Coagulation Factor Inhibitors and the Nijmegen Bethesda Assay

George A Fritsma MS, MLS
The Fritsma Factor, Your interactive Hemostasis Resource℠
Sponsored by Precision BioLogic
Dartmouth, Nova Scotia
george@fritsmafactor.com
www.fritsmafactor.com
Coagulation Factor Inhibitors

Bottom Line at the Start (BLATS); The Participant…

- Explains the origin of anti-factor VIII (FVIII inhibitor)
- Detects FVIII inhibitors using factor assays and mixing studies
- Measures FVIII inhibitors using the Bethesda titer, Nijmegen Bethesda assay, chromogenic Bethesda assay, enzyme immunoassay and fluorescence immunoassay
- Describes coagulation factor bypass therapy to resolve bleeding in inhibitor patients
- Describes immune tolerance induction therapy
- Lists new factor concentrates designed to prevent inhibitor formation
# 2-yo Hemophilic Boy

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB</td>
<td>11.8 g/dL</td>
<td>9.6–15.6 g/dL</td>
</tr>
<tr>
<td>PT</td>
<td>11.2 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT</td>
<td>65 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT</td>
<td>310,000/µL</td>
<td>150–400,000/µL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>390 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>

Inflamed, swollen knee and ankle
### Mixing Study: 2-yo Hemophilic Boy

**NP**: commercial pooled normal plasma from 20 normal donors with ~100 U/dL factor levels

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
<th>RI</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient PTT</td>
<td>63 s</td>
<td>25–35 s</td>
<td></td>
</tr>
<tr>
<td>Immediate PTT of patient/NP 1:1 mix</td>
<td>34.5 s</td>
<td>NP PTT 30 s</td>
<td>Limit: NP + 10%: Incomplete correction</td>
</tr>
<tr>
<td>PTT of Pt/NP 1:1 mix incubated 1 h at 37°C</td>
<td>47.9 s</td>
<td>NP PTT 35 s</td>
<td>Incubate mix and NP: Uncorrected</td>
</tr>
</tbody>
</table>

- R/O lupus anticoagulant (LAC): pediatric, bleeding
- Specific coagulation factor VIII (FVIII) inhibitor; Bethesda titer
Hemophilia A Symptoms
Spontaneous anatomic (soft-tissue) bleeds

- Bleeding at umbilical stump and circumcision
- Delayed bleeding triggered by injury
  - Joints, large muscles, body cavities, GI, soft tissue, tongue, kidney, testicles, brain, CNS
- Spontaneous bleeds, especially into joints
- Inflammation, hematomas, hemarthroses

<table>
<thead>
<tr>
<th></th>
<th>Severe</th>
<th>Moderate</th>
<th>Mild</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence</td>
<td>70%</td>
<td>15%</td>
<td>15%</td>
</tr>
<tr>
<td>FVIII U/dL</td>
<td>&lt; 1</td>
<td>1–5</td>
<td>6–30</td>
</tr>
<tr>
<td>Bleeding</td>
<td>Spontaneous</td>
<td>Minor trauma</td>
<td>Major trauma</td>
</tr>
</tbody>
</table>
Hemarthroses
Airway obstruction
40 YO Hemophilic

- Bleeding into ankle at midnight
  - Attempted DDAVP (Stimate®) inhaler
- Ran out of therapeutic FVIII concentrate
  - In US, Medicare subsidy via ~100 national hemophilia centers
  - Canadian Hemophilia Society Treatment Cntrs
- Night tech and on-call path resident
  1. Determine residual patient factor VIII activity
  2. Compute FVIII dosage from package insert to prevent potential thrombotic overdose and to avoid waste
  3. Order FVIII concentrate from transfusion service
  4. Reconstitute with sterile water, administer as IV push
  5. Subsequently determine therapeutic factor VIII level
If Factor Assay not Available

- When timing is critical, assume <1 U/dL FVIII activity or…
- Estimate residual FVIII from PTT:

<table>
<thead>
<tr>
<th>FVIII (U/dL)</th>
<th>PTT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>1</td>
<td>120</td>
</tr>
</tbody>
</table>

Example only, do not use. Varies by reagent sensitivity.

