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Affinity of Anti-Drug Antibodies in human plasma is a potential biomarker for immunogenicity assessment of therapeutic proteins: lessons learned from FVIII replacement therapy

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Outline

- **Introduction**
  - Severe Hemophilia A & FVIII-specific antibody responses
  - Model for immune regulation of FVIII-specific antibody responses
- **Affinity assessment of FVIII-specific antibodies**
  - Methodological approach & Method validation
  - Affinity of FVIII-specific antibodies in healthy individuals and severe hemophilia A patients
- **Longitudinal monitoring of FVIII-binding antibodies**
  - Proof of principle data
  - Hemophilia Inhibitor PUP Study
- **Take home messages**

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Severe hemophilia A hard facts

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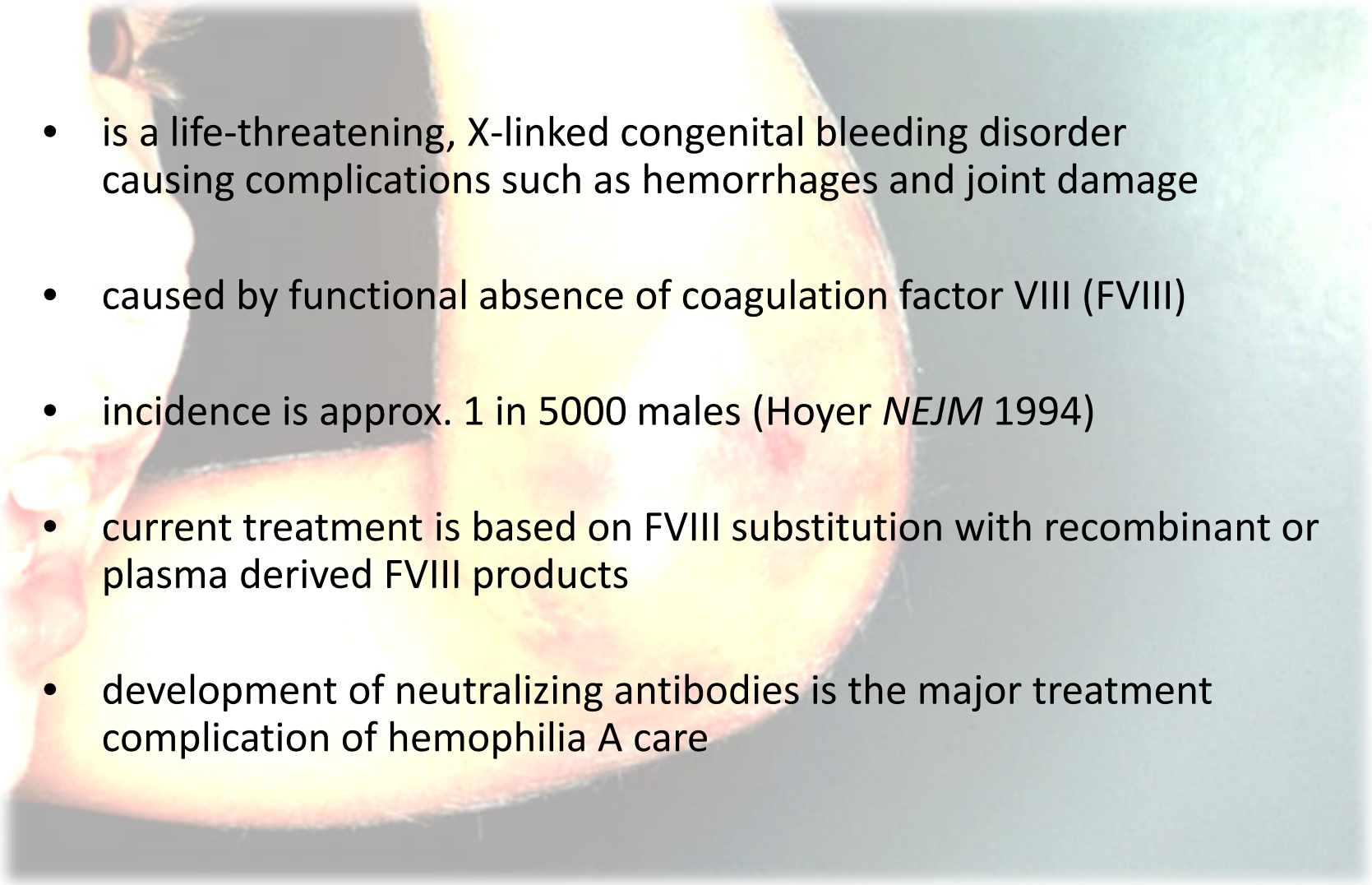


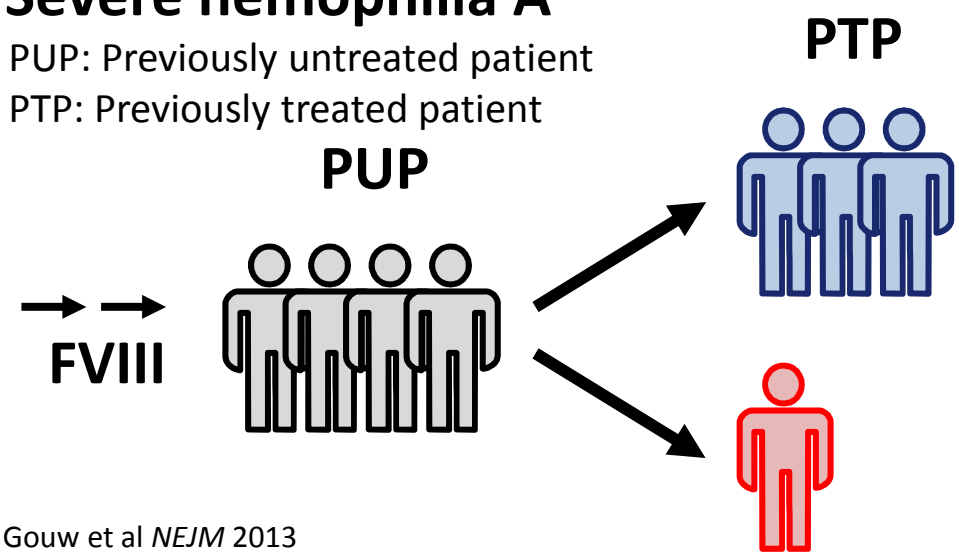
- is a life-threatening, X-linked congenital bleeding disorder causing complications such as hemorrhages and joint damage
- caused by functional absence of coagulation factor VIII (FVIII)
- incidence is approx. 1 in 5000 males (Hoyer *NEJM* 1994)
- current treatment is based on FVIII substitution with recombinant or plasma derived FVIII products
- development of neutralizing antibodies is the major treatment complication of hemophilia A care

