Inhibitor Testing: State of the Art

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Overview

Background

Inhibitor Testing Methods

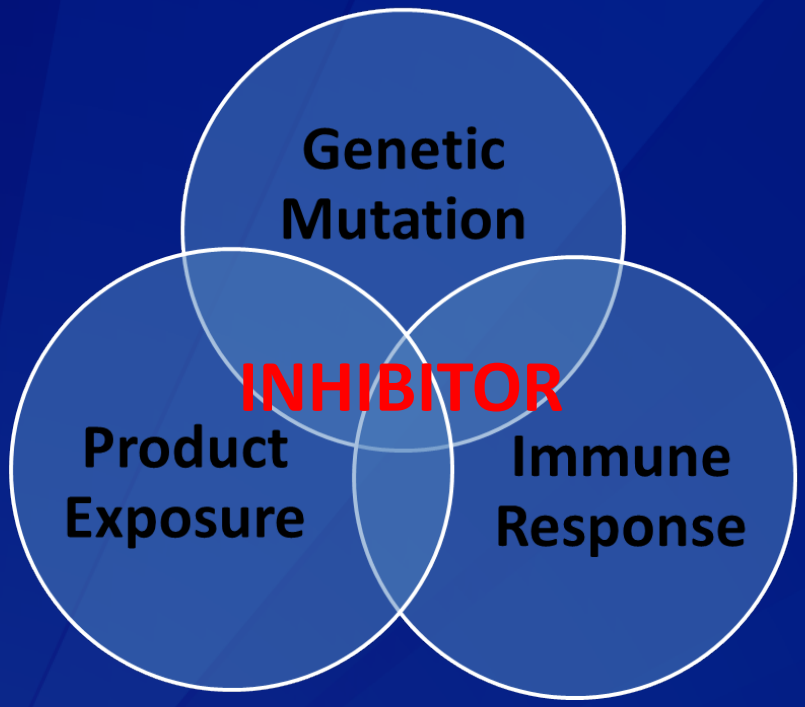
Hemophilia Inhibitor Research Study (HIRS)

National Inhibitor Surveillance (Community Counts)

What Are Inhibitors?

- Inhibitors are antibodies.
  - Our bodies make antibodies to fight diseases.
  - Antibodies are produced when the immune system does not recognize a protein entering the body.
  - Antibodies are part of the natural process to destroy a foreign substance.
- Inhibitors occur when the body does not recognize the normal clotting factor used for treatment.
  - Either the person with hemophilia (PWH) does not make any, or it is different from normal factor.
- Inhibitors act by combining with the factor and either blocking its action in clotting or removing it from the blood.

Risk Factors for Inhibitor Development



- **Genetic:**
  - Factor deficiency – FVIII>FIX
  - Severity of disease
  - Hemophilia gene defect
  - Family history of inhibitor
  - Race/ethnicity – Black and Hispanic rates two-fold higher than White
  - Immune response and modifying genes
- **Treatment-related:**
  - Frequency and intensity of exposure to factor products
  - Events surrounding treatment episodes
  - Type and structure of product used

Significance of Inhibitors

- Inhibitors often require a change in treatment.
  - Some inhibitors, called transient, disappear on their own.
  - Others may require the use of more factor but are not progressive.
  - The most significant inhibitors require :
    - use of a by-passing agent to produce clotting or
    - a process called immune tolerance induction to try to eliminate the inhibitor by giving frequent doses of clotting factor.
- Costs associated with inhibitors are staggering.
  - PWH with inhibitors are twice as likely to be hospitalized.
  - Cost of hospital care is 2-10 times greater.
  - Treating an inhibitor can cost up to $500,000 per year.
- Odds of death are 70% higher in inhibitor patients.
  - Analysis by Walsh et al of data collected by CDC Universal Data Collection (UDC) program
  - 42% of deaths in inhibitor patients were from bleeding, compared to 12% of deaths in non-inhibitor patients .