- Collect baseline specimen
  - Freeze, confirm next day shift with FVIII assay
- Maintain patient database
Plasma-derived FVIII Concentrates

- <25% of FVIII concentrate in industrialized countries
- Theoretical risk of HBV, HCV, HIV transmission 1/60,000
  - Human and animal plasma matrix
  - Purification: immunoaffinity column, solvent-detergent, Pasteurization, viral filtration, combinations
  - Hemofil-M, Monarc-M, Monoclate-P
- Seroconversions per 2003 CDC surveillance: 0
Recombinant FVIII Concentrates

- Serum or albumin in culture medium
  - Helixate, Kogenate, Recombinate
  - Abundant, used for prophylaxis
  - No HBV, HCV, HIV seroconversions
- No protein in culture or preparation
  - No theoretical viral risk: Advate
- B-domain-deleted FVIII concentrate
  - Human albumin in culture: ReFacto
  - No protein: Xyntha
  - FVIII clot assay unreliable
40 YO Receives Advate

- Peak: collected 15 m after administration
  - If peak reaches expected value, plan for next administration at 12 hours
  - Factor assay result: 30 U/dL
  - Should have reached 80 U/dL, what happened?
  - Suspect anti-FVIII inhibitor

- Trough: collect 12 h after administration
  - Reflects half-life, 50% of peak
  - Continue w/ half doses
One-stage Factor VIII Assay

1. Dilute pt plasma 1:10 in imidazole buffered saline (IBS)
2. Add FVIII-depleted reagent plasma 1:1 (FVIII DP)
   - Provides all factors except FVIII
   - Typical: 100 uL patient plasma dilution + 100 uL FVIII DP
3. Add PTT reagent, incubate 3 m
4. Add CaCl₂, record interval to clot formation
5. Compare result in seconds to calibration curve to derive plasma activity:

1% activity = 1 U/dL
### Factor VIII Assay Plasma Dilutions
Parallelism Indicates No Inhibitor

<table>
<thead>
<tr>
<th>Automated Plasma Dilution</th>
<th>Seconds</th>
<th>Raw Factor VIII Activity</th>
<th>Computed Factor VIII Activity (× dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 “undiluted”</td>
<td>90 s</td>
<td>20 U/dL</td>
<td>20 U/dL</td>
</tr>
<tr>
<td>1:20</td>
<td>105 s</td>
<td>10 U/dL</td>
<td>20 U/dL (parallel)*</td>
</tr>
<tr>
<td>1:40</td>
<td>107 s</td>
<td>5.5 U/dL</td>
<td>22 U/dL (parallel)</td>
</tr>
<tr>
<td>1:80</td>
<td>110 s</td>
<td>2.6 U/dL</td>
<td>20.8 U/dL (parallel)</td>
</tr>
</tbody>
</table>

* <10% difference from undiluted indicates parallelism, no inhibitor
**FVIII Assay Plasma Dilutions**

**non-Parallelism Indicates Inhibitor**

<table>
<thead>
<tr>
<th>Plasma Dilution</th>
<th>Seconds</th>
<th>Raw Factor VIII Activity</th>
<th>Computed Factor VIII Activity ($\times$ dilution)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 (undiluted)</td>
<td>95 s</td>
<td>10 U/dL</td>
<td>10 U/dL</td>
</tr>
<tr>
<td>1:20</td>
<td>99 s</td>
<td>8 U/dL</td>
<td>16 U/dL</td>
</tr>
<tr>
<td>1:40</td>
<td>107 s</td>
<td>5 U/dL</td>
<td>20 U/dL</td>
</tr>
<tr>
<td>1:80</td>
<td>108 s</td>
<td>4 U/dL</td>
<td>32 U/dL</td>
</tr>
</tbody>
</table>

- >10% difference from undiluted = non-parallel & rising, implies inhibitor

**Inhibitor: IgG alloantibody to FVIII concentrate**

- 30% prevalence, most arise in severe hemophilia
- Reflex to inhibitor assay

Why Measure Inhibitors?