Prevalence of FVIII neutralizing antibodies

Severe hemophilia A

PUP: Previously untreated patient

PTP: Previously treated patient

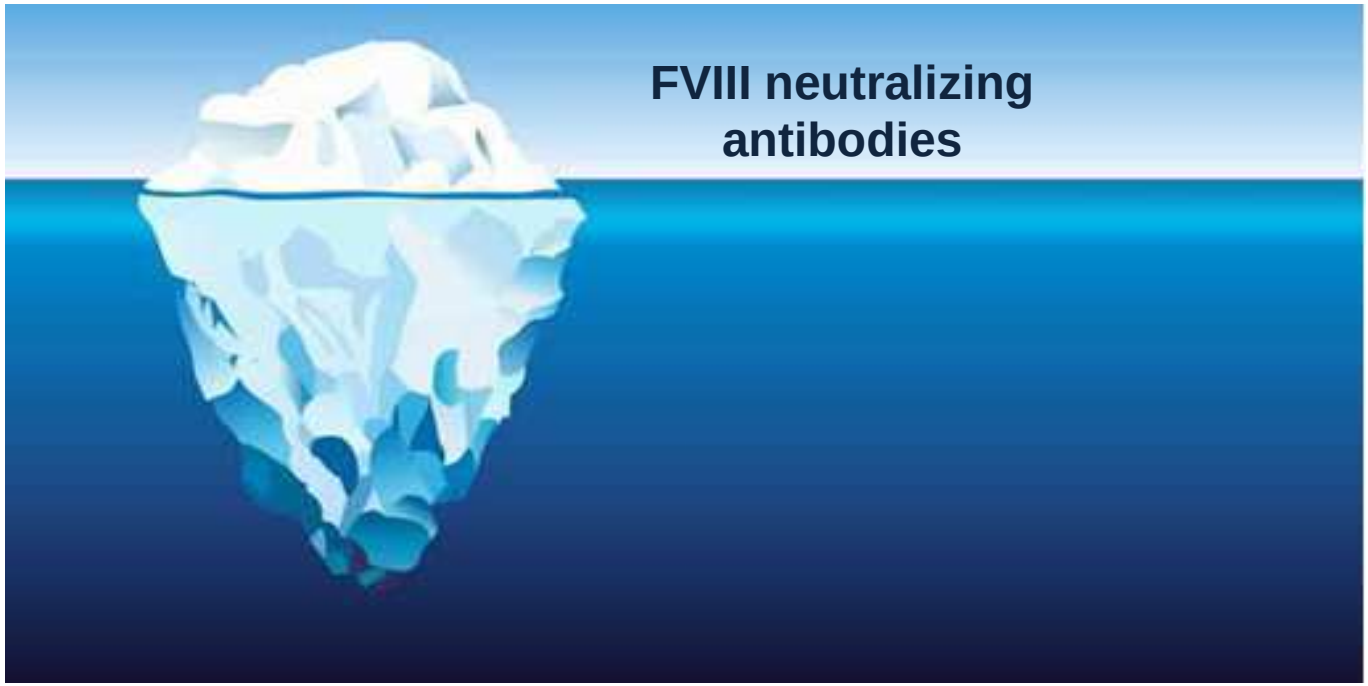


Gouw et al *NEJM* 2013

without FVIII neutralizing antibodies Approx. 70%

with FVIII neutralizing antibodies Approx. 30%

FVIII neutralizing antibodies are only the tip of the iceberg



Reipert B. Hematology, Am Soc Hematol Educ Program. 2014

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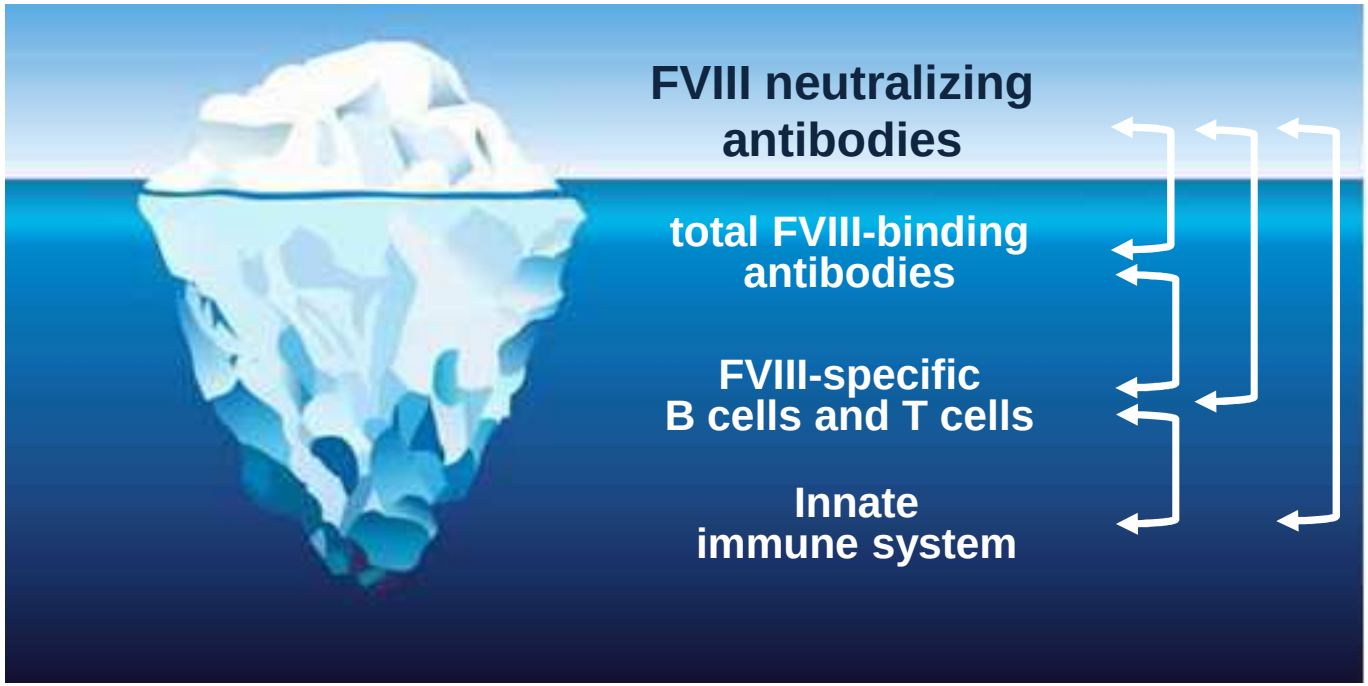

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Immunogenicity assessment of FVIII

FVIII-binding antibodies (ADA)

neutralizing antibodies
portion of binding antibodies that does neutralize FVIII biologic function

non-neutralizing antibodies
portion of binding antibodies that does not neutralize FVIII biologic function

Immunogenicity assessment of FVIII

FVIII-binding antibodies (ADA)

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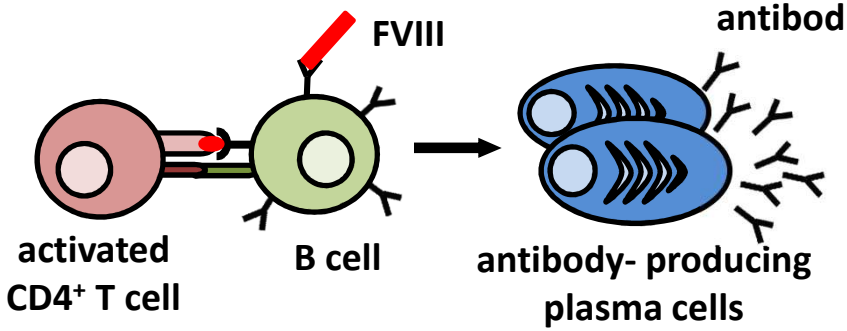
portion of binding antibodies that does not neutralize FVIII biologic function

Question in need to be addressed:

What is the difference between non-neutralizing antibodies found in healthy population and neutralizing antibodies found in patients?

