Laboratory Diagnosis of Inhibitors

M. Ahmadinejad MD. APCP
Iranian Blood Transfusion Organization
Functional inhibitors of hemostasis

are **Immunoglobulins** that interfere with blood coagulation

- **Lupus Anticoagulants** (Most frequent)
- **Inhibitors of individual coagulation proteins**
Individual Coagulation Factor Inhibitors Incidence

- **Factor VIII inhibitors** (First reported case = 1941)
  1) Healthy individuals
  2) Patients suffering from an autoimmune disease
  3) Patients with FVIII deficiency

Autoantibody = 1 in 1.48 million/year,
Alloantibody = 25-30 % (range of 8 % - 52 %) in severe cases
  3% in Moderate Hemophilia
  0.3% in Mild Hemophilia

- **Factor IX inhibitors**
  1.5-3 %, Possible reasons for lower incidence
  - Homology with the other vit- K-dependent clotting factors
  - Smaller size, may cross the placenta, inducing tolerance in developing fetus

- **Factor XI inhibitors**
  > 30 % In severe deficiency which is almost exclusively described in Azkenazi & Iraqi Jews, detected at routine screening because most do not bleed spontaneously.
• Multi-factorial (genetic and non-genetically)
• Despite improvements in understanding we remain unable to fully predict the immune response to the deficient factor
FVIII infusion

FVIII Mutation

APC

HLA Class II

Low risk

High risk

Immune System Challenge

Immune Response Genes

No anti-FVIII abs

- Neutralizing
- Non-neutralizing
Definitions

- **Titer level:**
  - Low Titer Inhibitors (LTI) $< 5$ NBU Low responders
  - High Titer Inhibitors (HTI) $> 10$ NBU High responders
  - Inhibitor Titer 5-10 NBU High or Low responders

- **Time of action:**
  - Immediate Acting Inhibitors
  - Time & Temperature-dependent Inhibitors

- **Kinetics:**
  - Type 1 (Simple Reaction Kinetics)
  - Type 2 (Complex Reaction Kinetics)
FVIII Inhibitors

• **Type 1 (Simple Reaction Kinetics)**
  – Many inhibitors
  – FVIII is permanently inactivated and the inhibitor is consumed (firm Ag & Ab bond)
  – Most alloantibody-inhibitors in Hemophilia A and some autoantibody-inhibitors in other persons

• **Type 2 (Complex Reaction Kinetics)**
  – React in a non-linear or complex way with FVIII
  – Some FVIII may still be measurable
  – FVIII inhibitors acquired by non-hemophilics
  – INHs in Mild/Moderate Hemophilia
  – Underestimation of inhibitor titer in Bethesda assay
Diagnosis and investigation of factor VIII and IX inhibitors

- In clinical practice, the diagnosis of an inhibitor is usually based on more than a single positive inhibitor titre; it includes the patient’s historical response to therapy and often PK studies.

- In clinical trials and surveillance programmes, however, such information may not be available, and there is a risk of miscounting of cases and mislabelling of patients due to false positive results.
Development of a neutralizing factor inhibitor is the most significant treatment complication in patients with hemophilia
- Decreases effectiveness of treatment
- Significantly increases treatment cost
- Life threatening

Laboratory plays an important role in providing a reliable and reproducible assay for the detection and quantitation of neutralizing factor inhibitors
- Monitoring and management of hemophilia care
- Evaluation of novel factor product safety
Laboratory methods

- **Screening**, based on aPTT
- **Simple INH screen** (more sensitive than Bethesda)
  
  (Patient plasma + FVIII/IX concentrate [final concentrate 1 iu/ml] 1 h in 37 °C) INH presence if Factor level < 90%

- **Quantitative Measurement** of FVIII Inhibitors
  - Functional assays
  - Immunologic assays
Table 18.5 Interpretation of the inhibitor screen based on the activated partial thromboplastin time

<table>
<thead>
<tr>
<th>TUBE</th>
<th>CONTENT</th>
<th>CLOTTING TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal plasma</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Patient's plasma</td>
<td>Long</td>
</tr>
<tr>
<td>3</td>
<td>50:50 mixture, patient: normal; incubated 2 h</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>50:50 mixture, patient: normal; no incubation</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Interpretation

