Inhibitor testing: Positive or negative? True or false?

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***With a lot of help from: Geoff Kershaw, Fiona Kwok, Roslyn Bonar**

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Talk outline:

- Overview of factor inhibitors
  - Significance & methodologies
- Quality control issues
  - External QC, assay & result variability, true and false positives and negatives

Types of factor inhibitors:

- Congenital/hereditary haemophilia
  - Develop in response to factor replacement therapy
  - Allo-antibodies (infused factor seen as a foreign protein)
  - Usually develop in Haemophilia A (incidence ~15-30%)
  - Serious complication/can compromise therapy
  - High responders, low responders
  - Risks/causes of inhibitor development not fully understood
    - severe > mild/mod
    - large deletions > inversions > nonsense > missense
    - family history, exposure days, mode of administration
    - MHC phenotype, ability to recognise FVIII as foreign and develop an immune response

Types of factor inhibitors:

- Acquired haemophilia
  - Autoantibodies; develop in individuals without a history of bleeding
  - Usually develop against factor VIII, but can also develop against other factors (eg FV)
  - Rare disease, 1-2 individuals/million persons/year
  - Associated with autoimmune and lymphoproliferative diseases, drug reactions, pregnancy/delivery.
  - In ~50% cases, no obvious underlying disease
  - Most patients are >60yrs of age
  - Patients often present with severe life-threatening bleeds: muscle, epistaxis, haematuria, GIT, intracerebral.
  - Otherwise chance finding (eg prolonged APTT, mixing studies)

Types of factor inhibitors:

- Acquired haemophilia
  - 10-20% mortality rate
  - Spontaneous remission 5-30% (esp. pregnancy related)
  - Complex (type 2) non-linear reaction kinetics
  - Titre can increase with dilution of patient plasma.
  - Hence, Bethesda assay may underestimate potency
  - No correlation between titre/FVIII/bleeding

FVIII inhibitor strength

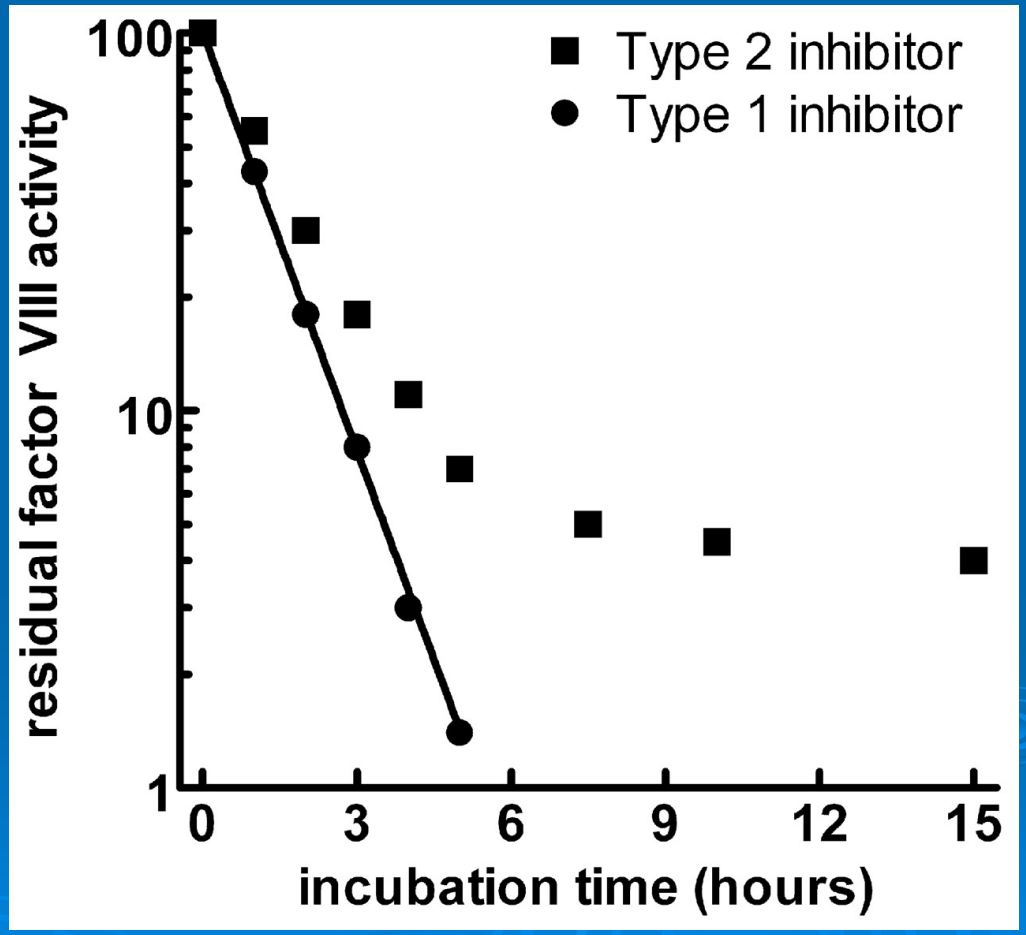
- ≤ 5 Bethesda units = ‘WEAK’ (low responders)
- > 5 Bethesda units = ‘STRONG’ (high responders)

- *Inhibitor strength can influence the approach to therapy*

Kinetics of type 1 and type 2 inhibitors against factor VIII.

- Type 1 inhibitors develop in patients with congenital haemophilia A and are generally alloantibodies that show complete neutralization of FVIII activity.

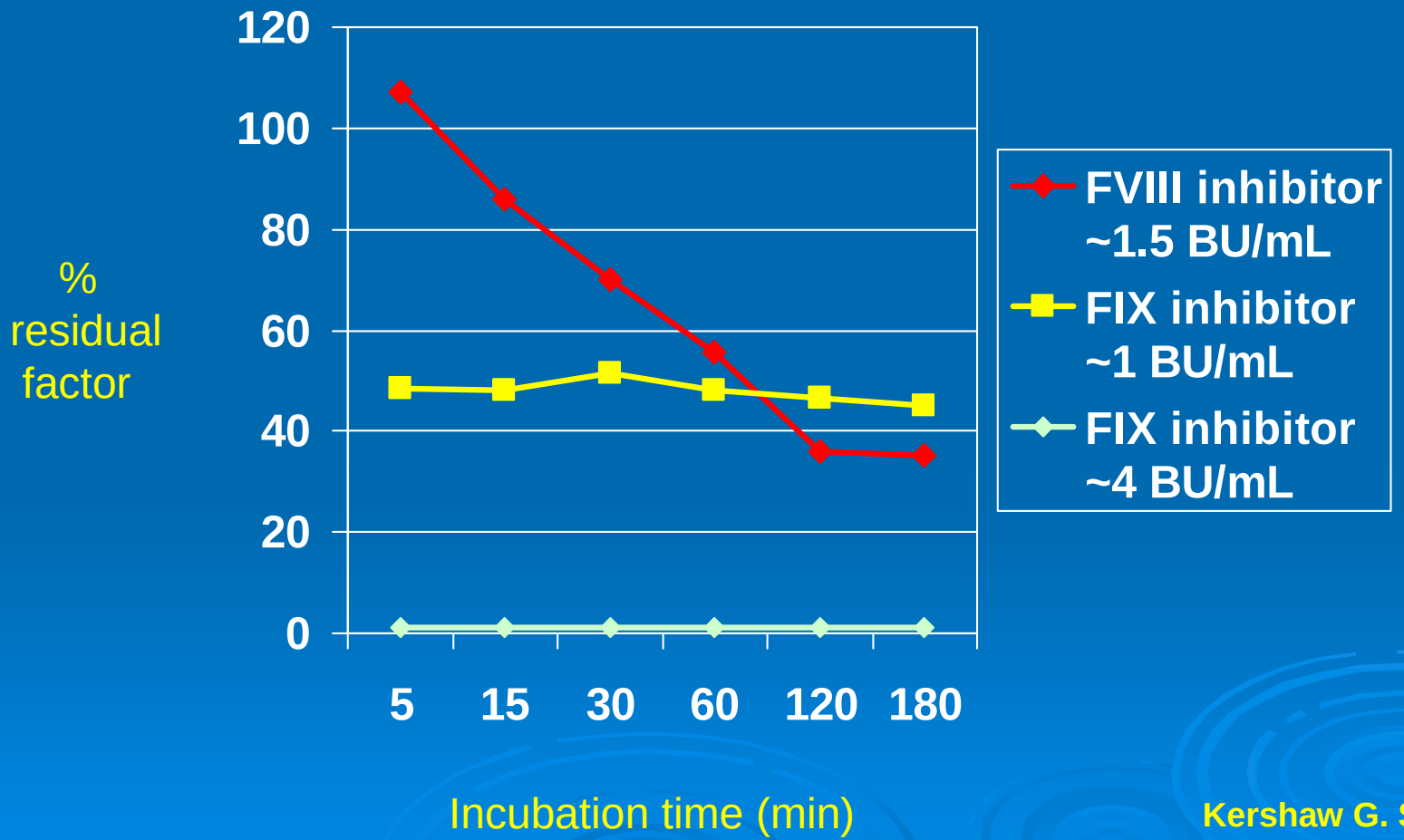
- Acquired inhibitors to FVIII show type 2 kinetics, with a rapid neutralization phase, followed by an equilibrium in which residual FVIII activity can be detected *in vitro*