Immune regulation of FVIII-specific antibody responses

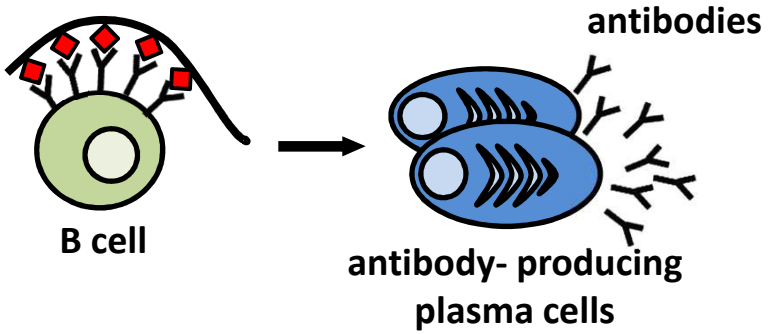
CD4⁺ T-cell dependent induction of antibodies



Affinity maturation: ++++

IgM ⟶ IgG (IgG1-4), IgA

CD4⁺ T-cell independent induction of antibodies



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Reipert et al
Current and Future Issues in Hemophilia Care
2011

Immune regulation of FVIII-specific antibody responses

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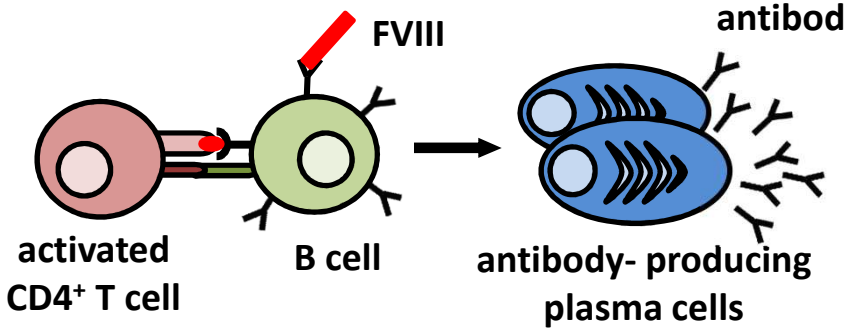

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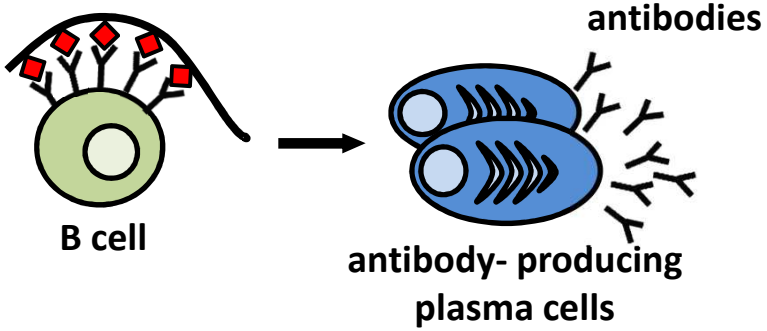

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Experimental approach:

- Detection of FVIII neutralizing antibodies (Bethesda Assay)
- **Ig isotype and IgG subclass determination of FVIII-specific antibodies (ELISA)**
- Affinity determination of FVIII-specific antibodies (Affinity ELISA)

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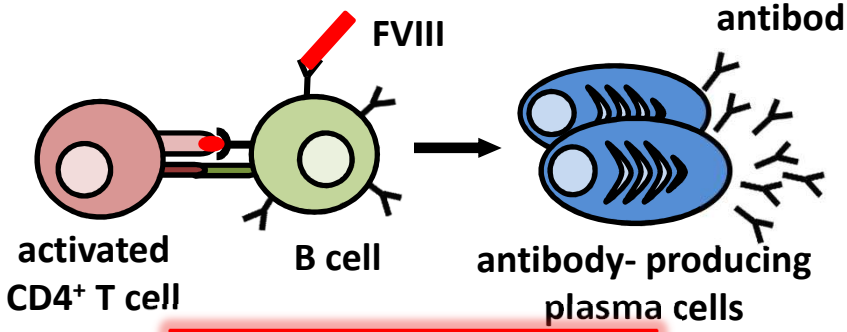

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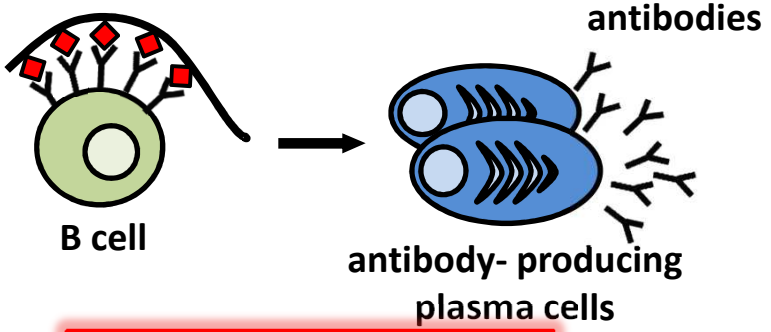

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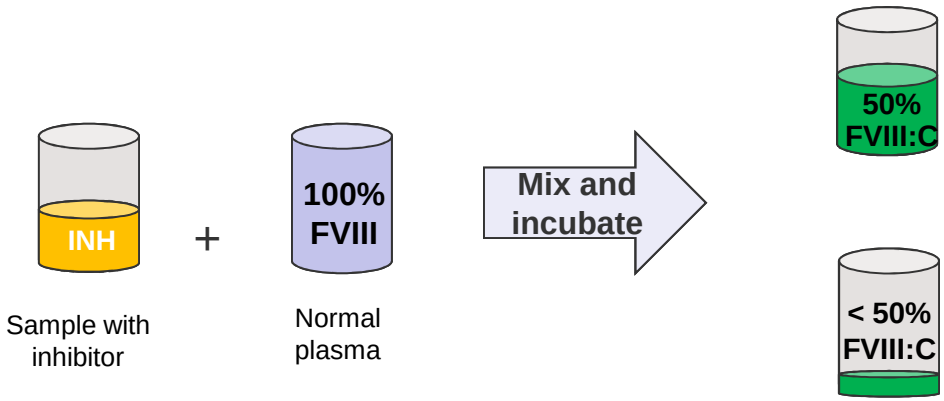
Test principle: Bethesda assay

Patient plasma (concentrated and diluted)
Mixed in 1:1 with normal human plasma (NHP)

→ incubated at 37℃ /2h

→ activity measured and compared to a control *(NHP mixed in 1:1 with buffer)*



No neutralizing antibody
→ 50 % FVIII activity measured

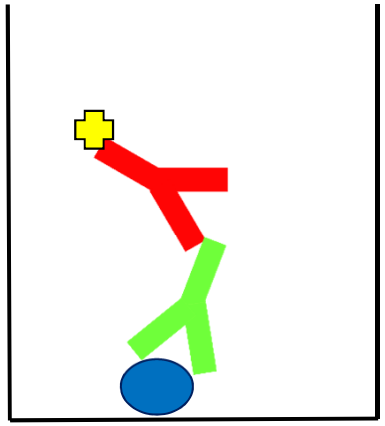
Neutralizing antibody present in the patient plasma → inhibition of FVIII function of NHP, thus resulting in decreased FVIII activity