- Deficiency
- Immediate Acting Inhibitor
- Time-dependent Inhibitor
Functional Quantitative Assays of FVIII Inhibitors

Oxford method

1959

Biggs et.al

Bethesda assay

1975

Kasper et.al

Nijmegen-Bethesda assay

1995

Verbruggen et.al

Gold Standard
Factor Inhibitor Assays

Classical Bethesda
Nijmegen Modified\(^1\)
Assay

- **Imidazole Buffer**
  - Neat
  - 1:2
  - 1:5
  - 1:10
  - 1:20

- **Patient Sample**

- **Buffered Normal Plasma**

- **Faktor deficient plasma**

- **Incubation at 37 °C**

- **Measure Factor VIII or FIX Activity**

\(^1\)Verbruggen, B et al
\(^2\)Kershaw GW et al *Thromb Res* 2013; 132:735
Calculation of the Nijmegen Bethesda Titer

1:1 Patient Mix

Post incubation at 37°C
Measure Factor Activity

1:1 Control Mix

Residual activity = \frac{\text{Sample Factor Activity}}{\text{Control Factor Activity}}

\text{NBU} = 2 \cdot \log[\% \text{Residual Activity}] - 0.301

1 \text{ NBU} = 50 \% \text{ residual Activity}
Determination of Accurate Nijmegen Bethesda Titer (NBU)

- If an inhibitor is suspected dilute patient plasma to obtain residual activity between 25 and 75%.
- Prepare sufficient dilutions of the patient sample to approach residual activity closest to 50%.
- Use the patient sample dilution that gives residual activity closest to 50% to calculate inhibitor titer.
Performance of Nijmegen Modified Bethesda Assay (NBA)

• EQAS programs (ECAT, NEQAS, RCPA QAP) report high inter-laboratory variability with CVs often greater than 30%.

• The percentage of false positive and negative results is unacceptably high.

• Surveys reveal that many laboratories use hybrid methods incorporating features of both the Bethesda and Nijmegen Modified assays.
The commonly used Nijmegen and Bethesda FVIII inhibitor assays exhibit a poor sensitivity for low-titre inhibitors.
Low Titer Inhibitor assay

(Verbruggen et.al 2012)

A new FVIII inhibitor method based on the Nijmegen assay with a detection limit as low as 0.03 BU mL⁻¹
Low-titre inhibitors, undetectable by the Nijmegen assay, reduce factor VIII half-life after immune tolerance induction

M. DARDIKH,* T. ALBERT,† R. MASEREEUW,‡ J. OLDENBURG,† I. NOVAKOVA,§ W. L. VAN HEERDE* and B. VERBRUGGEN*¶

*Department of Laboratory Medicine, Laboratory of Haematology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands; †Department of Experimental Haematology and Blood Transfusion, University Clinic Bonn, Bonn, Germany; ‡Department of Pharmacology and Toxicology, Radboud University Nijmegen Medical Center, Nijmegen; §Department of Haematology, Radboud University Nijmegen Medical Center, Nijmegen; and ¶Laboratory of Clinical Chemistry and Haematology, Jeroen Bosch Hospital, ‘s-Hertogenbosch, the Netherlands

Low-titre inhibitors, undetectable by the Nijmegen assay, reduce factor VIII half-life after immune tolerance induction

In Haemophilia Treatment Centre in Nijmegen:

*Abnormal high need for FVIII supply to prevent bleedings in the early post-ITI phase with formerly inhibitor positive patients.*

They hypothesized:

patients in the early post-ITI phase still have *low-titre inhibitors* that cannot be detected with the established assays, but contribute to rapid disappearance from the circulation of FVIII.
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Consecutive sample number</th>
<th>Date of sampling</th>
<th>Dosage (IU kg(^{-1}) BW)</th>
<th>Half-life of infused FVIII (h)</th>
<th>% FVIII level (time relapse after administration)</th>
<th>Expected FVIII-level (%) after indicated time relapse</th>
<th>Inhibitor titre (BU) (low titre assay)</th>
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<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>6 November 2002</td>
<td>25</td>
<td>4.0</td>
<td>2 (24 h)</td>
<td>&gt; 10</td>
<td>0.15</td>
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<tr>
<td>I</td>
<td>2</td>
<td>17 December 2003</td>
<td>28</td>
<td>15.9</td>
<td>37 (24 h)</td>
<td>&gt; 10</td>
<td>0.00</td>
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<tr>
<td>II</td>
<td>1</td>
<td>8 July 2004</td>
<td>55</td>
<td>4.6</td>
<td>–</td>
<td>–</td>
<td>0.16</td>
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<tr>
<td>II</td>
<td>2</td>
<td>4 April 2005</td>
<td>53</td>
<td>5.6</td>
<td>–</td>
<td>–</td>
<td>0.07</td>
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<tr>
<td>II</td>
<td>3</td>
<td>8 December 2006</td>
<td>25</td>
<td>6.2</td>
<td>5 (24 h)</td>
<td>&gt; 10</td>
<td>0.03</td>
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<tr>
<td>III</td>
<td>1</td>
<td>8 May 2002</td>
<td>25</td>
<td>3.1</td>
<td>3 (24 h)</td>
<td>&gt; 10</td>
<td>0.35</td>
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<tr>
<td>III</td>
<td>2</td>
<td>15 March 2005</td>
<td>25</td>
<td>3.1</td>
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<td>&gt; 10</td>
<td>0.01</td>
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<tr>
<td>IV</td>
<td>1</td>
<td>11 August 2009</td>
<td>111</td>
<td>–</td>
<td>5 (0.5 h)</td>
<td>&gt; 100%</td>
<td>0.46</td>
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<tr>
<td>IV</td>
<td>2</td>
<td>27 January 2010</td>
<td>105</td>
<td>–</td>
<td>5 (14 h)</td>
<td>&gt; 70%</td>
<td>0.11</td>
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<tr>
<td>IV</td>
<td>3</td>
<td>21 April 2010</td>
<td>87</td>
<td>–</td>
<td>8 (14 h)</td>
<td>&gt; 70%</td>
<td>0.08</td>
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<td>IV</td>
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<td>10 February 2011</td>
<td>167</td>
<td>–</td>
<td>56 (13 h)</td>
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<td>V</td>
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<td>–</td>
<td>2 (26 h)</td>
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<td>V</td>
<td>2</td>
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<td>83</td>
<td>–</td>
<td>14 (12 h)</td>
<td>&gt; 70%</td>
<td>0.05</td>
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<tr>
<td>V</td>
<td>3</td>
<td>4 March 2010</td>
<td>83</td>
<td>–</td>
<td>24 (11 h)</td>
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<tr>
<td>VI</td>
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<td>96</td>
<td>–</td>
<td>27 (16 h)</td>
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<td>&gt; 0.8</td>
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<tr>
<td>VI</td>
<td>2</td>
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<td>–</td>
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<tr>
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<td>3</td>
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<td>–</td>
<td>21 (12 h)</td>
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<td>0.02</td>
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<td>VII</td>
<td>2</td>
<td>6 April 2010</td>
<td>118</td>
<td>–</td>
<td>28 (12 h)</td>
<td>&gt; 70%</td>
<td>0.05</td>
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<td>VII</td>
<td>3</td>
<td>5 May 2010</td>
<td>118</td>
<td>–</td>
<td>38 (12 h)</td>
<td>&gt; 70%</td>
<td>0.01</td>
</tr>
</tbody>
</table>

BW, body weight; BU, Bethesda Units. Summary of the low-titre assay, FVIII activity and FVIII half-lives after FVIII administration in seven severe hemophiliacs with a history of FVIII inhibitors at the end of ITI treatment with tolerance development. Half-lives were calculated as described in the text. The FVIII activity was assayed with a one-stage assay. The ‘Expected FVIII levels’ were dependent on FVIII dose and time relapse after administration and were derived from data of inhibitor-free patients who also have been treated with identical doses of FVIII concentrates (J. Oldenburg, personal communication).
Low Titer Inhibitor assay

- These low-titre inhibitors decrease the half-life and the recovery of infused FVIII products

- LTI assay for monitoring patients suspected of having low-titre INH:
  
  *Increase the efficacy of the treatment*
  
  *Avoid frequent PK studies*
Alternative methods for Inhibitor Measurement

- **Chromogenic assays**
- **ELISA**
- **FLI (Fluorescence-base immunoassays)**
Thank you for your attention

Minoo Ahmadinejad MD.
minooam@gmail.com