Ma AD, et al. Hematology ASH 2006;432-437

Fast-acting vs slow-acting inhibitors

1:1 mixes of test plasma : pooled normal incubated at 37℃



Kershaw G. Semin Thromb Hemost, in press

Screening tests

1. PT
2. APTT
3. Fibrinogen
4. Thrombin Time
5. FBC/platelets film
6. MIX test with prolonged incubation
7. Factor assays

Inhibitor Titre (Bethesda) assays

Effect of FVIII inhibitors on APTT mixing tests

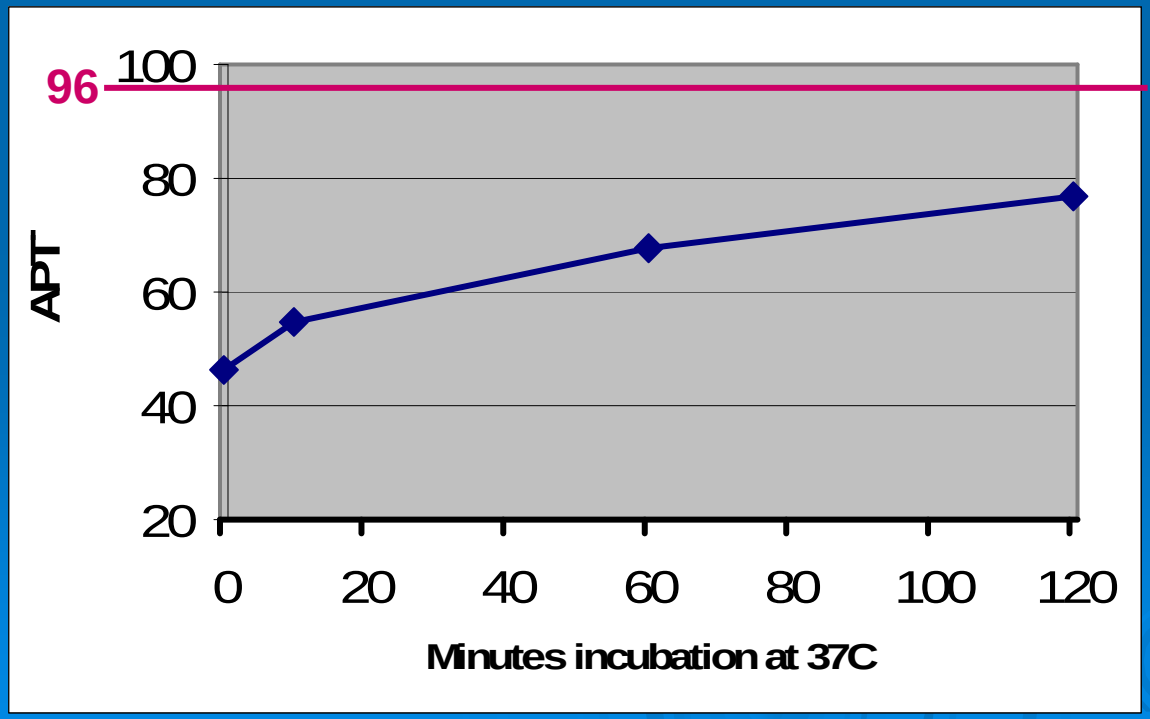
Case example: Titre = 59 BU/mL,

Pool normal APTT = 29 sec

Patient APTT = 96 sec

Immediate mix = 46 sec

2 hour 37ºC mix = 76 sec (still rising)



Kershaw G. Semin Thromb Hemost, in press

Factor inhibitor Assays

- Bethesda assays are the most widely used for quantitation
- Original assay according to Kasper et al, 1975
  - *defined 1 BU as inhibitor strength that reduced FVIII:C in a plasma pool by 50%*
- Nijmegen modification (Verbruggen et al, 1995)
  - *introduced to improve specificity, especially at lower end of activity range*
  - *additional refinements (Verbruggen et al, 2001, 2002)*
- ELISA

Original vs Nijmegen modified Bethesda Assays

1. Normal plasma pool (NPP)

Original assay: - unbuffered

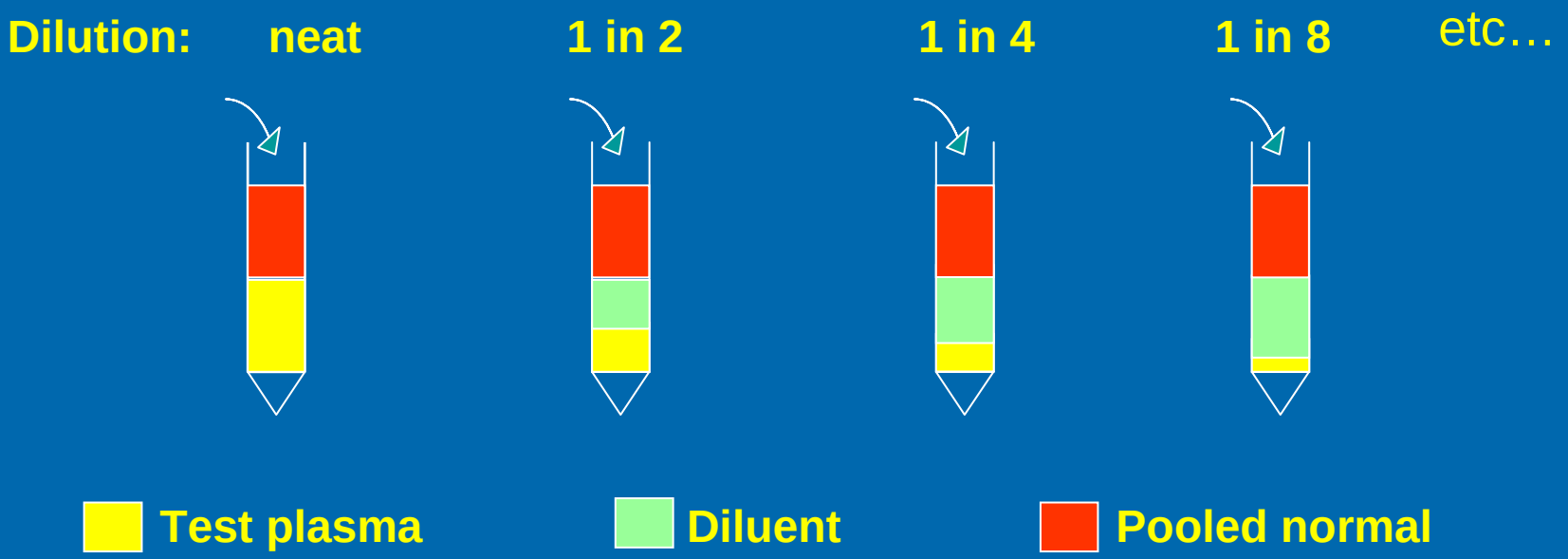
Nijmegen: - buffered to pH 7.4 with 0.1M imidazole / HCl

2. Control mixture

Original assay: - NPP plus equal volume of imidazole buffer of pH 7.4.