Quantity of neutralizing antibody is expressed as BU/ml

1 BU/ml = the amount of the inhibitor which decreases the FVIII activity to 50 % in the assay mixture compared to the control sample

Methodological approach: Anti-FVIII IgM, IgA, IgG1-4 direct binding ELISA

1) Screening assay



1) Coating of ELISA plate with FVIII

2) Incubate plasma sample containing FVIII-binding antibodies

3) Detect FVIII-binding antibodies using enzyme-conjugated secondary antibody

Whelan et al *Blood* 2013

Methodological approach: Anti-FVIII IgM, IgA, IgG1-4 direct binding ELISA

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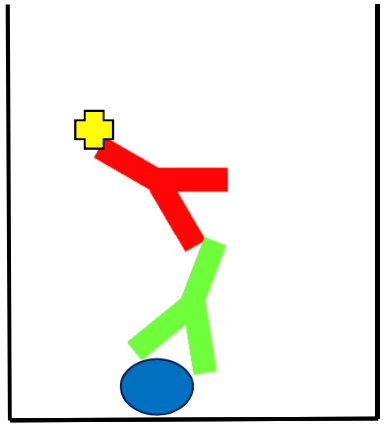
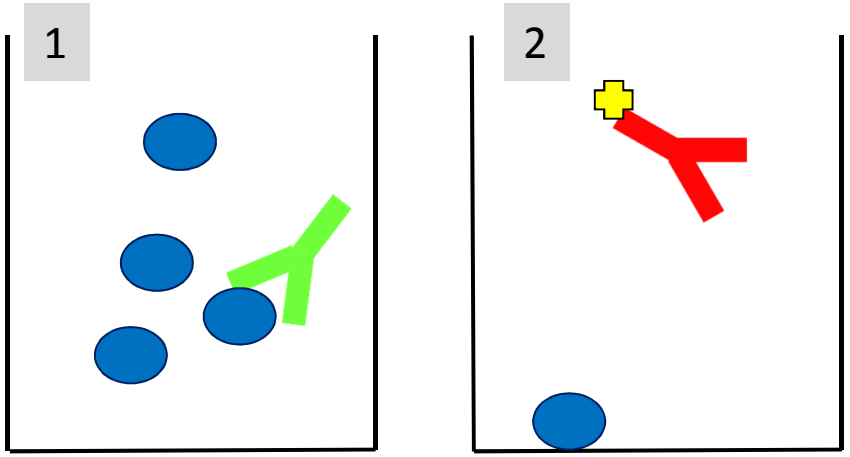


1) Coating of ELISA plate with FVIII
2) Incubate plasma sample containing FVIII-binding antibodies
3) Detect FVIII-binding antibodies using enzyme-conjugated secondary antibody

2) Confirmatory assay



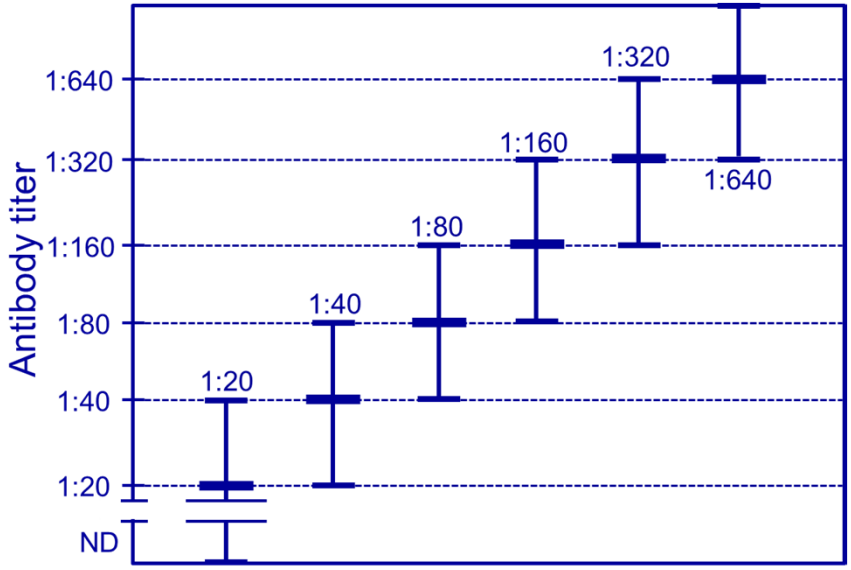
1) Pre-incubation of plasma sample with FVIII
2) Apply pre-incubated sample to screening assay

→ FVIII-antibody complex is not detectable in screening resulting in a lower signal in the ELISA assay

Whelan et al *Blood* 2013

Confirmatory assay

Validation demonstrated a variability of the ELISA assay platform of ± 1titer step.
Therefore, differences ≤ 2 titers steps may be due to variability.



Antibody titer needs to be decreased by at least three titer steps after competition to confirm specificity of the ELISA assay

Whelan et al.
Blood 2013

Methodological approach: Anti-FVIII Affinity ELISA

Analysis of the affinity of antibodies against FVIII in human plasma

Assay specificities:

- no need for IgG isolation → artefacts (McMahon *JI Methods* 2010)
- low plasma sample consumption
- Individual assessment of Ig isotypes and IgG subclasses
- quantitative measurement of apparent affinity
- address polyclonality of human plasma

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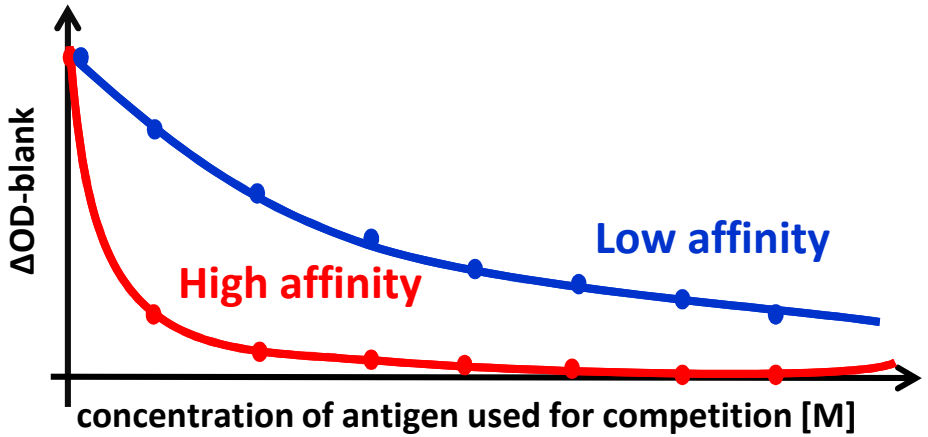
Affinity ELISA

- competition ELISA with multiple concentrations of FVIII
- can detect up to two affinity populations of FVIII-specific antibodies



Bobrovnik et al J. Mol. Recognit. 2010

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Affinity of FVIII-specific antibodies reveals major differences between neutralizing and non-neutralizing antibodies

Christoph J. Hofbauer[1], Shawn F.J. Whelan[1], Maria Hirschler[1], Peter Allacher[1], Frank M. Horling[1], John-Philip Lawo[1], Johannes Oldenburg[2], Andreas Tiede[3], Christoph Male[4], Jerzy Windyga[5], Andreas Greinacher[6], Paul N. Knöbl[7], Gerald Schrenk[1], Jadranka Koehn[1], Friedrich Scheiflinger[1], and Birgit M. Reipert[1],*