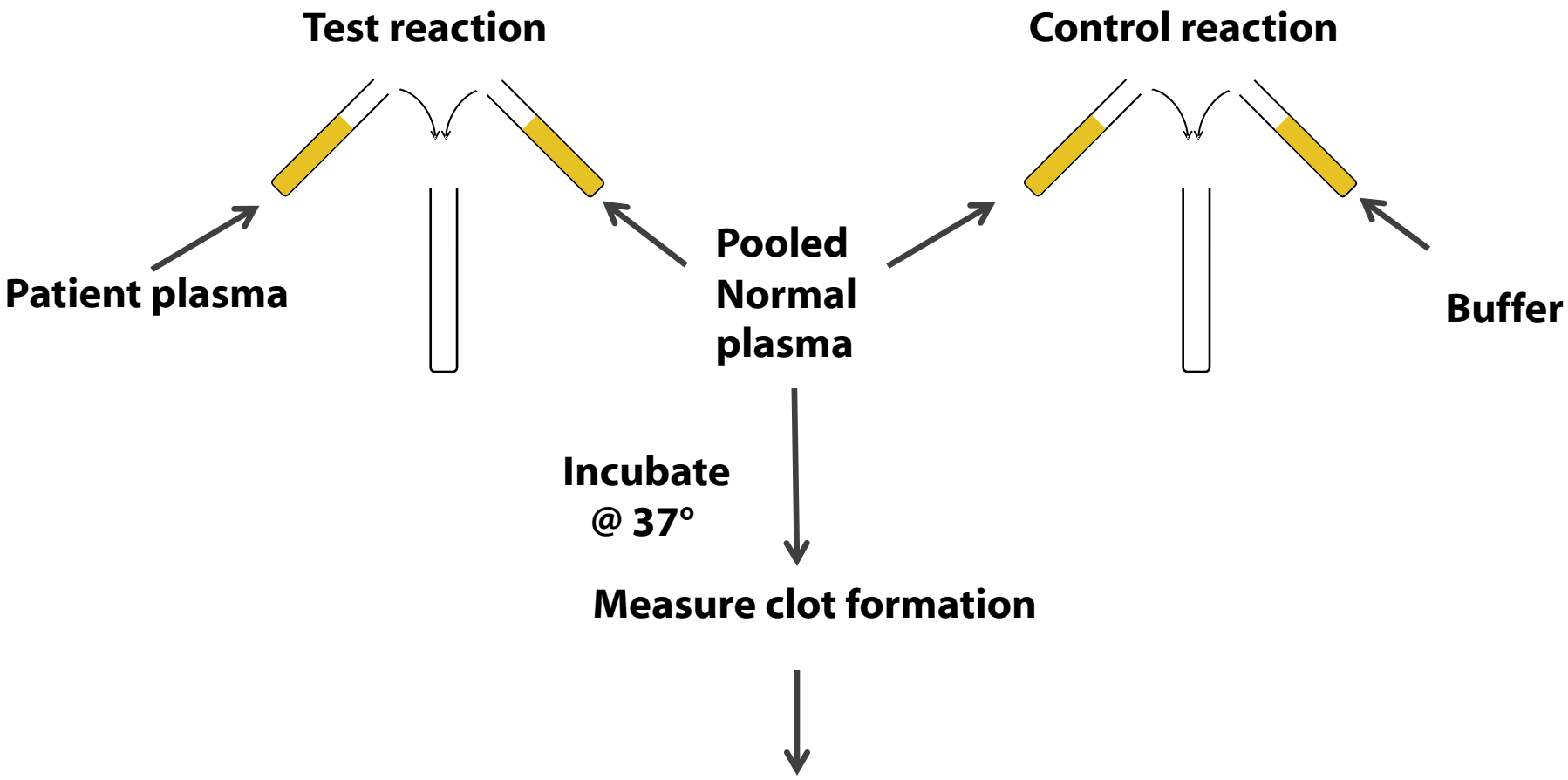
Inhibitor Testing Issues

- Proportion of people with severe hemophilia receiving an inhibitor test ranged across US centers from 0-100%, averaging 46% (UDC data).
- Inhibitors detected early are more easily treated by immune tolerance induction.
- Limitations to doing routine inhibitor testing in the U.S. (expert panel)
  - Requirement for "wash out" for testing
  - Lack of available laboratory expertise
  - Payment issues
  - High rate of false positive tests
- False positive rate is as high as 32%. The CV among laboratories is near 50%. (Favaloro et al. 2014, *Haemophilia* 20: Suppl 4)

Inhibitor Testing Issues

- Accurate inhibitor measurement is important for:
  - Clinical care
  - Evaluation of product safety
  - Assessment of population trends
- Clinically, inhibitor diagnosis is based on:
  - Laboratory findings
  - Response to therapy
  - Pharmacokinetic studies
- For surveillance and clinical trials, tests must be:
  - Accurate and reproducible
  - Usable during treatment
  - Confirmed, to minimize false positive results

Principle of Inhibitor Assay



History of Inhibitor Measurement

- 1959: First report (Biggs and Bidwell)
  - Two-stage assay for Factor VIII
- 1975: Bethesda Assay (Kasper *et al*)
  - One-stage assay for Factor VIII
  - Two hour incubation with pooled normal plasma
  - Established "Bethesda unit" for measurement
- 1995: Nijmegen Assay (Verbruggen *et al*)
  - Modified Bethesda Assay using buffered pooled normal plasma and dilution with factor VIII-deficient plasma
  - More sensitive and specific
  - "Gold standard" for inhibitor testing
- 2012: North American Specialized Coagulation Laboratory Association (NASCOLA) Survey (Pruthi *et al*)
  - 20% using the Nijmegen assay
  - 10% using the classical Bethesda assay
  - 70% using buffered normal plasma (hybrid)