Nijmegen: - NPP plus equal volume of FVIII-deficient plasma

Bethesda assays – composition of patient dilutions



	Diluent	Pooled normal
Original assay	Imidazole buffer	Unbuffered
Nijmegen assay	FVIII-deficient plasma	Buffered to pH 7.4

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Bethesda assays – composition ‘control’ mixture

Original Bethesda assay

1 volume unbuffered pooled normal

1 volume imidazole buffer pH 7.4

Nijmegen assay

1 volume buffered pooled normal pH 7.4

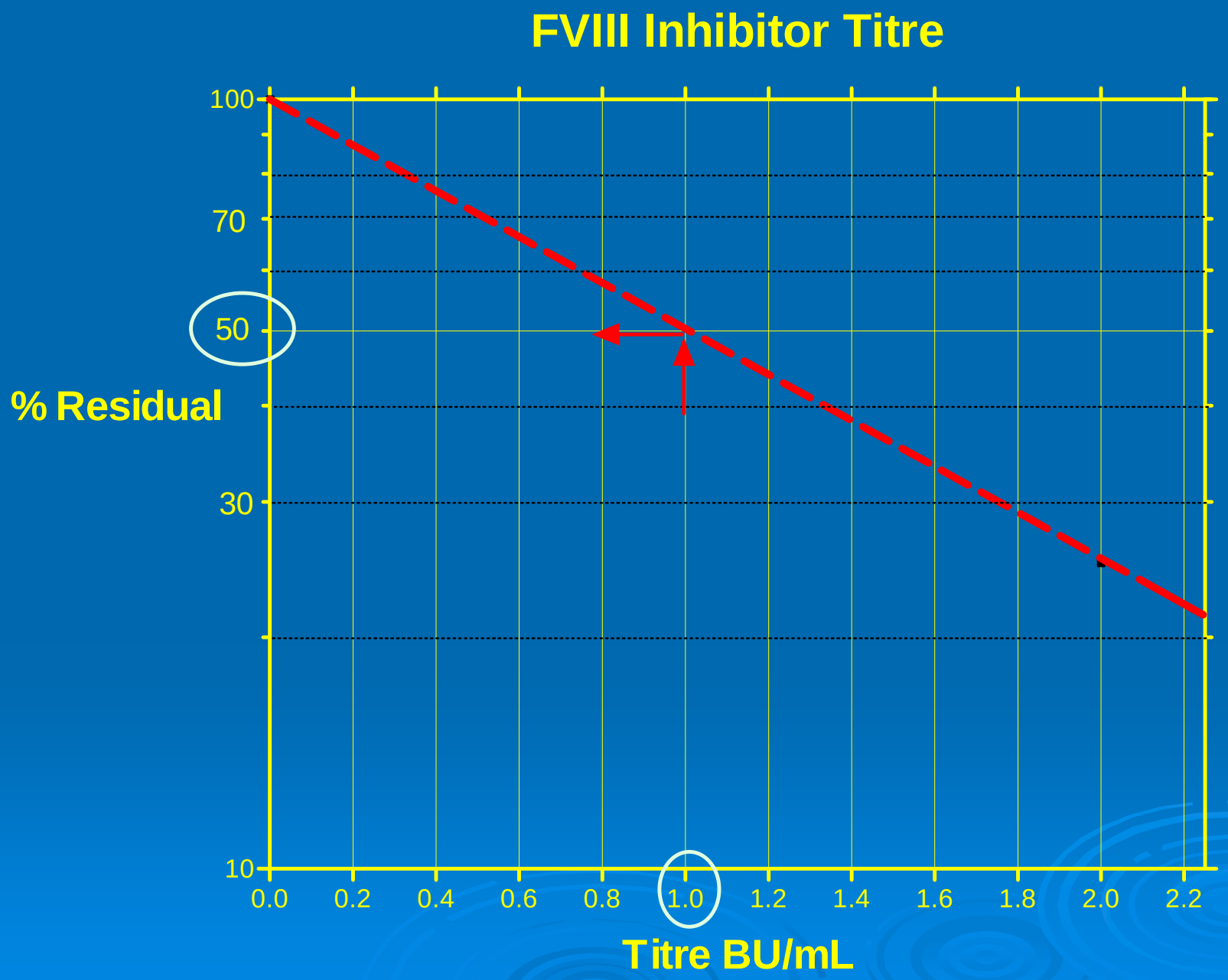
1 volume FVIII deficient plasma (or 4% albumin)

NB: The control mixture has about 50% FVIII:C at the start of the incubation

Kershaw G. Semin Thromb Hemost, in press

FVIII inhibitor method:

1. Test plasma (option: heat inactivated 30 min at 56˚C, hard spun. Removes endogenous Fibrinogen and FVIII:C)
2. NPP (Neijmen: Buffer to pH 7.4 with Imidazole + HCl)
3. 'Control' plasma (NPP + Imidazole (Bethesda) or + FVIII-def. Plasma (Neijmen) using 1:1 mix)
4. Make serial dilutions of test plasma from neat to 1/*X* (Bethesda using Imidazole; Neijmen using FVIII-def. Plasma)
5. Mix equal volumes of test plasma dilutions with (Neijmen: buffered) NPP
6. Incubate all samples at 37 degrees for 2 hours, then measure residual FVIII in the mixture (compared to 'control' plasma representing 100% residual FVIII).
7. The amount of FVIII remaining from the NPP in test samples after 2 hours incubation is inversely proportional to the inhibitor titre.


FVIII Inhibitor Titre
100
70
50
30
10
% Residual
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Titre BU/mL

Comparative inhibitor detection: original vs Neijmen modified Bethesda assay

	n	original	modified
Severe H-A, no Hx inhibitor	10	0.5-0.9	0.0
Mild H-A, no Hx inhibitor	22	0.5-0.9	0.0
H-A with inhib. under treatment	6	0.5-1.9	0.6-1.7
H-A with strong inhibitor	7	13-315	10-320

Verbruggen et al. Thromb Haemost 1995; 73:247-51

Bethesda assays vs ELISA

Bethesda assays detect antibodies that inhibit FVIII function

ELISAs (immunoassays) may detect both inhibitory and non-inhibitory antibodies.

Examples:

A. 8 of 21 (38%) –ve by Bethesda were +ve by ELISA (BJH 1996; 93:688-93)

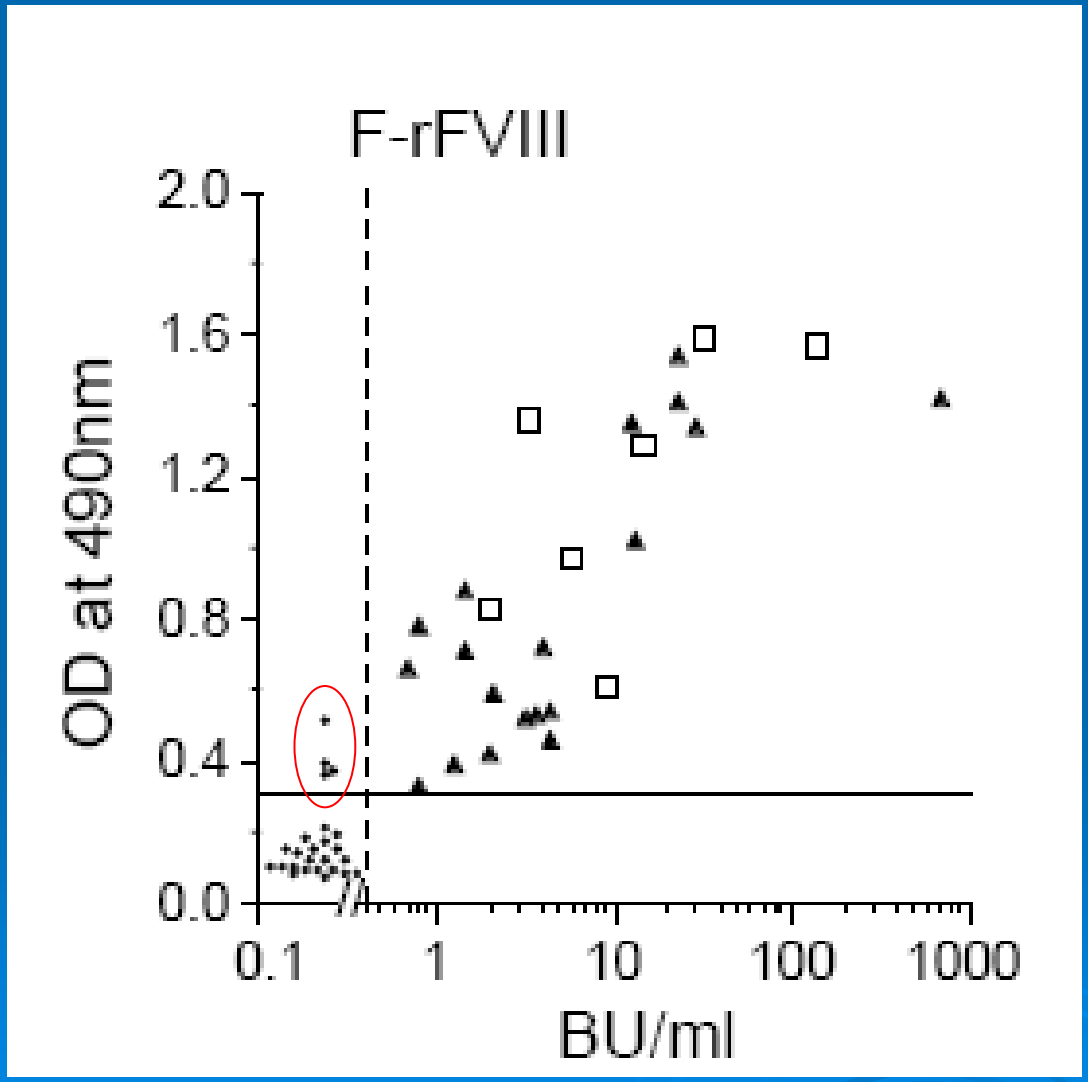
B. 4 of 26 (15%) -ve by Bethesda were +ve by ELISA (JTH 2003 1:2548-53)