- Confirm refractory response to factor therapy
- Monitor efficacy of prophylactic FVIII or FIX therapy
- Monitor factor bypass preparation efficacy and safety
- Distinguish low response from high response patients
  - Low responders: treat with factor concentrate
  - High response when bleeding: treat with bypass preparations
  - High response when not bleeding: immune tolerance therapy
- Detect factor-induced anamnesis
- Monitor immune tolerance therapy efficacy
- Establish prevalence and population trends
  - Hemophilia severity, correlate to mutation, correlate to therapy

Courtesy of Connie Miller, PhD, CDC
Limitations of Inhibitor Measurement

- Median 40% of severe hemophilia patients in US hemophilia treatment centers are tested for inhibitors
- Require therapeutic “wash-out” before sampling
- Local laboratory expertise and experience
- Median 32% FP rate, 5% FN rate, interlab CV 50%
- The need for method standardization

- Favaloro et al. Haemophilia 2014; 20: Suppl 4
### CVs of NASCOLA Inhibitor Measurement

<table>
<thead>
<tr>
<th>Year</th>
<th>Expected</th>
<th>Average</th>
<th>Min</th>
<th>Max</th>
<th>CV</th>
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<tbody>
<tr>
<td>2010</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>5.0</td>
<td>178</td>
</tr>
<tr>
<td>2011</td>
<td>0</td>
<td>0.4</td>
<td>0</td>
<td>2.9</td>
<td>150</td>
</tr>
<tr>
<td>2010</td>
<td>0.5-1.0</td>
<td>0.5</td>
<td>0</td>
<td>2.0</td>
<td>83</td>
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<tr>
<td>2011</td>
<td>0.9</td>
<td>1.3</td>
<td>0</td>
<td>3.7</td>
<td>46</td>
</tr>
<tr>
<td>2012</td>
<td>0.6</td>
<td>4.0</td>
<td>0</td>
<td>25.5</td>
<td>40</td>
</tr>
<tr>
<td>2010</td>
<td>1-1.5</td>
<td>1.0</td>
<td>0</td>
<td>2.0</td>
<td>48</td>
</tr>
<tr>
<td>2011</td>
<td>2.0</td>
<td>2.8</td>
<td>0</td>
<td>15.0</td>
<td>47</td>
</tr>
<tr>
<td>2012</td>
<td>2.0</td>
<td>3.3</td>
<td>0</td>
<td>15.0</td>
<td>48</td>
</tr>
<tr>
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<td>1.3</td>
<td>0</td>
<td>2.7</td>
<td>46</td>
</tr>
<tr>
<td>2010</td>
<td>3.5</td>
<td>5.0</td>
<td>0.5</td>
<td>30.0</td>
<td>36</td>
</tr>
<tr>
<td>2011</td>
<td>3.9</td>
<td>4.0</td>
<td>0</td>
<td>25.5</td>
<td>40</td>
</tr>
<tr>
<td>2012</td>
<td>5.0</td>
<td>7.5</td>
<td>0</td>
<td>30.0</td>
<td>39</td>
</tr>
</tbody>
</table>

Variations That Affect Inhibitor Measurement

- Residual patient plasma coagulation factor
- Presence of anticoagulants: dabigatran, heparin
- Presence of other inhibitors: lupus anticoagulant
- Differences in inhibitor epitope specificity
- Elevated coagulation factors shorten PTT results
- Inhibitor kinetics: neutralizing and non-neutralizing