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4 Department of Paediatrics, Medical University of Vienna, Vienna, Austria;
5 Institute of Hematology and Transfusion Medicine, Warsaw, Poland;
6 Institute for Immunology and Transfusion Medicine, Greifswald Medical School, Greifswald, Germany;
7 Department of Internal Medicine I, Medical University of Vienna, Vienna, Austria

Validation plan for Anti-FVIII Affinity ELISA

- **3 representative samples:**
  - ○ anti-FVIII IgG1 MAb in negative control plasma
  - △ anti-FVIII IgG1 positive plasma from healthy donor
  - ◇ Mix of A and B

- **total IgG Intra-Assay variability**
  - 6 replicates within 1 run
- **total IgG Inter-Assay variability**
  - 6 individual runs (3 operators on 2 days)
- **total IgG robustness towards freeze/thaw cycles**
  - Standard sample + 5 additional freeze/thaw cycles
- **total IgG vs. IgG1 Intra-Assay stability**

Acceptance criteria: Coefficient of Variation (CV) < 25%

- **Comparison of different technology platforms using anti-FVIII IgG1 MAb**

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Validation of anti-FVIII Affinity ELISA

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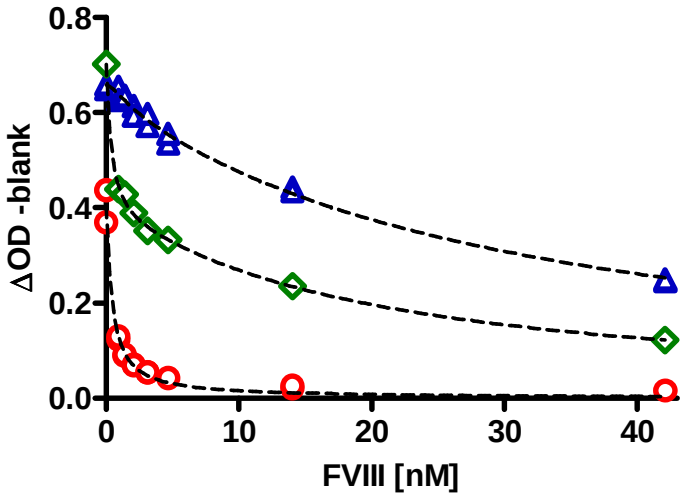

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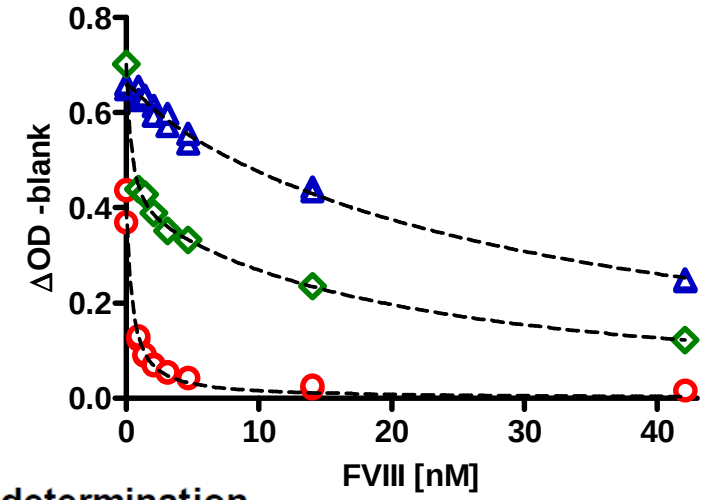

Table 1: Model selection for apparent affinity determination

Sample	$R^2 > 0.7$		Lower Limit of 95% CI > 0		F-test	Selected Model	Population 1 K_A [M^{-1}]	Population 2 K_A [M^{-1}]
	M1	M2	M1	M2				
○	Yes	Yes	Yes	No	NA	M1	$4.9x10^9$	NA
△	Yes	Yes	Yes	No	NA	M1	$9.1x10^7$	NA
◇	Yes	Yes	Yes	Yes	M2	M2	$5.4x10^9$	$9.9x10^7$

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Validation of anti-FVIII Affinity ELISA

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3 representative samples:

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- ◇ – Mix of A and B

Acceptance criteria: Coefficient of Variation (CV) < 25%

Affinity ELISA validation summary, n=6,CV [%]

Assay	Validation criteria	○	△	◇	
				Population 1	Population 2
total IgG	Intra-Assay stability	10%	5%	15%	9%
	Inter-Assay stability	18%	11%	17%	5%
	Robustness (Freeze/Thaw)	20%	4%	8%	8%
IgG1	Intra-Assay stability	5%	2%	5%	11%

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Affinity ELISA shows good comparability to alternative technologies

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MAb	competition based		direct binding		
	Affinity ELISA	SET	SPR C1 Fc capture	SPR CM5 Fc capture	BLI Strep Biotin-FVIII
K_A [M^{-1}]	$5.3x10^9$	$4.2x10^9$	$1.5x10^9$	$5.1x10^7$	$2.3x10^8$

SET = Solution Equilibrium Titration

SPR = Surface Plasmon Resonance

C1, CM5 = commercially available Biacore Chip technologies

Fc capture = commercially available kit for antibody immobilization

BLI = Biolayer Interferometry

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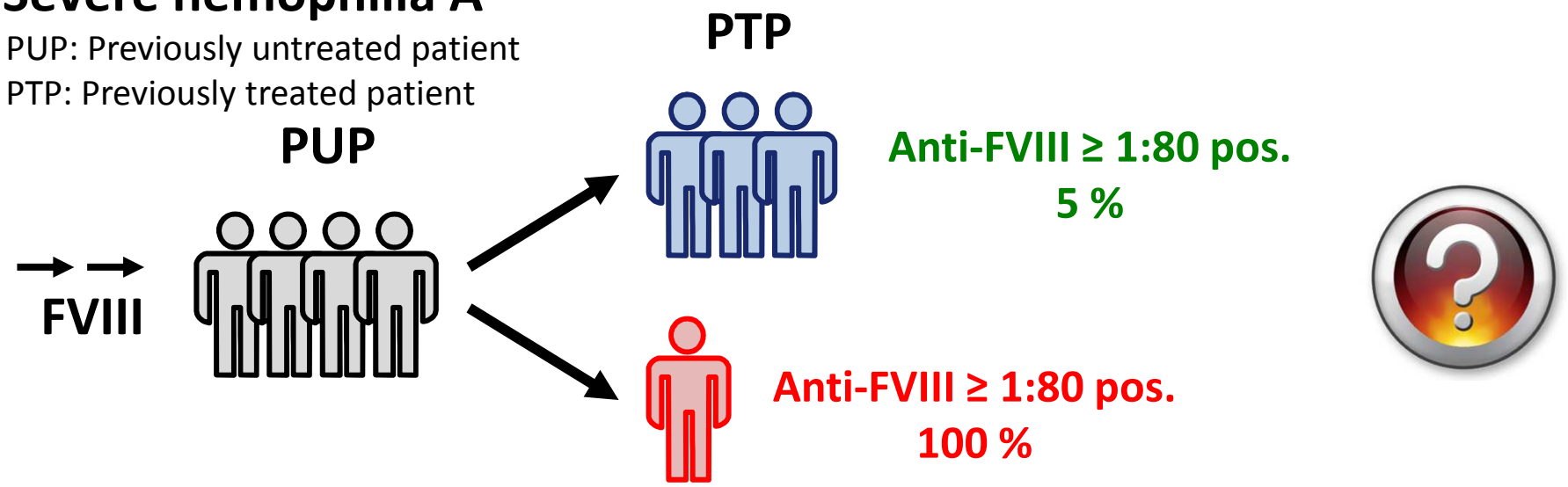
Affinity of FVIII-specific antibodies?