Factor VIII Inhibitor Measurement Methods

Functional Assays

Antibody Detection Assays

Factor VIII Inhibitor Measurement Methods

Functional Assays

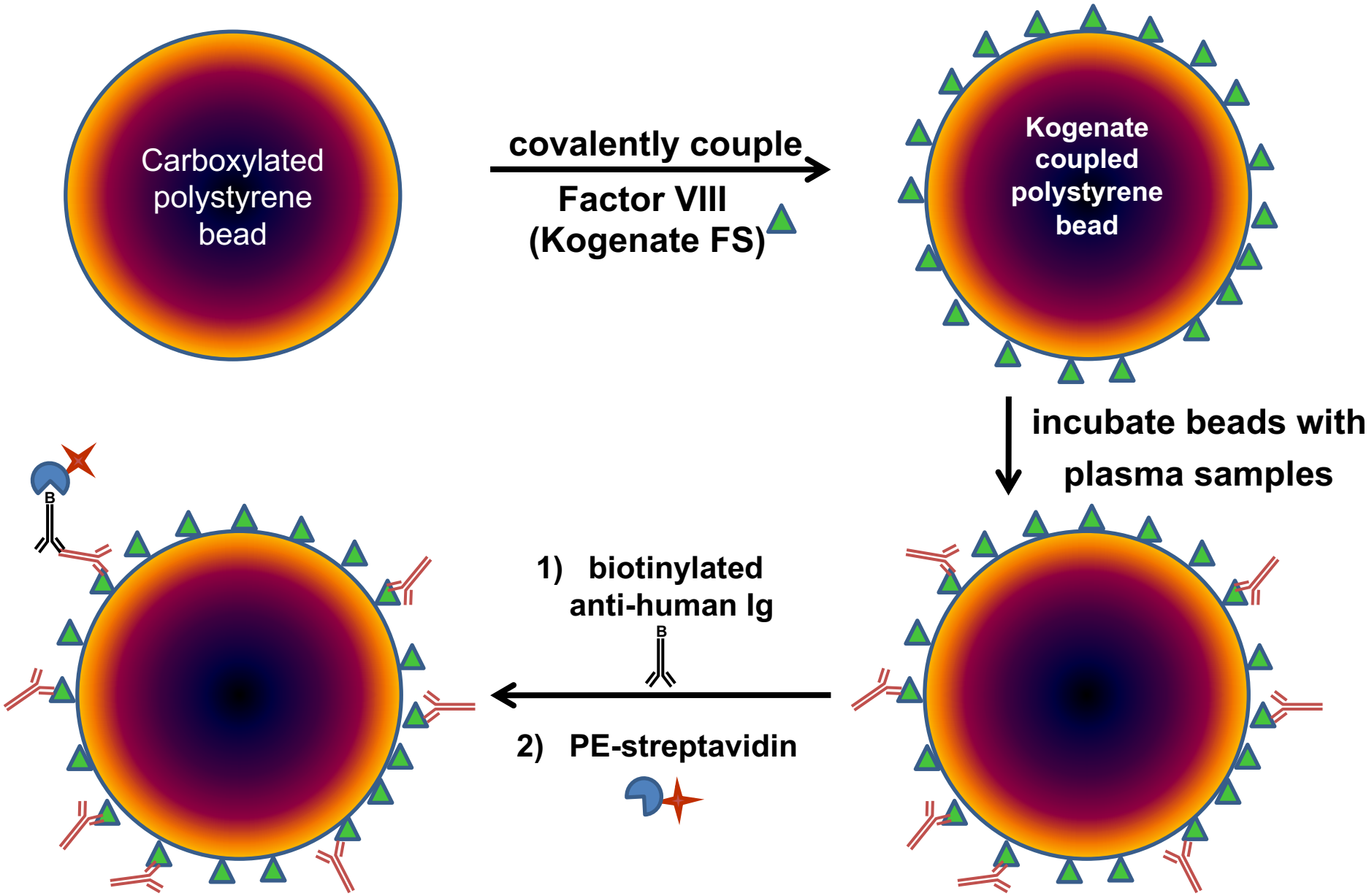
- Nijmegen-Bethesda Assay (NBA)
  - Measures inhibition of clot formation
  - Limitations:
    - Non-specific endpoint (fibrin clot)
    - Plasma components from multiple individuals
    - Influenced by LA, heparin, non-specific inhibitors
    - Insensitive (low-titer modification)
    - In vitro kinetics poorly understood
- Chromogenic Bethesda Assay (CBA)
  - Measures inhibition of FXa generation
  - Not influenced by LA, heparin, non-specific inhibitors
  - More specific for FVIII inhibitors

Factor VIII Inhibitor Measurement Methods

Antibody Detection Assays

- Detect both inhibitory and non-inhibitory antibodies
- Not primary assays: require follow-up with functional assays for confirmation and quantitation
- More sensitive than functional assays
- May be used to confirm FVIII reactivity
- Enzyme linked immunosorbent assay (ELISA)
  - Antibody binding to FVIII immobilized on a plastic surface
  - Commercially available
- Fluorescence-based immunoassay (FLI)
  - Antibody binding to FVIII immobilized on fluorescent beads
  - Krudysz-Amblo *et al*. Blood 2009; 113: 2587.

Fluorescence-based immunoassay for detecting anti-Factor VIII antibodies in plasma



Hemophilia Inhibitor Research Study (HIRS)

- Study conducted by the CDC at 17 US hemophilia treatment centers, beginning in 2006, funded by the CDC Foundation with grants from Pfizer and Baxter
- Prospective treatment data collected on 1163 patients from 17 sites
  - Followed for 3329 person-years
  - Records for 113,205 exposure days
- 23 new inhibitors detected: 9 at enrollment, 14 during follow up
- Population at risk for inhibitors includes all patients:
  - One-third of new inhibitors were in non-severe patients.
  - One-half were over age 5.
  - One quarter had >150 exposure days.
  - 61% had no clinical effect at detection.

Soucie MJ *et al*. A study of prospective surveillance for inhibitors among persons with hemophilia in the United States. *Haemophilia* 20: 230–237, 2014.

HIRS Inhibitor Testing

- 2590 specimens for FVIII and 567 for FIX inhibitor testing
- Laboratory goals:
  - To adapt NBA for high-throughput testing
  - To establish quality control
  - To determine cut-off for a positive inhibitor
  - To evaluate alternative methods
- Key Findings:
  - Developed and validated a modified NBA allowing testing of infused patients
  - Developed a means of confirming newly detected inhibitors using alternative testing methods
  - A proportion of low titer inhibitors are false positives.