- Favaloro et al. Haemophilia 2014; 20: Suppl 4
PTT Mix Inhibitor Kinetics

- **Immediate mix full correction**
- **Immediate mix partial correction**
- **Second order kinetics, non-neutralizing**
- **First order kinetics, neutralizing**

Incubation time:
- 0 m
- 25 m
- 50 m
- 75 m
- 100 m

Seconds:
- 0
- 10
- 20
- 30
- 40
- 50
- 60
- 70
- 80
- 90
- 100

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PrecisionBioLogic
Inhibitors Effects

- Severe hemophilia A: 30%, moderate, 10%
- Hemophilia B: 1–3%
- Render replacement therapy ineffective
- Most occur in early age factor administration
- Raise rates of hemarthrosis
- More catastrophic bleeds, mortality
- Delay physical maturation
- Raise healthcare costs five-fold

Inhibitor Measurement Methods

- Functional, clot-based factor activity inhibition
  - Bethesda titer, 1975
  - Nijmegen Bethesda assay (NBA), 1995
- Functional: chromogenic Bethesda assay (CBA)
- Qualitative immunoassay
  - Solid state enzyme immunoassay (EIA, ELISA)
  - Flow-based fluorescence immunoassay (FLI)
- CDC modification of the NBA, 2012

Courtesy of Connie Miller, PhD, CDC
1975 Bethesda Titer

1. Prepare serial twofold patient plasma dilutions in imidazole buffered saline (IBS)
2. Mix patient plasma dilutions 1:1 with commercial NP
3. Prepare 1:1 NP/IBS for normalization
4. Incubate patient dilutions and 1:1 NP/IBS 37°C 2h
5. Add FVIII-depleted plasma to all dilutions after incubation
6. Perform FVIII assay on all dilutions using PTT methodology
7. Report Bethesda units (BU)/mL as reciprocal of dilution that neutralizes 50% FVIII compared to the incubated 1:1 NP/IBS
   - NP is the source for FVIII, should be ~100 FVIII U/dL
   - Incubated NP/IBS compensates for FV & FVIII deterioration

Serial dilutions of patient plasma in IBS

Patient mix
Mix 1 part patient dilution with 1 part NP

NP/IBS 1:1 mix

Incubate 37°C/2h, perform FVIII assay

Express reciprocal of patient dilution that yields 50% FVIII as BU/mL


NP/IBS 50% FVIII

Patient or control dilution 50% FVIII

BU = reciprocal of dilution
Chromogenic Bethesda Assay

1. Prepare pt plasma *and control* dilutions as for Bethesda titer
2. Mix pt plasma and control dilutions 1:1 with NP, incubate 2 h.
3. Dilute incubated pt/control plasma/NP 1:31 in IBS.
4. Add reagent: bovine FX, FIXa, thrombin, CaCl$_2$, & PL.
5. Incubate 90s at 37°C, generates FXa
6. Add FXa chromogenic substrate with thrombin inhibitor and stopping buffer to measure FXa surrogate to FVIII activity
7. Measure FXa: read \( \Delta A/m \) at 405 nm.
8. 1 chromogenic Bethesda unit (CBU) = level of inhibitor/mL of patient plasma that inactivates 50% of FVIII in 1 mL NP.
9. Limit: \( \geq 0.5 \) CBU = positive
FXa activity and inversely proportional to patient anti-VIII

- Gilles et al. Blood 1993;82 2452
- Blanco et al. Haematologica 2002;87:271
Bethesda Titer Vs Chromogenic Bethesda Assay

**Bethesda Titer**
- Variable non-specific endpoint: clot detection
- Insensitive to inhibitors ≤ 0.5 BU/mL
- Interference from lupus anticoagulant
- Therapeutic A/Cs interfere: heparin and dabigatran

**Chromogenic Bethesda**
- Quantitative endpoint detection system
- Sensitive to inhibitors at 0.1 BU/mL
- Specific for factor inhibitors
- No LAC interference, distinguish LAC from inhibitor