Severe hemophilia A

PUP: Previously untreated patient
PTP: Previously treated patient



Healthy

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Distinct patterns for Ig isotypes and IgG subclasses of FVIII-specific antibodies in healthy individuals and in severe hemophilia A patients

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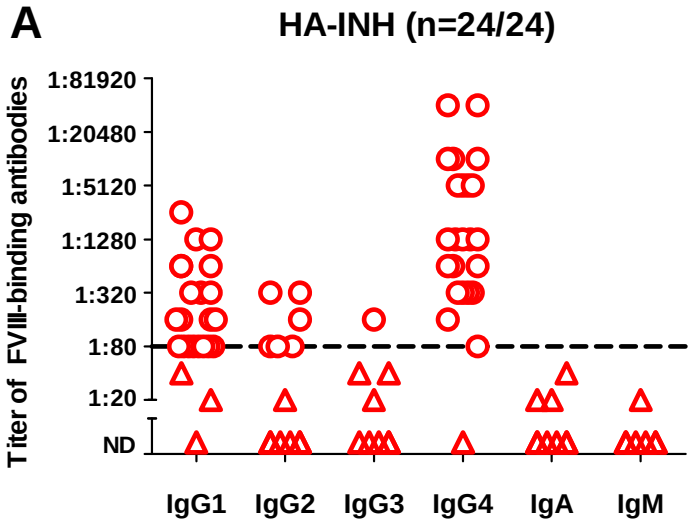
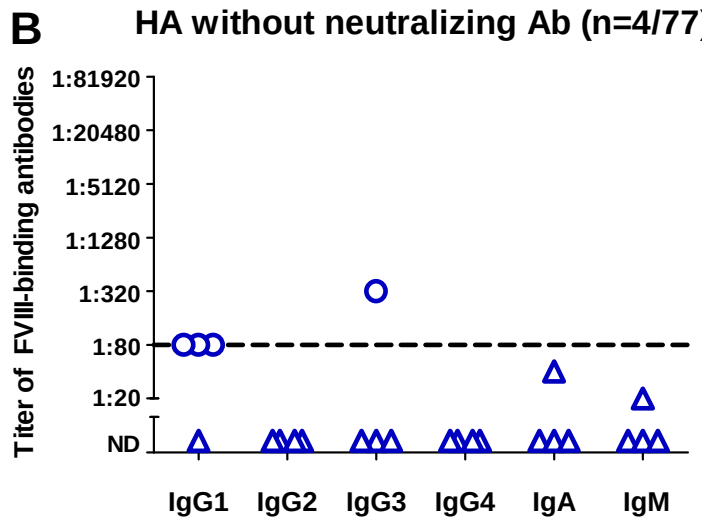
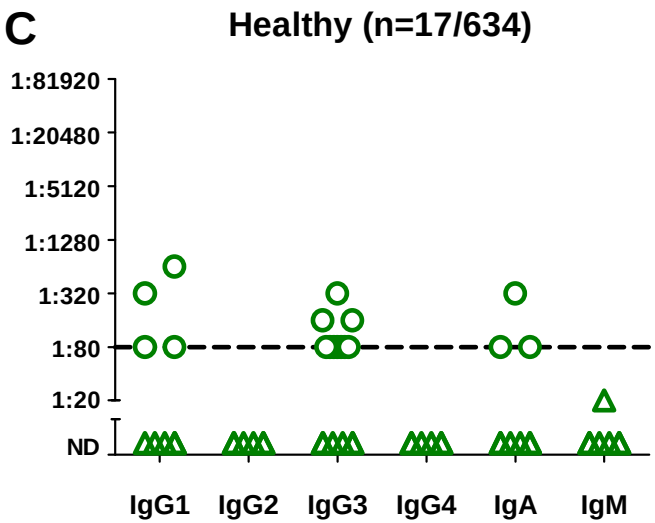


HA = severe Hemophilia A
LLOD = lower limit of detection
1:20 - LLOD for binding antibody
1:80 - LLOD for specific antibodies





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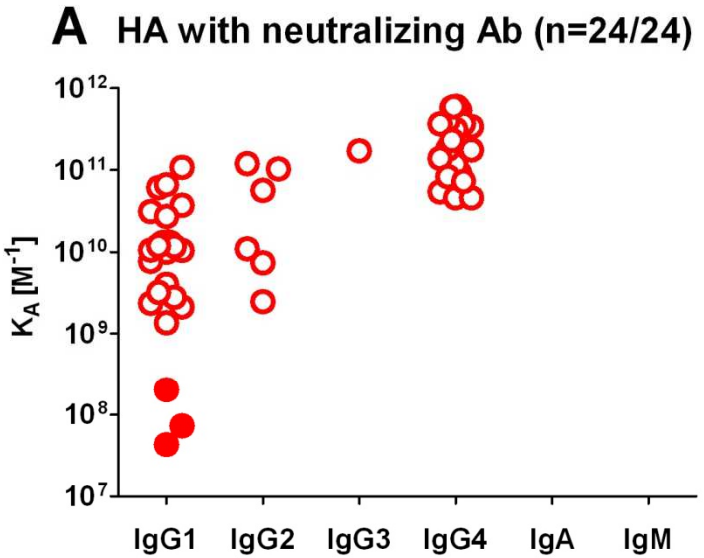
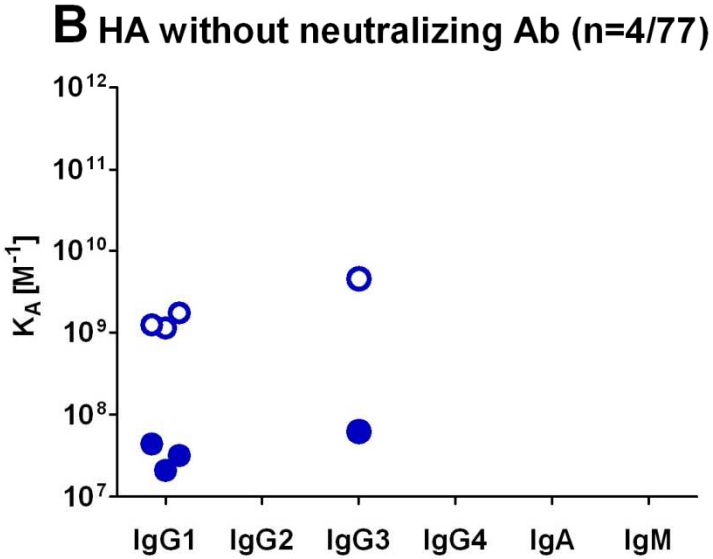
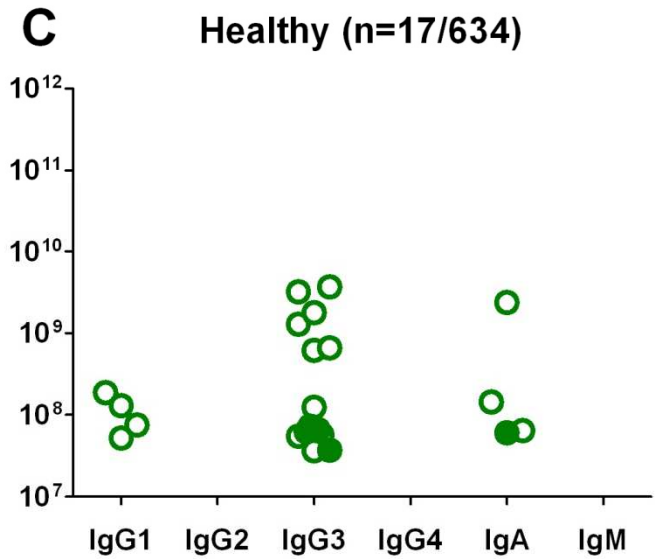