?false positives by ELISA or true negatives by Bethesda

Bethesda assays vs ELISA

- Dazzi et al; BJH 1996; 93:688-93: 8 of 21 (38%) –ve by Bethesda were +ve by ELISA
  - Most considered ‘specific’ using pre-incubation with rFVIII to abrogate ELISA binding (ie lower or normal OD obtained using test serum post rFVIII pre-dilution).

Correlation between ELISA and Bethesda assays for inhibitor detection



Ling et al, JTH (2003) 1:2548-53

- □ Acquired Haemophilia A, n=7
- ▲ Hereditary Haemophilia A with positive Bethesda assay, n=19
- ● Hereditary Haemophilia A with negative Bethesda assay, n=26

4 of 26 samples –ve by Bethesda were +ve by ELISA. These 4 did not have clinical suspicion of FVIII inhibitor

Case Study: Weak inhibitor

Sample Dilution	% Residual FVIII	Titre BU/mL
Neat	3	
1/2	22	
1/5	**69**	**0.54x 5 = 2.7**
1/10	86	

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Case Study: Strong inhibitor

Sample Dilution	% Residual FVIII	Titre BU/mL
Neat	5	
1 / 2	9	
1 / 5	19	
1 / 10	26	
1 / 20	35	
1 / 50	44	1.18 x 50 = 59
1 / 100	65	

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Case Study: Auto-anti FVIII, Complex kinetics

Sample Dilution	% Residual = (B/A)*100	Titre BU/mL
Neat	36	
1 / 2	44	1.2 x 2 = 2.4
1 / 5	48	1.1 x 5 = 5.5
1 / 10	50	1.0 x 10 = 10.0
1 / 20	52	.95 x 20 = 19
1 / 50	56	1.18x50 = 59

Kershaw G. Semin Thromb Hemost, in press

Acquired haemophilia – APTT does not predict inhibitor titre

(example of 6 cases of FVIII inhibitors in non-haemophiliac patients)

ID	Inhibitor titre at diagnosis (BU/mL)	APTT (25-37sec)	%FVIII (70-220)
1	8.4	68	3
2	103	104*	<1
3	96	96	<1
4	21	83**	2
5	4.1	108*	4
6	172	81**	3

N. Viiala (2008), submitted

Quality control for FVIII inhibitor assays

Internal

1. Minimum: Factor assay QC as in routine factor assays
2. Optimum: Positive control with each assay if available

External

RCPA (Australia)

ECAT (Netherlands)

RCPA QAP factor inhibitor exercise 2005

- 8 samples comprising 2 true positives and 6 ‘others’
- Intent was to be intellectually challenging & provide true factor inhibitor samples plus samples reflecting potential sample collection/processing artifacts that might otherwise give rise to false inhibitor identification.
- Perceived degree of difficulty per sample varied.
- Easy: LA positive and ‘Vit-K/OAT-like’ (common).
- Moderate: Heparin contaminated normal sample, aged normal plasma, normal serum (each not uncommon).
- Challenging: FV inhibitor (rare); FVIII inhibitor (common, but sample was defibrinogenated).
- Difficult: EDTA normal sample (very rare).

Favaloro EJ, et al. Thromb Haemost, 2006; 96:73-78.
Favaloro EJ, et al. Pathology, 2007; 39:504-511.

RCPA QAP factor inhibitor exercise 2005

- Factor inhibitor exercise 2005
  - True factor inhibitor samples were correctly identified by 63% (FV) and 46% (FVIII) participants, but reported (detected) inhibitor levels varied widely.
  - Most laboratories correctly identified: Heparin contaminated sample (68%), normal (aged) plasma (87%), normal serum sample (90%), positive LA (98%), 'Vit-k/OAT' (93%).
  - Only one laboratory correctly identified the EDTA sample.

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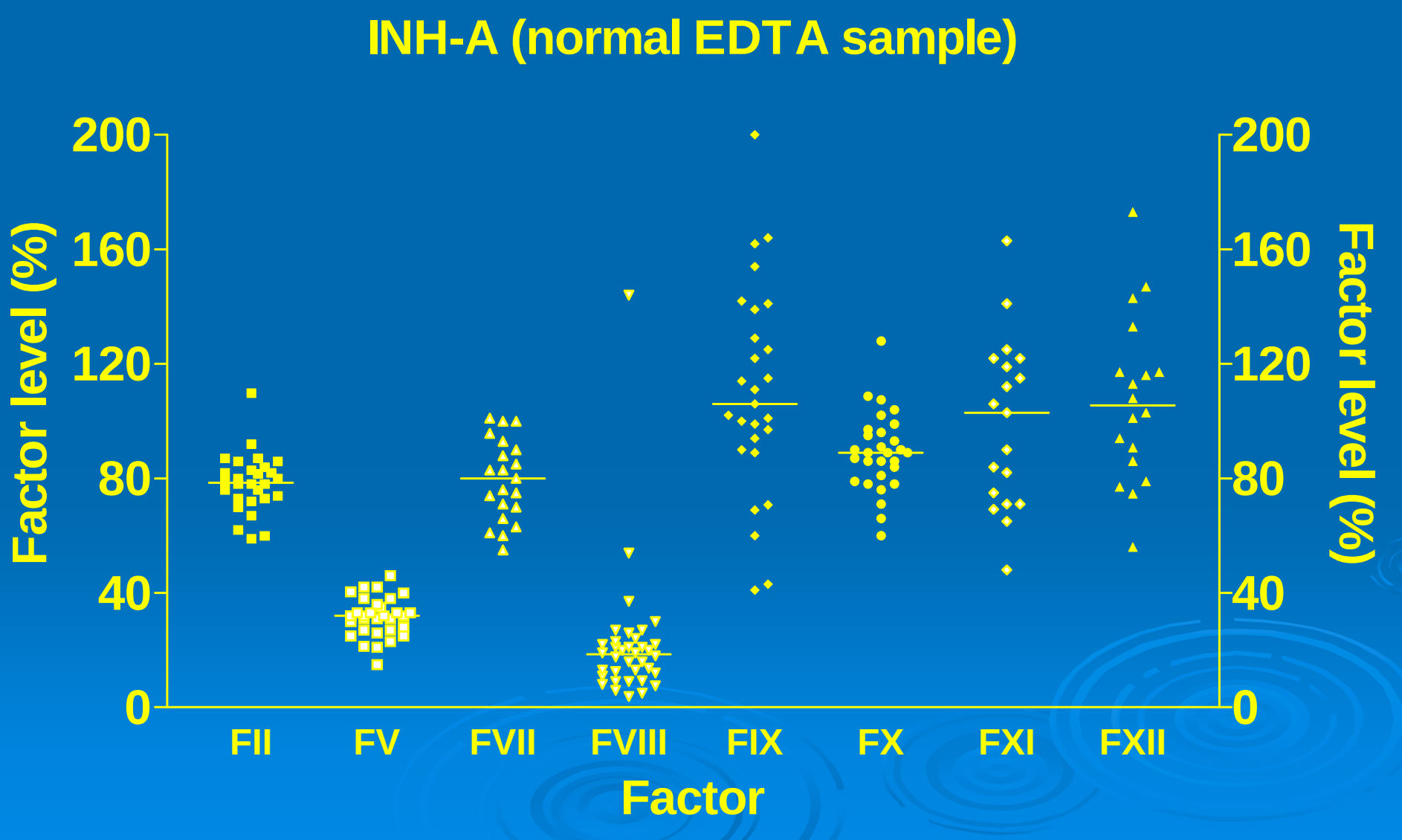
RCPA QAP factor inhibitor exercise 2005