Immunoassay Measurement

- **EIA**: FVIII antigenic target immobilized in well
- **FLI**: FVIII target immobilized on fluorescent beads
- More sensitive than functional assays
- Detect neutralizing & non-neutralizing inhibitors
- Detect non factor-inhibiting immunoglobulins
- Non-specific, require functional assay follow-up
- Confirm FVIII reactivity in clot-based assays and distinguish isotypes IgG₁, IgG₂, IgG₄

Solid-phase Enzyme Immunoassay

1. Add test plasma
2. FVIII-coated microtiter well
3. Add conjugate
4. Anti-FVIII inhibitor binds solid-phase FVIII
5. Add substrate
6. Measure pNA color intensity

pNA-conjugated mouse anti-human IgM or IgG; isotype-specific
Fluorescence-based immunoassay detects anti-FVIII immunoglobulins

1. Carboxylated polystyrene bead
2. Covalently couple FVIII (Kogenate FS)
3. Incubate beads with test plasma
4. Anti-FVIII inhibitor
5. Anti-FVIII binds Kogenate FS

Measure fluorescence

1. Biotinylated anti-human Ig
2. Phycoerythrin-streptavidin

Courtesy of Connie Miller, PhD, CDC
FVIII IgG Binding Domains: A₂>C₂>A₃

- A₂ binds FIXa
- A₃ binds FIXa
- B domain deleted from many rFVIII preps
- C₂ binds VWF
Inhibitor Isotype by Fluorescence Immunoassay

- Non-hemophilia subject samples
  - 5% IgG₁ and IgG₂; 2% IgG₃ and IgG₄
- 491 samples from 371 hemophilia A patients
  - 41% IgG₁, 17% IgG₂, 6% IgG₃, 27% IgG₄
- Many patients with multiple isotypes
- Inhibitor-positive hemophilia A patient isotypes
  - 14% single, 84% multiple, 2% no IgG by FLI
- 7 patients developed NBA-positive inhibitors
  - 5 had prior IgG₁ antibodies, added IgG₄

Nijmegen Bethesda Assay
1995 Nijmegen Bethesda Titer

1. Serially dilute patient plasma in FVIII-DP
2. Mix dilutions 1:1 with IBS-pH 7.4 stabilized NP
3. Incubate 37°C 2h, perform PTT-based FVIII assay
4. Report Nijmegen Bethesda units (NBU)/mL as reciprocal of dilution that neutralizes 50% FVIII

“Hybrid” assay uses IBS-pH 7.4 NP but substitutes IBS for FVIII-DP to reduce expense

CDC Standard NBA Protocol Updates

- Ship patient specimens on cold packs, not frozen
- Use IBS-pH 7.4 reagent normal plasma (NP) to stabilize FVIII
- Heat specimens 56°C 30” & centrifuge to remove FVIII
  - Residual FVIII from recent prophylaxis or on-demand Rx
  - Non-neutralizing antibody leaves behind residual VIII
  - Heat improves specificity for low-titer inhibitors
  - Factor IX titer, 58°C for 90”?
- Dilute heated pt plasmas and unheated 1 BU positive control in bovine serum albumin (BSA) or FVIII-DP, not IBS
- Confirm all <2 BU/mL samples with alternate assay
  - Miller CH, Adcock DM. The need for standardization of factor inhibitor assays. 2016 THSNA Poster
CDC-modified Nijmegen Bethesda Assay

1. Heat patient plasma 56°C for 30” and centrifuge
2. Serially dilute heated patient plasma in BSA or FVIII-DP
3. Serially dilute unheated commercial 1.0 NBU-positive control in BSA or FVIII-DP
4. Mix dilutions 1:1 with IBS-pH 7.4 NP
5. Incubate 37°C 2h, perform PTT-based FVIII assay
6. Convert residual FVIII activity to NBU/mL using NBA nomogram and multiply the NBU/mL value by the dilution factor