FVIII Inhibitor Method Validation

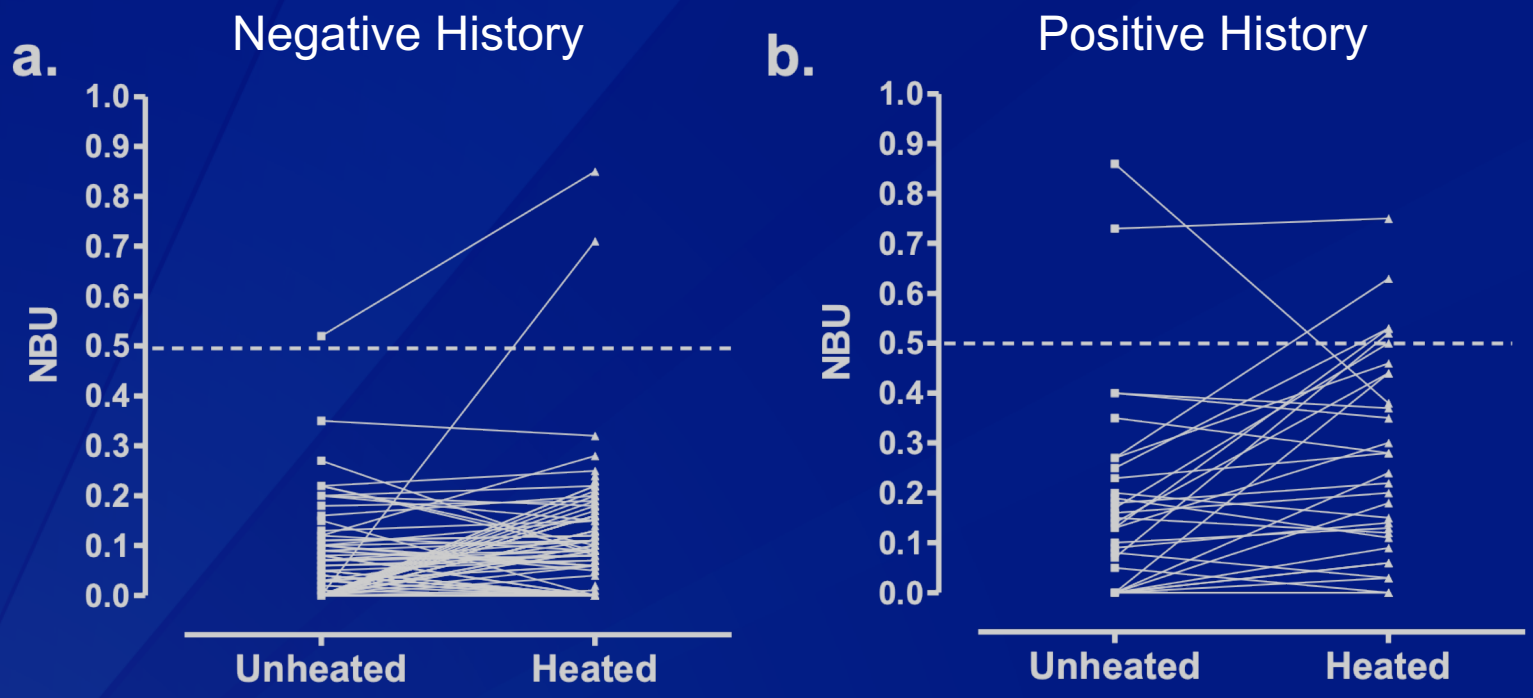
Each modification to the Nijmegen-Bethesda assay was validated.

- **Shipping on cold packs vs. frozen: n=50, r=0.998**
- **Use of commercial vs. locally prepared imidazole-buffered normal pool plasma: r=0.97**
- **Use of buffer instead of FVIII deficient plasma for dilution: n=71, r=0.99 (not adopted)**
- **Elimination of FVIII contamination:**
  - **Measurable FVIII in 126/228 (55%) of frozen specimens**
  - **Due to prophylaxis, episodic treatment, or ITI**
  - **If FVIII is not accounted for, residual activity will be 100% or higher leading to NBU of 0.**
  - **Instituted heating step, 56° for 30 minutes then centrifugation**
  - **Measured FVIII activity and FVIII antigen after heating, <1%**

Miller CH, et al. Validation of Nijmegen-Bethesda assay modifications to allow measurement during replacement therapy and facilitate inhibitor surveillance. JTH 2012; 10: 1055-61.

Change in Inhibitor Titer after Heating Plasma

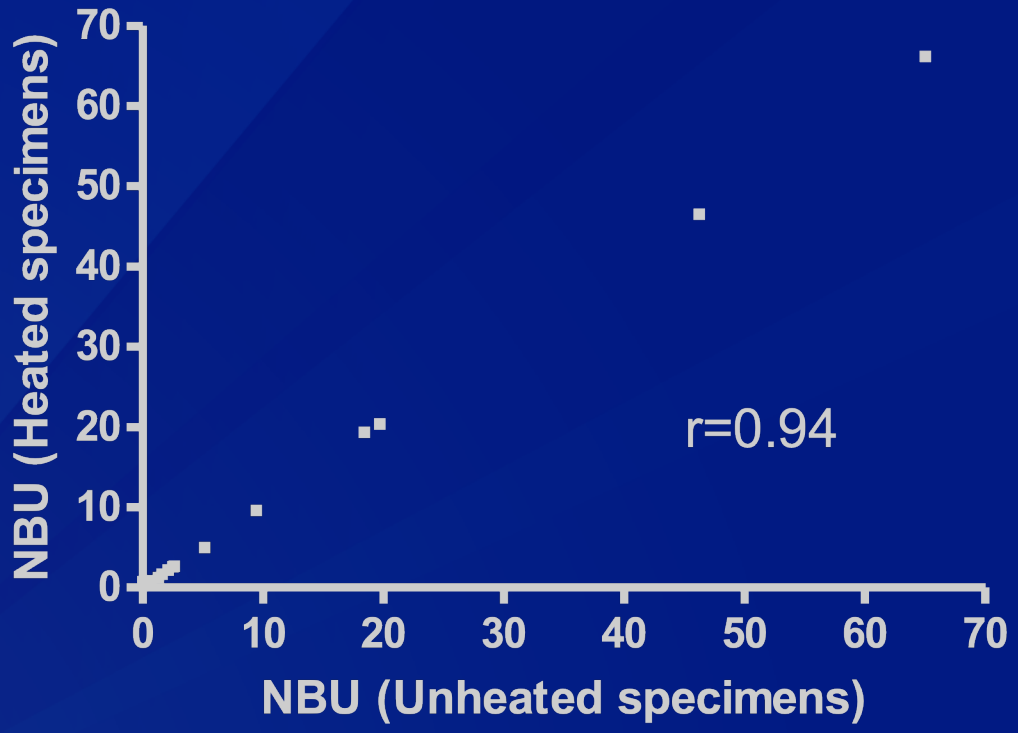
Specimens with NBU <1.0



1/159 with negative history (0.6%) and 5/30 with positive history (16.7%) increased above cut-off, *P*=0.0004

Change in Inhibitor Titer after Heating Plasma

21 specimens with positive NBU



Modified Nijmegen-Bethesda Method

Heat patient and control plasmas to 56°C and centrifuge.**

Dilute patient plasma in FVIII-deficient plasma,* if an inhibitor is expected.