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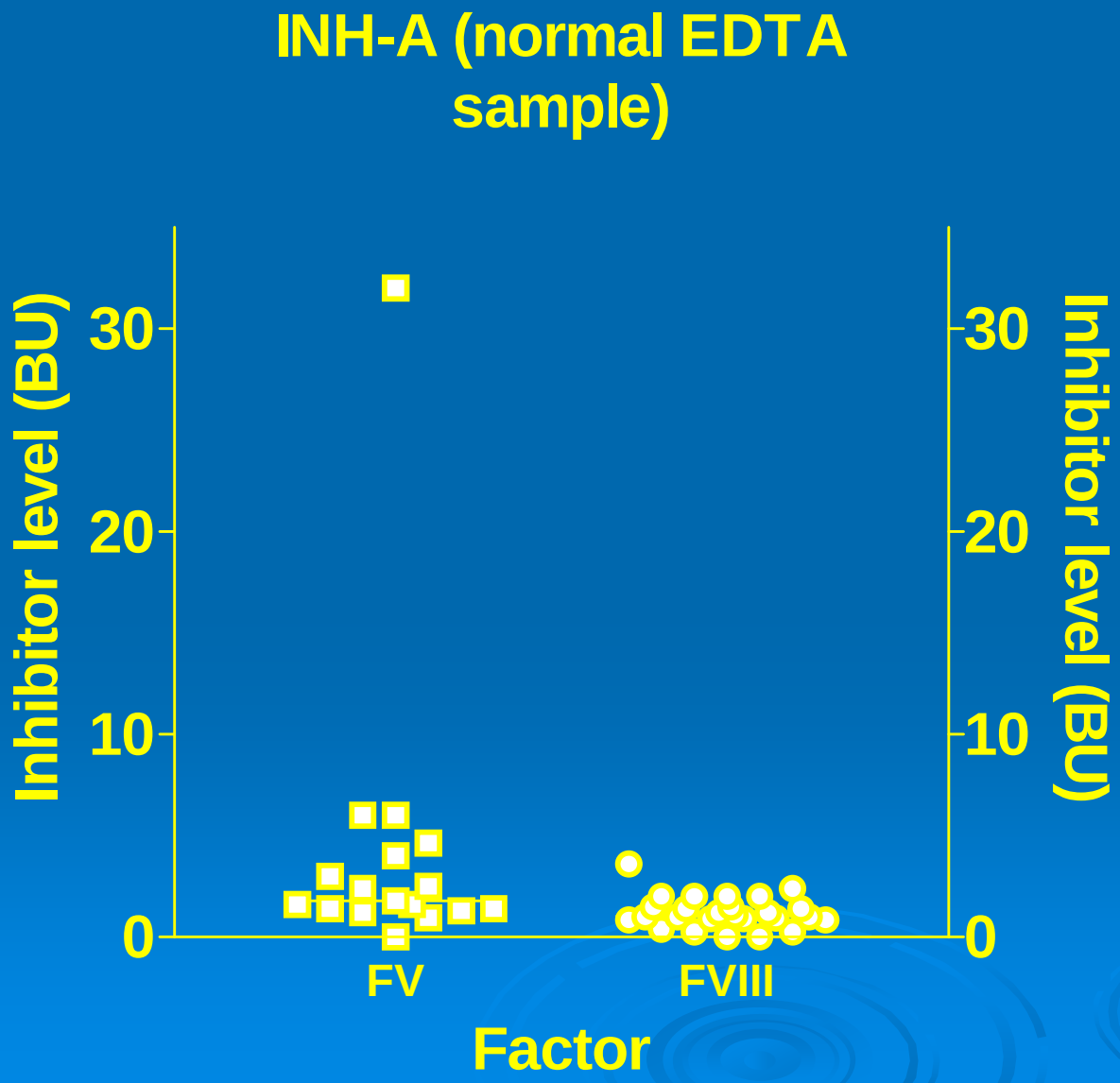

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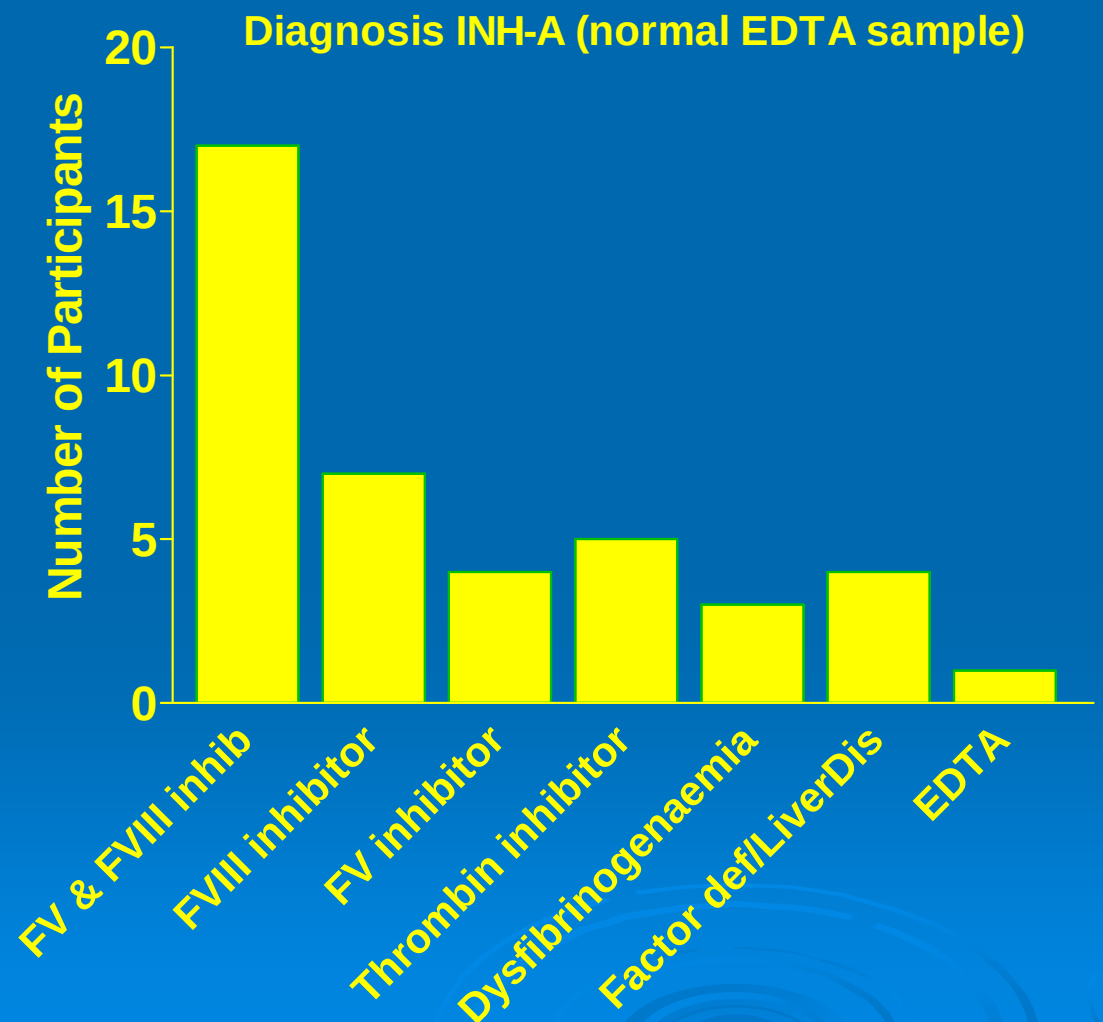

