- Communication with coagulation laboratory performing assay:
  - Contact activator used for OSA FVIII:C in local laboratory: kaolin (underestimates rFIXFc)
- Mailed out sample to central laboratory performing OSA FVIII:C with different contact activator (silica)
  - Confirmed original pharmacokinetic study results
Pitfalls of Variability of Performance of OSA With New Modified Concentrates

• Underestimation of true factor level
  – Potential consequence:
    • Overdosing; cost and thrombotic complications

• Overestimation of true factor level
  – Potential consequence:
    • Underdosing; increase risk of bleeding and morbidity
Potential Solutions

• **Individualized calibrators**
  – Requirement for multiple product-specific calibrators
  – Reference lab challenge: identification of purpose of FVIII/FIX assay request (diagnostic vs monitoring, which factor concentrate?)

• **Chromogenic assays**
  – Limited availability of FDA-approved kits for FVIII
    • No FDA-approved FIX kits available
  – Each lab would have to validate kit as a laboratory-developed test (LDT)
  – Reference lab challenge: identification of purpose of FVIII/FIX assay request (diagnostic vs monitoring, which factor concentrate?)

• **Correction factor**
  – Multiply OSA FVIII:C by 2 to get “true” FVIII level
  – FDA labeling of a currently approved modified rFVIII
  – Important for hemophilia care providers to be aware of such recommendations
Conclusions

- The OSA and CSA assays have different performance characteristics
  - Risk for misclassification/potential for missed diagnosis of non-severe hemophilia A
- Variable results when measuring factor levels of current and new modified rFVIII and rFIX products
  - Overestimation or underestimation, depending on reagent and factor product
- Accurate PK measurements of current and new modified recombinant factors are essential for proper dosing and monitoring of factor activity levels
  - Underdosing puts patients at increased bleeding risk, and overdosing increases cost of care and puts patients at higher thrombosis risk
- Awareness of this variability and of the correction factors for currently approved factor products is important for optimal care of hemophilia
Please visit www.bloodcmecenter.org for free CE activities in hemophilia and rare bleeding disorders