Patient Mix	Control Mix
1 part patient or dilution	1 part FVIII-deficient plasma*

plus

1 part imidazole-buffered normal pooled plasma (BNPP)*

Incubate for 2 hours at 37°C
Measure Factor VIII activity

Patient mix/control mix X 100 = % residual activity (RA)
Convert RA to NBU by formula
Adjust for dilution, if necessary

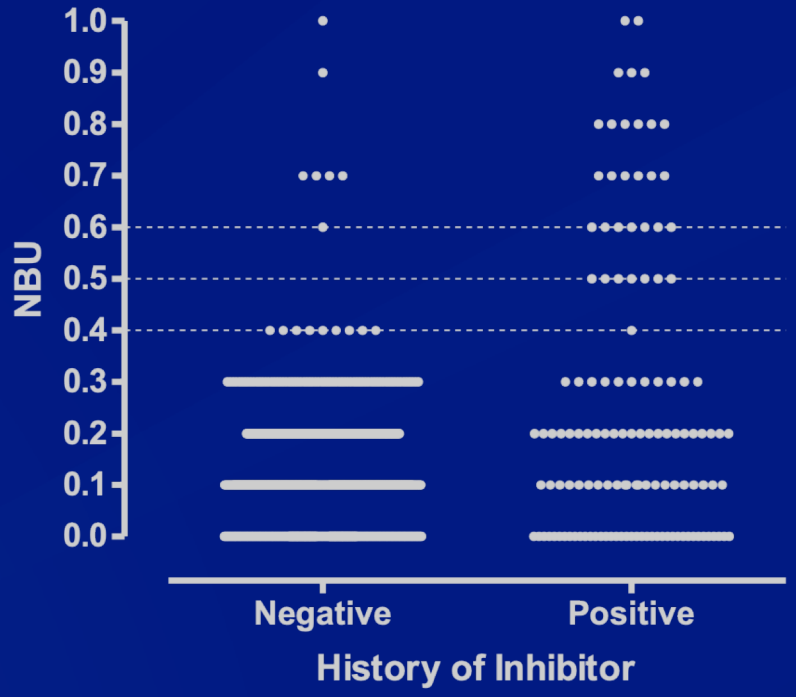
*Nijmegen modifications
**CDC modification

Controls: Negative control CV 9.8% (n=117)
Positive control CV 10.3% (n=114)

FVIII Inhibitor Cut-off

a. At enrollment (n=644)

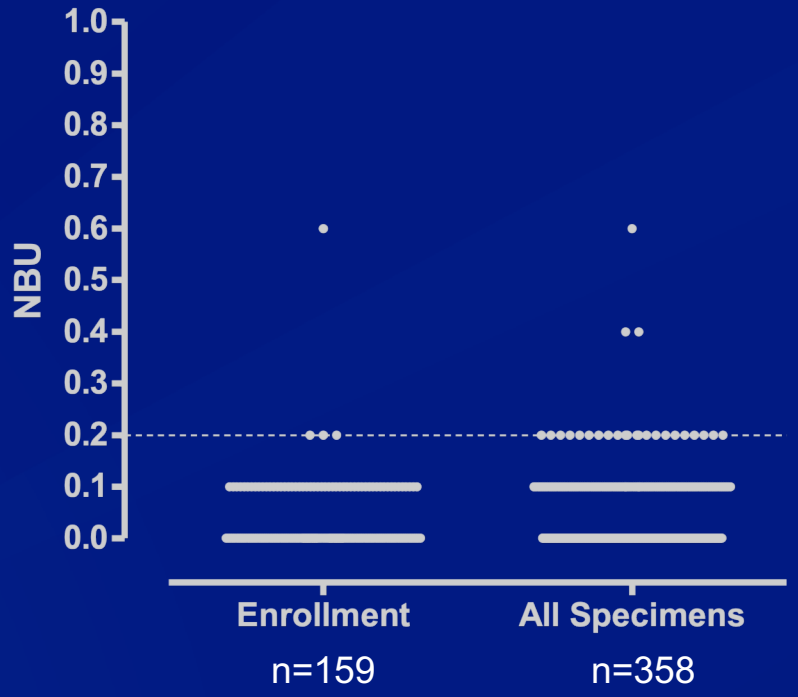
b. All specimens (n=1259)



Cutoff of ≥0.5 for positive inhibitor misclassified fewer specimens than a cut-off of ≥0.6.

FIX Inhibitor Assay

- Heating:
  - 1 of 17 specimens changed with heating (0 to 0.6 NBU)
- Establishment of inhibitor cut-off:
  - All with negative history of inhibitor had NBU ≤0.2.
- Controls:
  - Negative control: CV = 6.8%
  - No positive control