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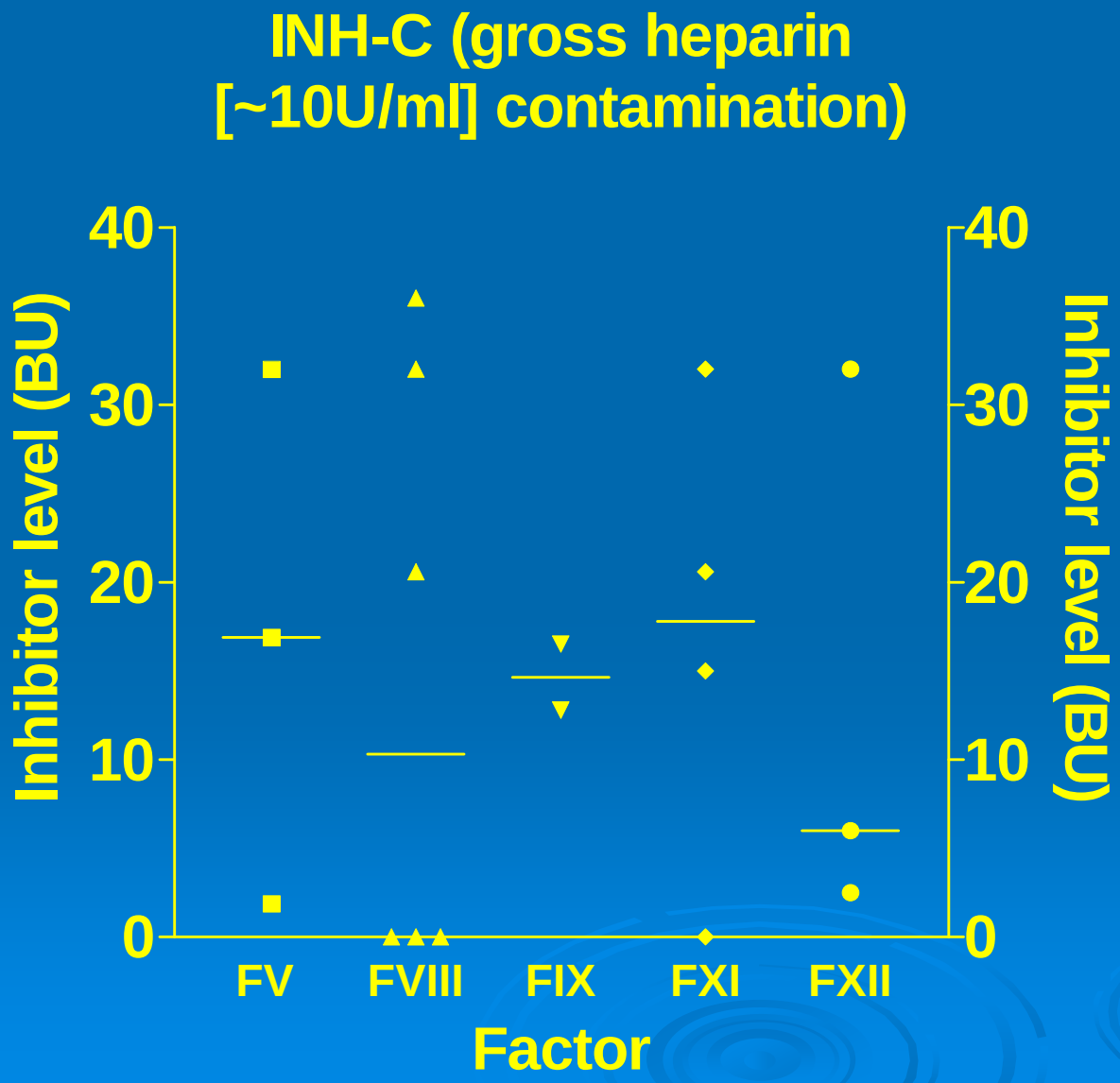

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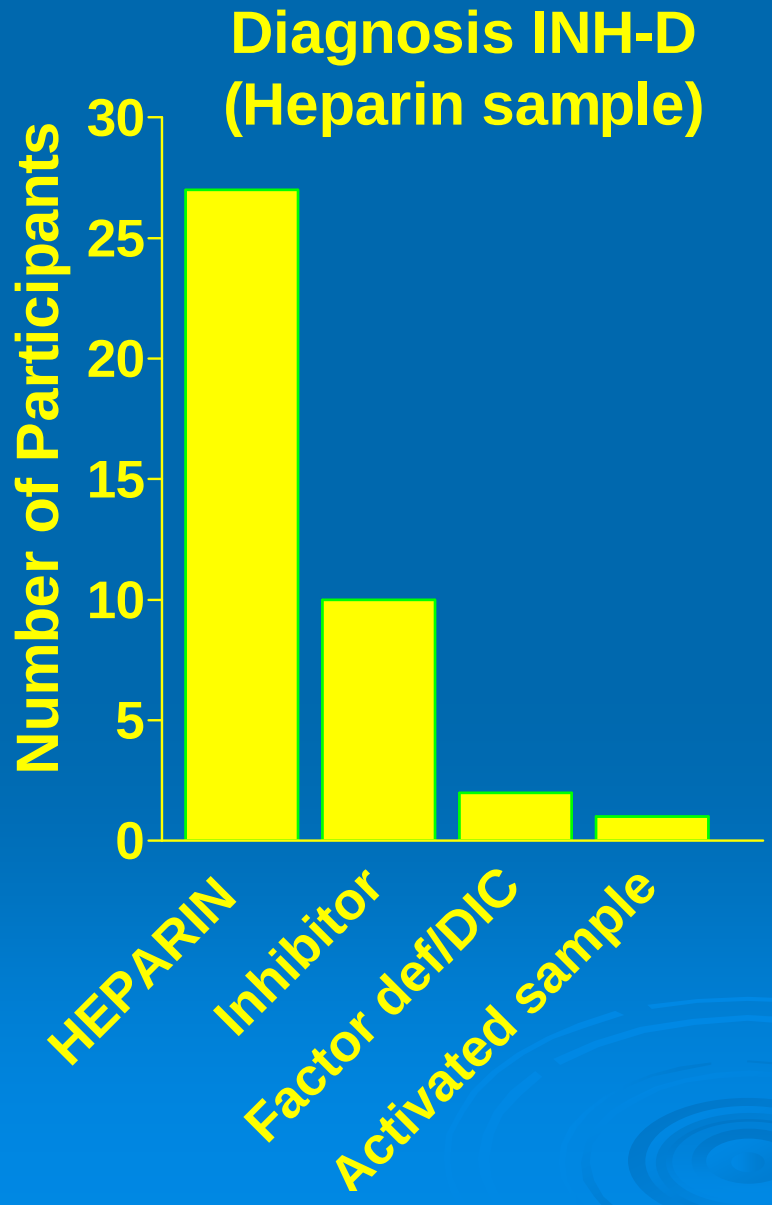