HA = severe Hemophilia A

○○○ 1st Antibody population

●●● 2nd Antibody population





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FVIII-specific antibodies in HA patients with neutralizing Ab are of higher affinity compared to HA without neutralizing Ab and Healthy

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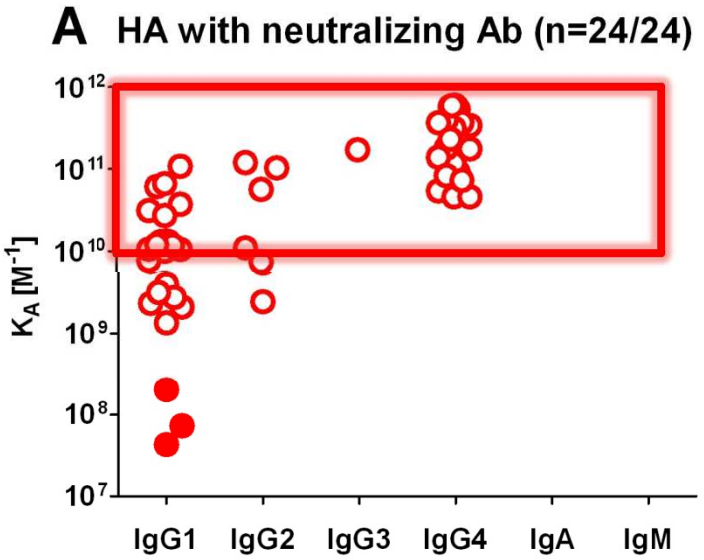
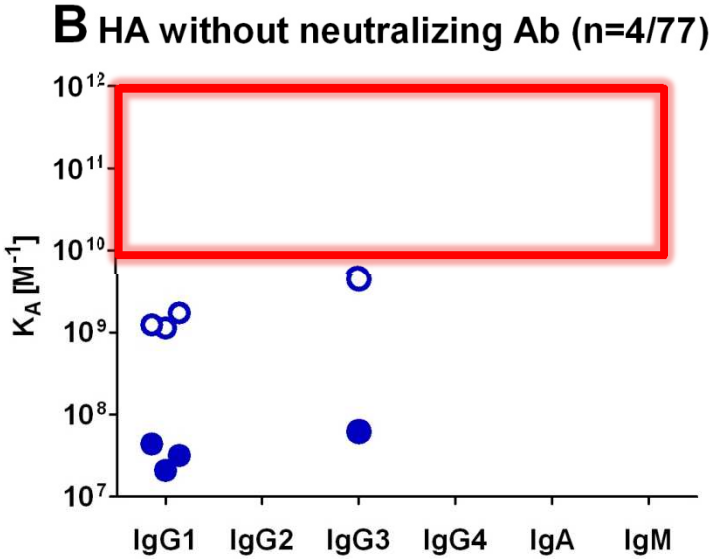
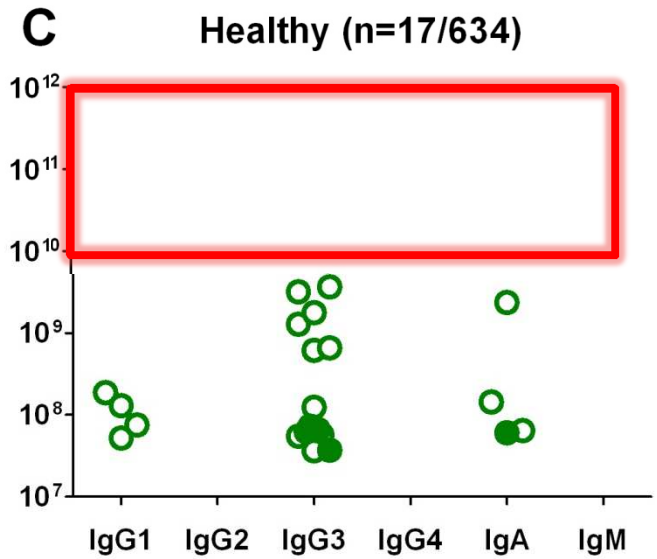


HA = severe Hemophilia A

1st Antibody population

2nd Antibody population





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Longitudinal monitoring of FVIII-binding antibodies

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Healthy individual

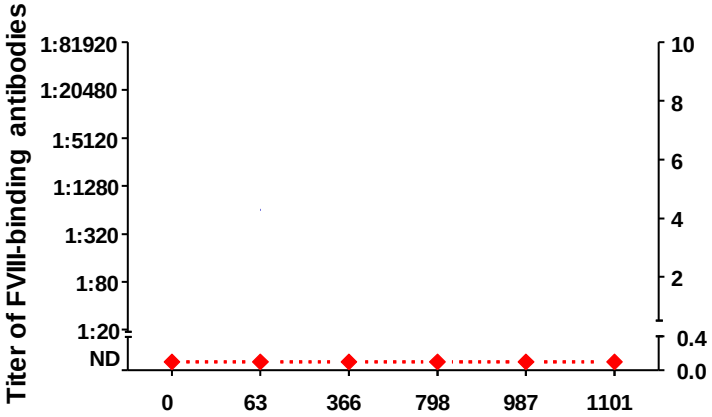
- 22 year old, healthy female donor
- No history of coagulation disorders
- Regular plasma samples for almost 3 years
- No FVIII neutralizing antibodies

Severe hemophilia A patient

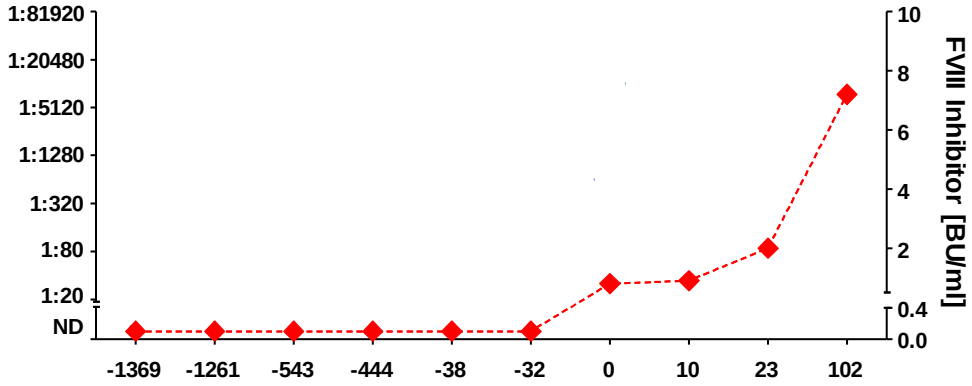
- 29 year old severe hemophilia A patient on daily FVIII prophylaxis
- progressive athropathy of the ankles and the right knee
- Bethesda assays during prophylactic treatment were negative
- FVIII neutralizing antibodies were first diagnosed after a FVIII wash-out period of 72 hours