Inhibitor Method Comparison

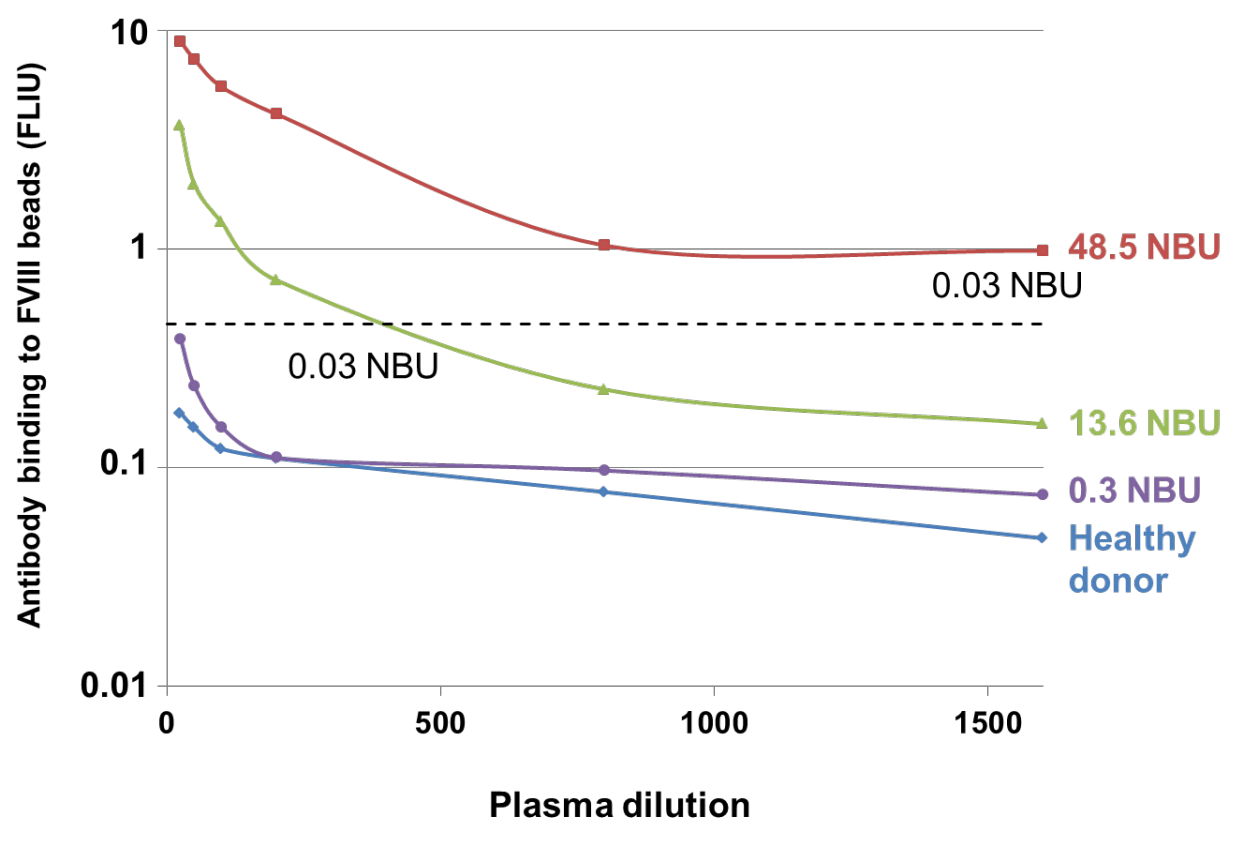
- 3 methods used in HIRS:
  - Functional clot-based assay:
    - Modified Nijmegen-Bethesda assay (NBA)
  - Functional chromogenic assay:
    - Identical to NBA except FVIII method (CBA)
  - Antibody detection method:
    - Fluorescence immunoassay (FLI)

Inhibitor Method Comparison

NBA Result (n)	CBA Negative n (%)	CBA Positive n (%)
NBA Negative (883)	880 (99.7)	3 (0.3)
NBA Positive (122)	37 (30.3)	85 (69.7)
≥ 2.0 NBU (42)	0	42 (100)
0.5-1.9 NBU (80)	37 (46.2)	43 (53.8)

- 1005 specimens compared in NBA and CBA
- 37 specimens (4%) were NBA positive and CBA negative, all 0.5-1.9 NBU.
  - 5 had positive DRVVT
  - 13 had non-time-dependent inhibition

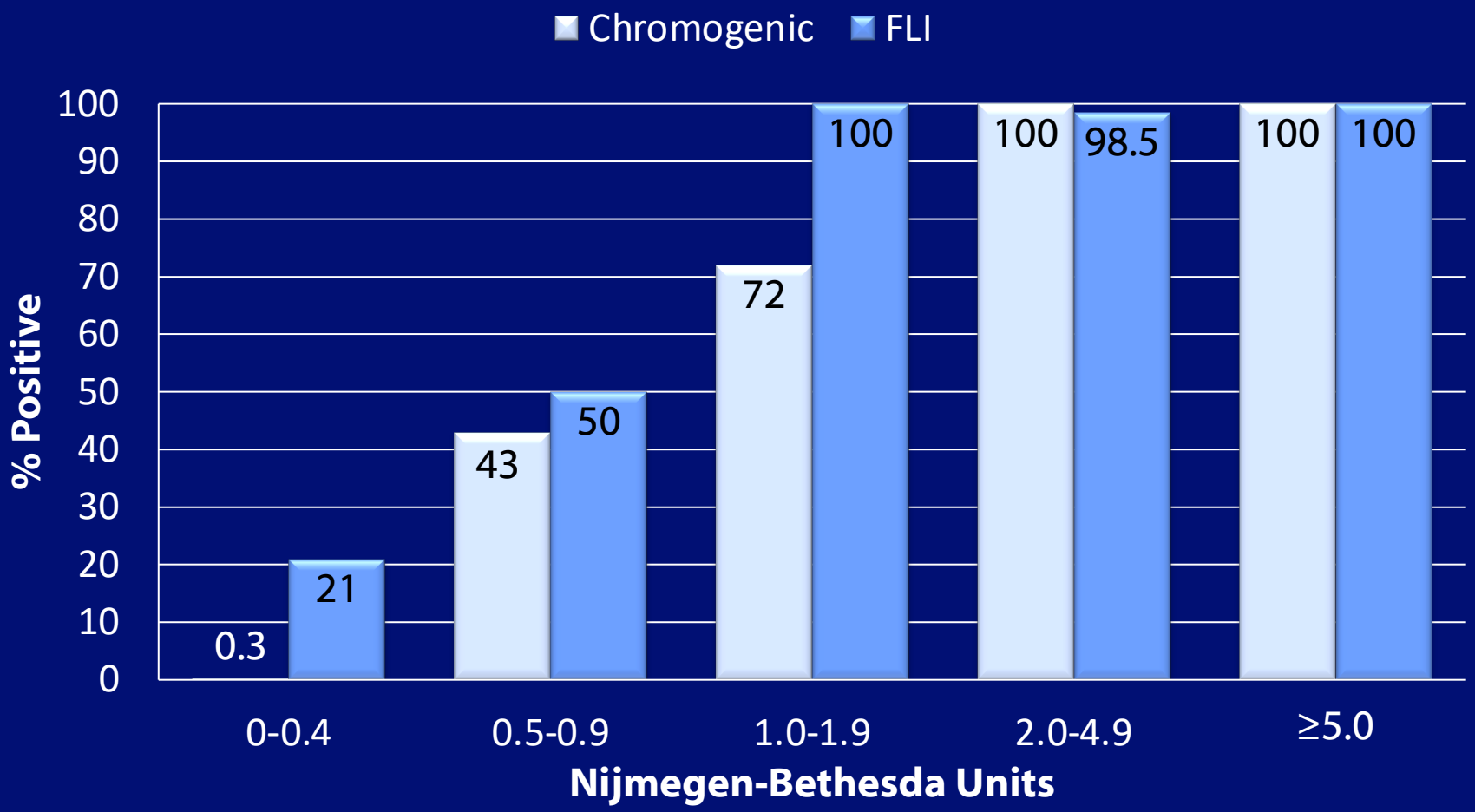
Fluorescence Immunoassay (FLI) for Antibodies to Factor VIII