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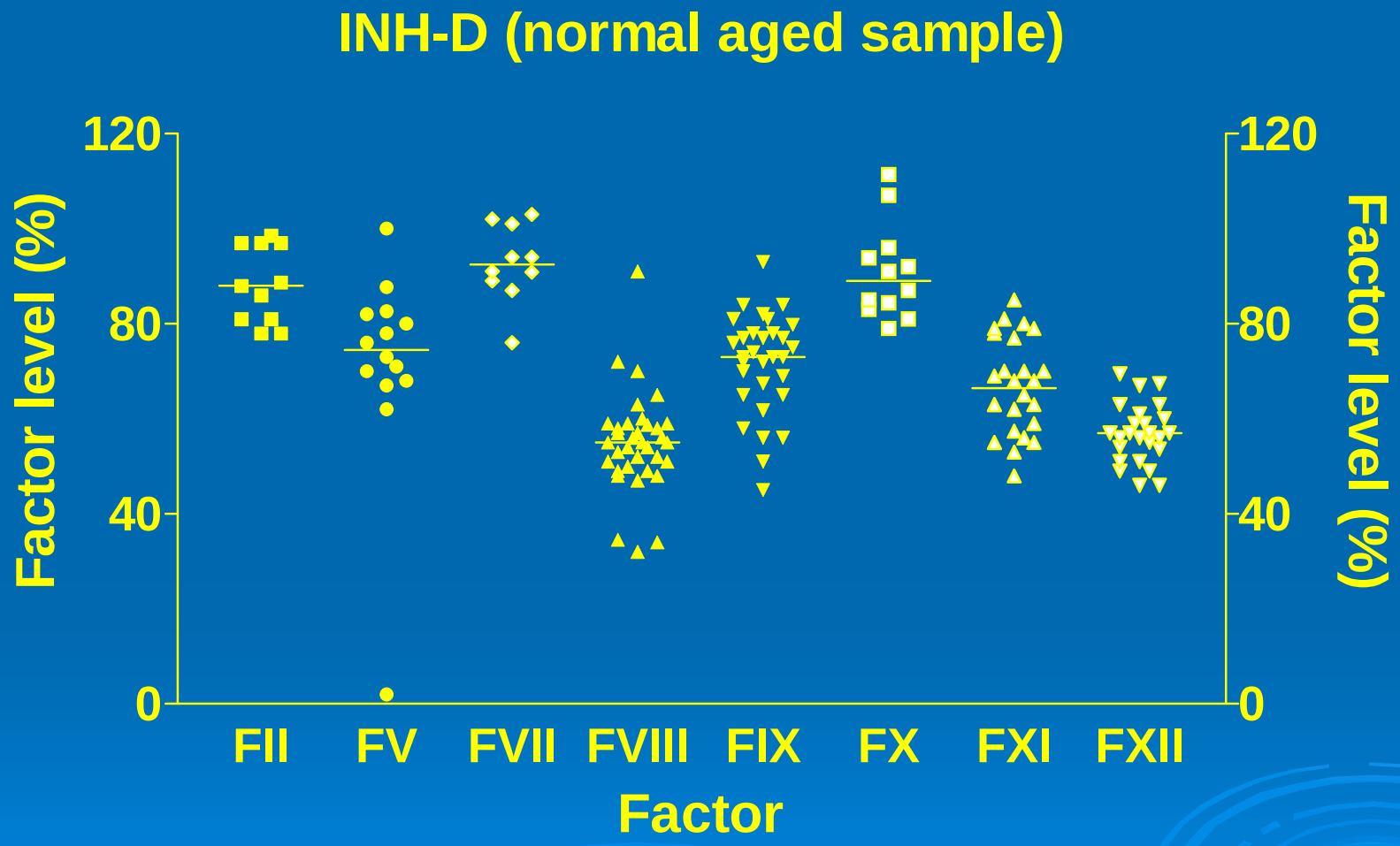

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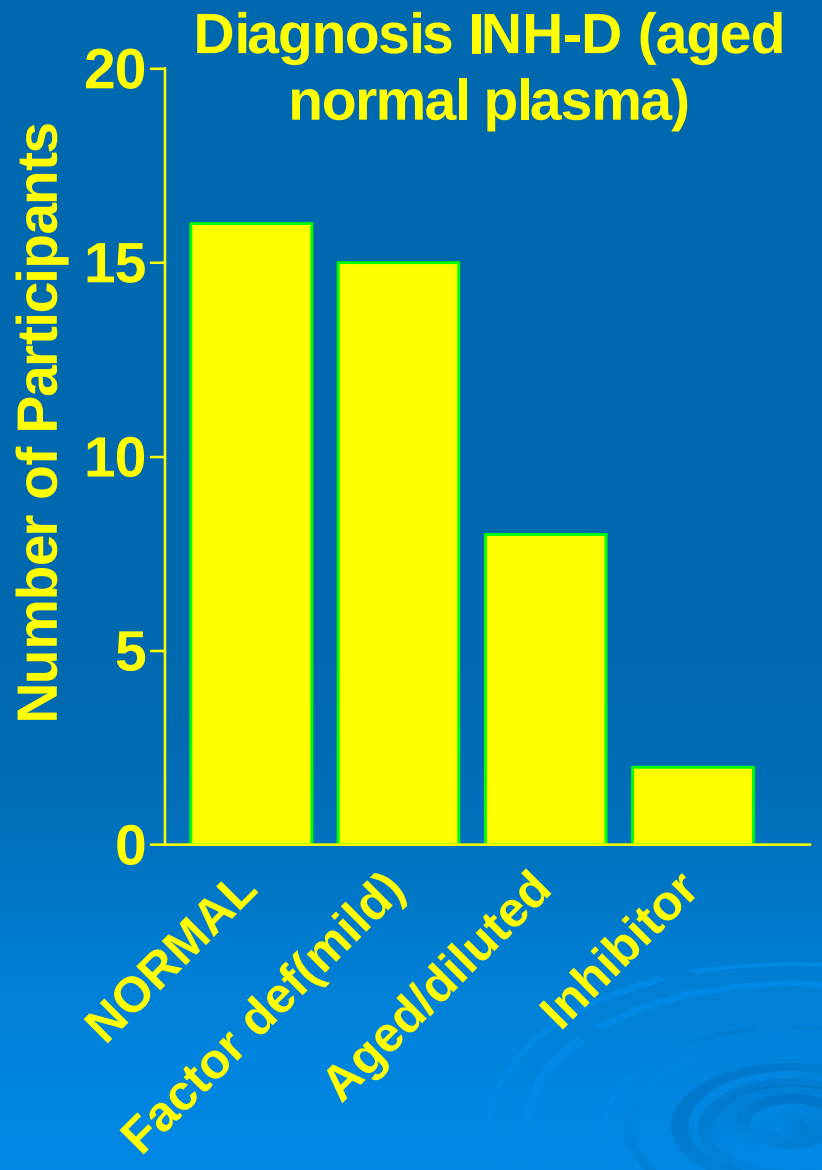

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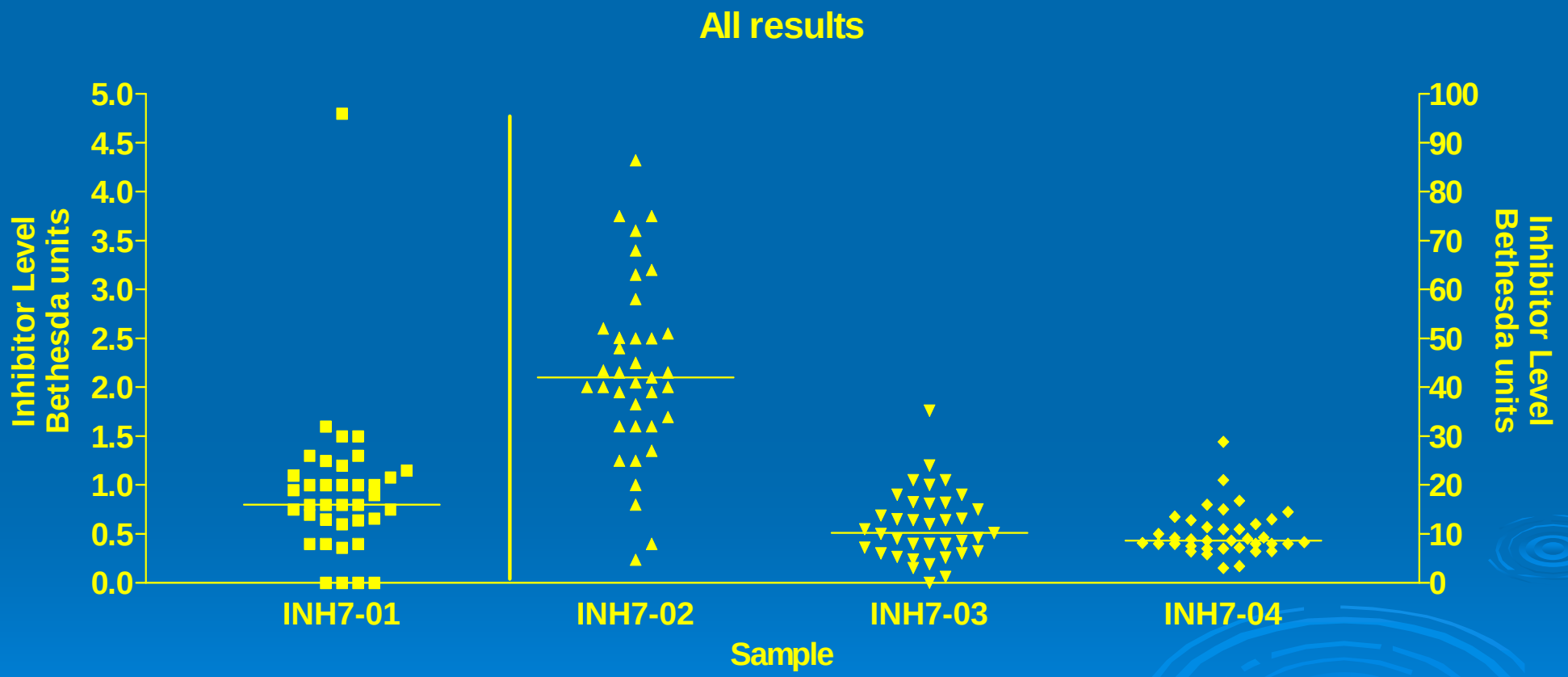
RCPA QAP factor inhibitor exercise 2005