Heat patient plasma 56°C/30m & cfg

Prepare dilutions of heated pt plasma and 1 NBU control in BSA or FVIII-DP

Patient and 1 NBU control mix
Mix 1 part patient or control dilution with 1 part IBS-pH 7.4 NP

Mix 1 part IBS-pH 7.4 NP with 1 part BSA or FVIII-DP

Incubate 37°C/2h, measure FVIII activity

Patient mix FVIII/NPmix FVIII X 100 = % residual activity (RA)

Nijmegen-Bethesda Units (NBU) = (2–log %RA)/0.301
Read from nomogram

Courtesy of Connie Miller, PhD, CDC
The Value of Heating

- 126 (55%) of 228 severe HA samples had measurable FVIII.
  - All from patients treated with FVIII within 72 h of specimen collection
  - These had residual activity of >100 U/dL and a false inhibitor titer of 0
- Of 159 presumed inhibitor neg samples, 120 had unheated NBU of 0.
  - After heating, 45 (37.5%) remained 0
  - But 74 (61.7%) rose from 0 to 0.1–0.2 NBU, one rose from 0 to 0.7 NBU
- Of 30 documented inhibitor pos samples with results <0.5 NBU at enrollment, 5 (16.7%) rose to >0.5 NBU after heating.
- FVIII was in samples of patients infused within 24 h of collection.
  - in 15 severe patients, all had FVIII before heating and <1 U/dL after.
  - In 7 moderate patients, FVIII decreased to <1 U/dL after heating.

Purpose of NBA When Bleeding

- If ≤5 NBU, use high-dose FVIII concentrate
- If >5 NBU, prothrombin complex concentrate (PCC, 1980)
  - \( \text{BaSO}_4 \) extracted human plasma; II, VII, IX, X: Proplex
  - Activated PCC: FEIBA, Autoplex
- FEIBA or Autoplex
  - 50 U/kg/12 h prophylactic
  - 70 U/kg/8 h in hemorrhage
  - Limit 200 U/kg/24 h to avoid DIC
- No direct monitor: repeat NBA, perform generalized coag evaluation using PTT

Ludlam DA, Morrison AE, Kessler C. Treatment of acquired hemophilia. Semin Hematol 1994;31 (Sup 4) 16–19
FEIBA Alternatives: NovoSeven, Obizur

- **NovoSeven (1999):** rFVIIa: 90 µg/kg, 6 h FVII half-life
  - Binds cell-bound tissue factor, no DIC risk
  - No direct monitor: repeat NBA, perform generalized coag evaluation using PTT

- **Obizur (2014):** recombinant porcine sequence FVIII
  - Indication: acquired hemophilia
  - Unlikely to cross-react with anti-FVIII IgGs
  - Initial dose 200 U/kg
  - Titrate dose and frequency based on FVIII recovery levels and clinical response
  - Monitor with chromogenic FVIII
NBA Purpose When Not Bleeding

- ≤5 NBU/mL in adults or ≤10 in peds, “low responders”
  - Use standard prophylactic FVIII concentrate Rx
- >10 NBU/mL, “high responder”
- If 5–10 NBU but consecutive Rx generates anamnestic pharmacokinetic response: “high responder”
- Immune tolerance FVIII therapy for high responders

Immune Tolerance Induction (ITI) Rx

- Success rate 60%. Patient is good candidate when...
  - Historical peak < 200 NBU, < 10 BU at ITI initiation
  - < 2 years from inhibitor identification to ITI initiation
  - Age < 8 years; lower peak titer during ITI
- ITI: use 85–200 NBU FVIII/kg/day
  - Monitor using NBA; 0.6 NBU/mL = “negative”
  - 20% drop at 6 months Rx = satisfactory
  - Use maintenance dose throughout life
  - D/C if no response after 3 periods of 6 m