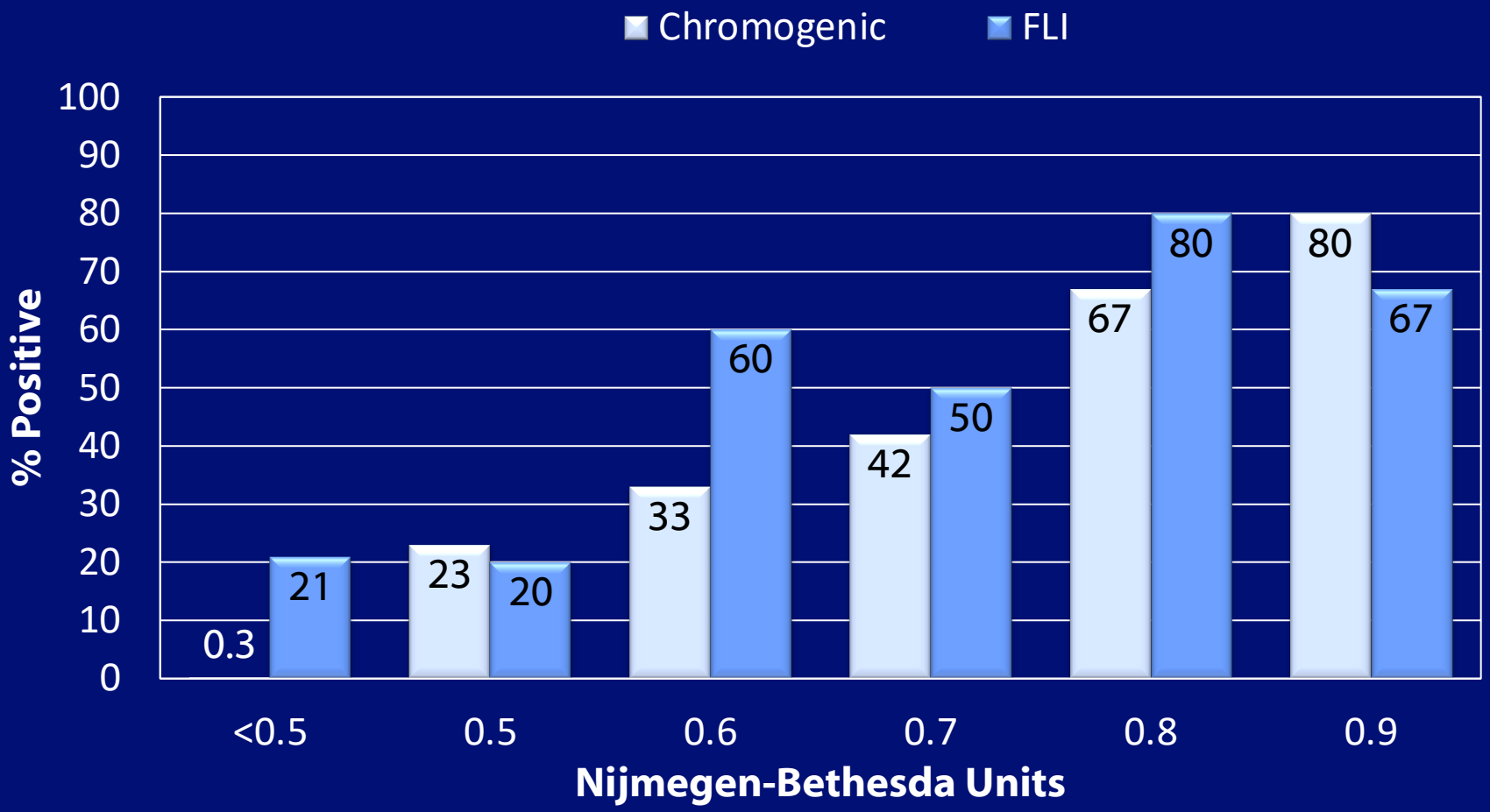
Performed on 272 specimens
Sensitivity: 0.03 NBU

FLI positive in:
98% of CBA positive specimens
82% of NBA positive specimens

Comparison of Chromogenic and FLI Results to Clotting NBU in Study Specimens



Comparison of Chromogenic and FLI Results to Very Low Titer Clotting NBU



Inhibitor Method Comparison

- Conclusions:
  - NBA, CBA, and FLI agree on specimens ≥2.0 NBU.
  - FVIII specificity could not be demonstrated for 26% of inhibitors <2.0 NBU using 2 tests with different mechanisms.
  - Low titer inhibitors detected in clot-based assays should be repeated by testing a new specimen and confirmed in tests more specific for FVIII.
  - 21% of NBA-negative patients have anti-FVIII antibodies.