- Conclusions:
  - Laboratories usually get it right, but sometimes get it ‘wrong’.
  - False positives & false negatives.
  - Even if they get it ‘right’, wide variation in detected inhibitor levels.
  - More common presentations more often correctly identified.
  - No laboratory correctly identified all samples (the lab that correctly identified the EDTA sample, also correctly identified all other samples except the ‘Vit-k/OAT’ sample - identified FX deficiency).
  - Occasionally, laboratories ‘see’ factor inhibitors when no such inhibitor exists.

Favaloro EJ, et al. Thromb Haemost, 2006; 96:73-78.
Favaloro EJ, et al. Pathology, 2007; 39:504-511.

RCPA QAP FVIII Inhibitor Exercise 2007



Source: RCPA Haematology QAP Inhibitor Exercise Part 2 report

External QAP - FVIII Inhibitor titres

RCPA-QAP; n=~35

2007 INH-	Titre Median BU/mL	CV %
1	0.8	**84**
2	42	**43**
3	10.2	**61**
4	8.6	**48**

Compare with ~CV of:

VWF:Ag (10%)

VWF:CB (20%)

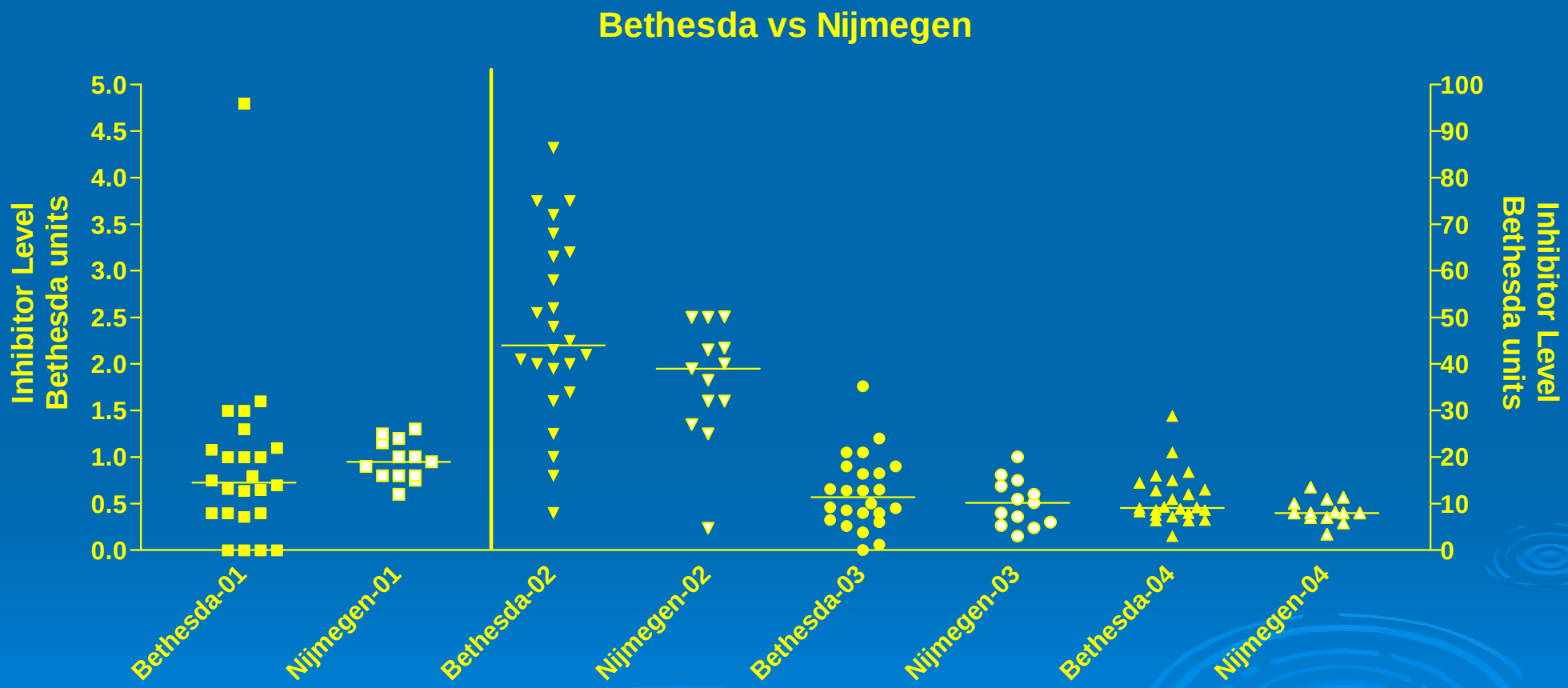
VWF:RCo (20%)

Factor assays: (15%)

Protein C: (6%)

Hb: (1.2%)

RCPA QAP FVIII Inhibitor Exercise 2007



Source: RCPA Haematology QAP Inhibitor Exercise Part 2 report

External QAP - FVIII Inhibitor titres

ECAT

sample	Titre (Mean BU/mL)	CV %	CV % (Bethesda vs Neijmen)
06.31	1.3	**41.8**	**43.4 v 28.6 ****
06.64	1.7	**51.5**	**53.4 v 45.0 ****
07.31	1.0	**44.6**	**47.0 v 40.0 ****
07.32	0.5	**67.9**	**67.2 v 68.3**
07.65	1.3	**36.1**	**35.9 v 37.8**
07.66	3.1	**38.3**	**35.9 v 45.0**
08.33	3.0	**28.2**	**28.5 v 25.0 ****
08.34	1.3	**38.5**	**41.0 v 28.8 ****

Sources of variation in inhibitor titre assays

- Type of assay: Nijmegen vs Original assay vs other
- Type of FVIII deficient plasma used: (haemophiliac/immunodepleted; + / - VWF)
- Source of pooled normal plasma: (in-house normal donors; patient donors; FFP; commercial pool)
- Level of FVIII in pooled normal plasma
- Pre-treatment of sample & presence/absence of FVIII in sample
- Normal assay variables of 1-stage FVIII assay (calibrator, APTT reagent, def. plasma, citrate conc. 3.2 v 3.8%)

2005 ECAT survey : TYPE OF NORMAL PLASMA USED

	Home-made Non-buffered	Home-made Buffered	Commercial Non-buffered	Commercial buffered
Bethesda assay, n=93	24	9	15	44
Nijmegen assay, n=30	3	8	2	17
Other n=12	1	3	1	7

Values given are numbers of participants

58% of Bethesda assay users indicated they used buffered normal pooled plasma!!

Thank you:

ECAT – Piet Meijer
RCPA QAP – Roslyn Bonar
RPA – Geoff Kershaw
Westmead – Fiona Kwok