New Factor Formulations

PrecisionBioLogic

ALPROLIX®

IDELVION®
Coagulation Factor IX (Recombinant), Albumin Fusion Protein

ELOCTATE™
[Antihemophilic Factor (Recombinant), Fc Fusion Protein]
Extended Half-life Factor VIII: Eloctate

- Recombinant B-domain deleted Fc fusion factor VIII
- Extended by Fc receptor and IgG recycling pathway
  - 96 HA adult males with >12 annual bleeds, 3–4 doses/week
  - rFVIIIFc half-life 19 h vs rFVIII 12h; 1.6–3.6 annual bleeds
- Prophylactic Rx interval up to 5 days versus 3–4 doses/week
- Monitor using clot-based FVIII assay with non-kaolin-based PTT
- Improved monitoring using chromogenic FVIII assay for all B-domain-deleted FVIII preparations

### Additional rFVIII Preparations

<table>
<thead>
<tr>
<th>Name</th>
<th>MFR</th>
<th>Description</th>
<th>Progress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax 111</td>
<td>Baxter</td>
<td>rVWF (not rFVIII)</td>
<td>At FDA</td>
</tr>
<tr>
<td>Kovaltry</td>
<td>Bayer</td>
<td>Full-length rFVIII with no human or animal proteins, Reduced inhibitors, normal to slightly extended half-life</td>
<td>Approved</td>
</tr>
<tr>
<td>NovoEight</td>
<td>Novo Nordisk</td>
<td>Pegylated, plasma/albumin free, full-length rFVIII, up to 7.5 d frequency</td>
<td>At FDA</td>
</tr>
<tr>
<td>NuWiq</td>
<td>Octapharma</td>
<td>Pegylated plasma/albumin free, full-length rFVIII, 1.5 X Advate half-life</td>
<td>At FDA</td>
</tr>
<tr>
<td>Bay 94-9027</td>
<td>Bayer</td>
<td>rFVIII covalently bonds VWF reduces clearance, extends half-life; no inhibitors</td>
<td>Phase II</td>
</tr>
<tr>
<td>Bax 855</td>
<td>Baxter &quot;Baxalta&quot;</td>
<td>Bispecific protein mimics FVIII cofactor, activates IX &amp; X, bypasses inhibitors, SC 1/wk, generates no immune response</td>
<td>FDA breakthrough status</td>
</tr>
<tr>
<td>rFVIII single-chain</td>
<td>CSL Behring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE 910</td>
<td>Chugai &amp; Genentech</td>
<td></td>
<td></td>
</tr>
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</table>
Extended Half-life Factor IX

- Recombinant Fc fusion factor IX Alprolix, FDA-approved 2014
  - rFIXFc half-life 70–80 h versus 24 h, 7–10 day intervals
  - Monitor using FIX assay with non-kaolin-based PTT
  - Chromogenic FIX valid but not available in the US
  - Patients <12 years old: 40–55 U/kg 7 day interval
  - Patients ≥12 years old: 25–40 U/kg 7 day interval
  - For ≥12 YO if controlled, go to 14-d interval at 50–75 U/kg

Biogen

PrecisionBiologic
BioMarin BMN270 FVIII Transfer Trial

- B-domain-reduced FVIII gene with minimal glycosylation
- Vector optimization: adenovirus-associated vector “8” with adequate capacity for the FVIII gene
  - University College London
  - St. Jude’s Research Hospital
- Phase 1 & 2 trial completed
- Preparing phase III trial

- McIntosh J, Lenting PJ, Rosales C, et al. Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. Blood. 2013 25;121:3335-44
### BioMarin Phase 1 and 2 Results