Miller CH *et al*. Comparison of clot-based, chromogenic and fluorescence assays for measurement of factor VIII inhibitors in the U.S. Hemophilia Inhibitor Research Study (HIRS). *Journal of Thrombosis and Haemostasis* 11:1300-9, 2013.

Immune Tolerance Induction (ITI) Therapy Monitoring

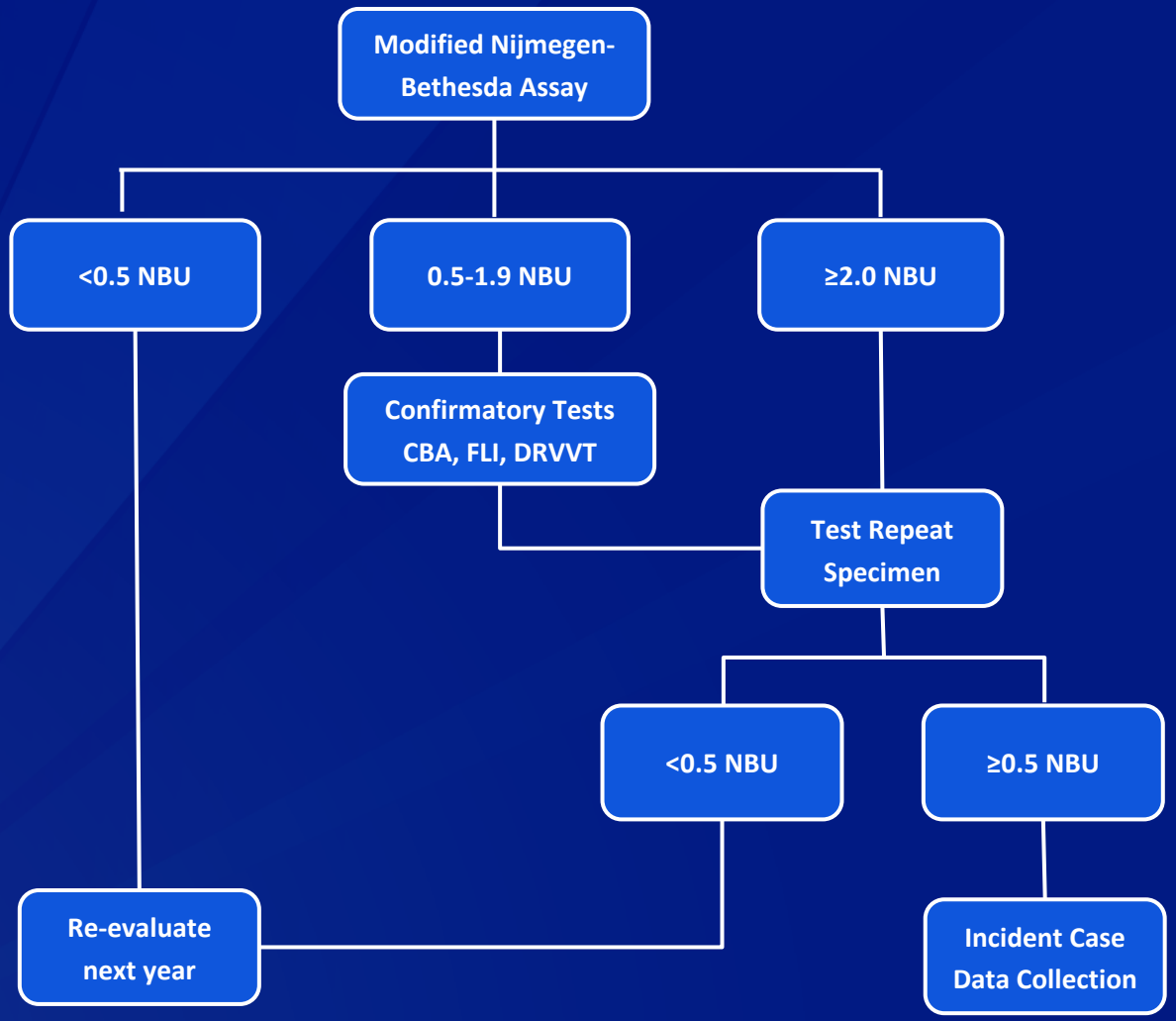
- 38 patients who had been on ITI in HIRS
- Positive FLI was present in:
  - 0 of 15 patients with successful ITI
  - 5 of 5 patients with failed ITI
  - 61% of 18 patients with ongoing ITI
- Differs from previous reports of positive antibodies after successful ITI
- Suggests that this might be a useful test for monitoring ITI

Low Titer Inhibitor Method

- NBA modified to increase sensitivity
  - Patient plasma concentrated
  - Ratio of plasma to normal pooled plasma 3:1 instead of 1:1
  - Uses chromogenic assay to measure Factor VIII
- Limit of detection 0.03 Bethesda units
- Identifies presence of inhibitors after successful ITI which reduce half-life and recovery (n=7)

Dardikh *et al*. Low-titre inhibitors, undetectable by the Nijmegen assay, reduce factor VIII half-life after immune tolerance induction. *J Thromb Haemost* 2012; 10: 1335-44.

U.S. Inhibitor Surveillance Testing



Community Counts Surveillance Testing 5/31/2014

- Inhibitor testing: 646 specimens
  - Elevated inhibitor titer frequency
    - Hemophilia A 42/506 8%
    - Hemophilia B 3/140 2%
    - VWD Type 3 1/8 13%

Inhibitor "Outbreak" Investigation

- 4 new inhibitors in hospitalized previously treated patients over 14 months - ? product-related
- Investigation compared:
  - 2 time periods (no inhibitors previous 3 years)
  - Patients developing and not developing inhibitors while hospitalized
- Conclusions:
  - Incidence did increase
  - Not product- related
  - Inhibitor patients had more factor and more hospital days, increased odds of infection, continuous infusion, and product switch
  - A specific cause could not be determined.

Ghaji *et al.* Manuscript in preparation

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National Center on Birth Defects and Developmental Disabilities

Division of Blood Disorders