#### 4/20/16: 8 Severe Hemophilics

<table>
<thead>
<tr>
<th>Dose</th>
<th>Week</th>
<th>%</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>6X10^{12} vg/kg</td>
<td>20</td>
<td>&lt;1</td>
<td>Severe</td>
</tr>
<tr>
<td>2X10^{13} vg/kg</td>
<td>16</td>
<td>2</td>
<td>Moderate</td>
</tr>
<tr>
<td>6X10^{13} vector genomes/kg</td>
<td>16</td>
<td>57</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>60</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>8</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>21</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>Mild</td>
</tr>
</tbody>
</table>

Prednisolone controls liver toxicity as measured by ALT
Gene Transfer Therapy May Reduce Inhibitor Formation: Animal Models

Target Antithrombin to Decrease Inhibition

- Silencing RNA (siRNA): synthetic RNA complementary to mRNA sequence, blocks mRNA translation
- siRNA-AT3 binds antithrombin mRNA and silences hepatic antithrombin production
- siRNA can be produced against any gene product: first accomplished in petunias

- Margaret Ragni, MD, MPH, University of Pittsburgh, THSNA, Chicago 4/14/16
Crosslinked Fibrin

Fibrinogen

Fibrin Polymer

Crosslinked Fibrin

Extrinsic

VIIa

TF

VIIIa

IXa

Thr

Fibrinogen

Fibrin Polymer

Crosslinked Fibrin

Intrinsic

XIIa

Pre-K

HMWK

VIIa

IXa

Xa

Va

XIIa

Common

Figure courtesy of Margaret G. Fritsma
siRNA AT3 in Primates

Graph showing the relative plasma AT activity over time in response to different doses of siRNA. The graph indicates that the activity decreases with treatment and then recovers during the recovery phases.
siRNA AT3 in Humans

Thrombin Generation by % AT Lowering

![Graph showing Thrombin Generation by % AT Lowering](image)

**Healthy Volunteers**
- N=4
- AT Lowering < 25%
- N=24

**Patients with Hemophilia**
- AT Lowering 25-50%
- N=21
- AT Lowering 50-75%
- N=18
- AT Lowering > 75%
- N=9

PrecisionBioLogic
Annual Bleed Rate in Humans Treated with siRNA AT3

Bleed Events by % AT Lowering

<table>
<thead>
<tr>
<th>AT Lowering</th>
<th>N</th>
<th>ABR, Mean (SEM)</th>
<th>ABR, Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25%</td>
<td>24</td>
<td>34 ± 10</td>
<td>13</td>
</tr>
<tr>
<td>25-50%</td>
<td>21</td>
<td>20 ± 7</td>
<td>11</td>
</tr>
<tr>
<td>50-75%</td>
<td>18</td>
<td>14 ± 4</td>
<td>10</td>
</tr>
<tr>
<td>&gt;75%</td>
<td>9</td>
<td>6 ± 3</td>
<td>0</td>
</tr>
</tbody>
</table>

Conclusions: With AT lowering by quartile, <25% to >75%, there is reduction in ABR.
siRNA AT3 Phase I Safety

- No discontinuation
- Mild adverse events
  - Transient erythema and pain at injection site, resolved at 24 hours
  - Headache
- Bleeds treated with standard therapy
- No antibody formation
- Normal LFTs, CBC, PLTs, FG, EKG
Coagulation Factor Inhibitors

Bottom Line at the End (BLATE); The Participant…

• Explains the origin of anti-factor VIII (FVIII inhibitor)
• Detects FVIII inhibitors using factor assays and mixing studies
• Measures FVIII inhibitors using the Bethesda titer, Nijmegen Bethesda assay, chromogenic Bethesda assay, enzyme immunoassay and fluorescence immunoassay
• Describes coagulation factor bypass therapy to resolve bleeding in inhibitor patients
• Describes immune tolerance induction therapy
• Lists new factor concentrates designed to prevent inhibitor formation
Thanks for listening!

The Fritsma Factor,
Your interactive Hemostasis Resource
Sponsored by Precision BioLogic
george@fritsmafactor.com
www.fritsmafactor.com