Longitudinal monitoring of FVIII-binding antibodies

Healthy individual



Severe hemophilia A patient



FVIII inhibitor

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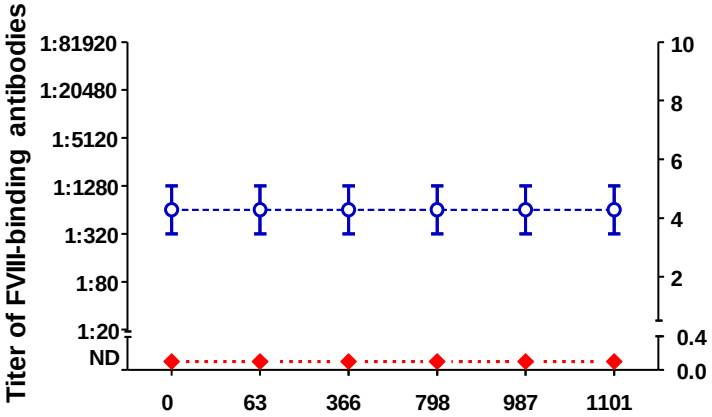
Longitudinal monitoring of FVIII-binding antibodies

Healthy individual



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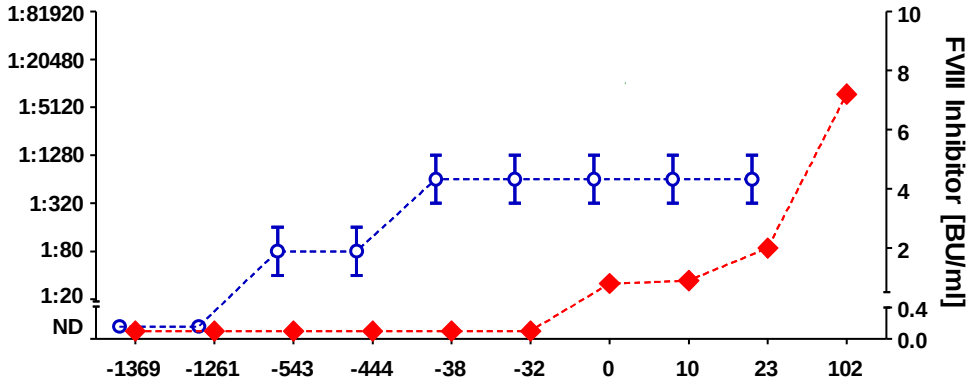

FVIII inhibitor

FVIII-binding IgG1

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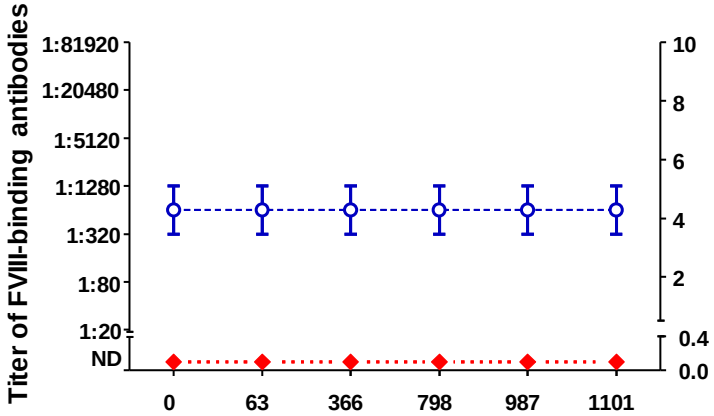
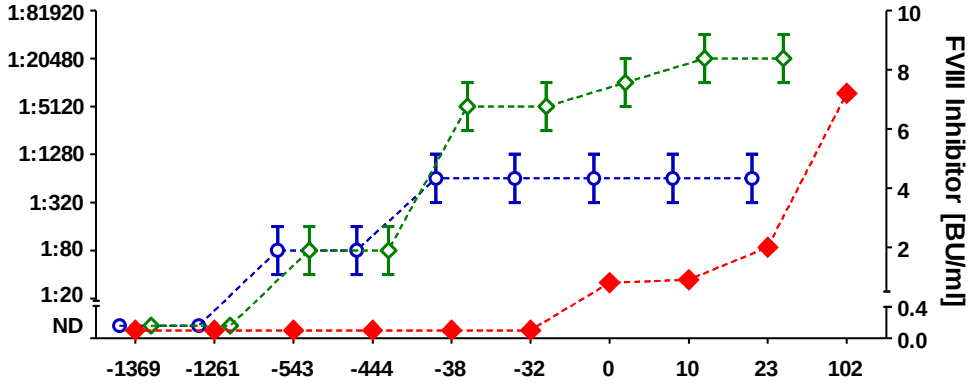
Blood 2015

Longitudinal monitoring of FVIII-binding antibodies

Healthy individual



Severe hemophilia A patient



FVIII inhibitor

FVIII-binding IgG1

FVIII-binding IgG4

Hofbauer et al.
Blood 2015

Longitudinal monitoring of FVIII-binding antibodies

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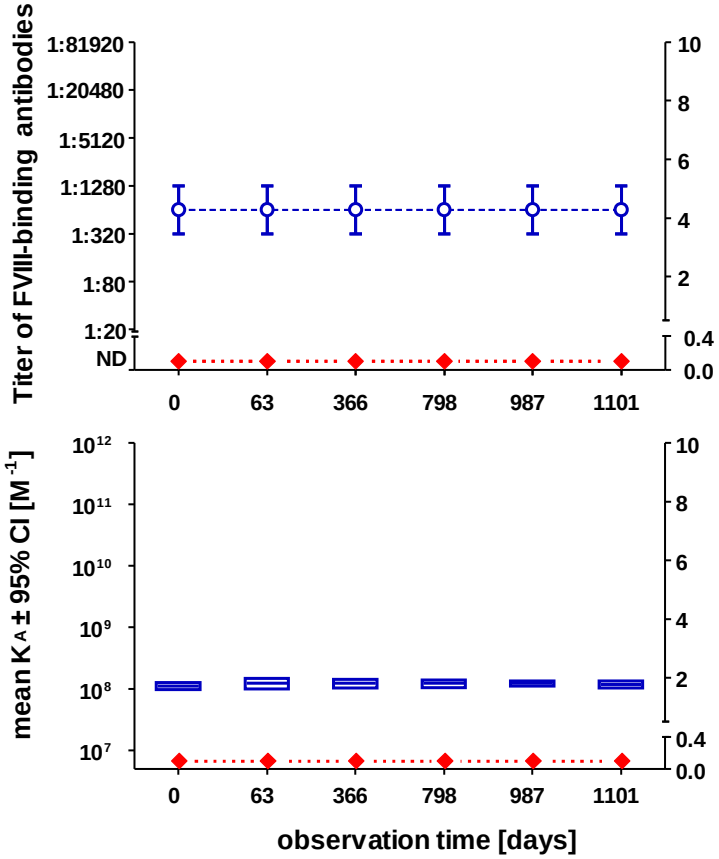
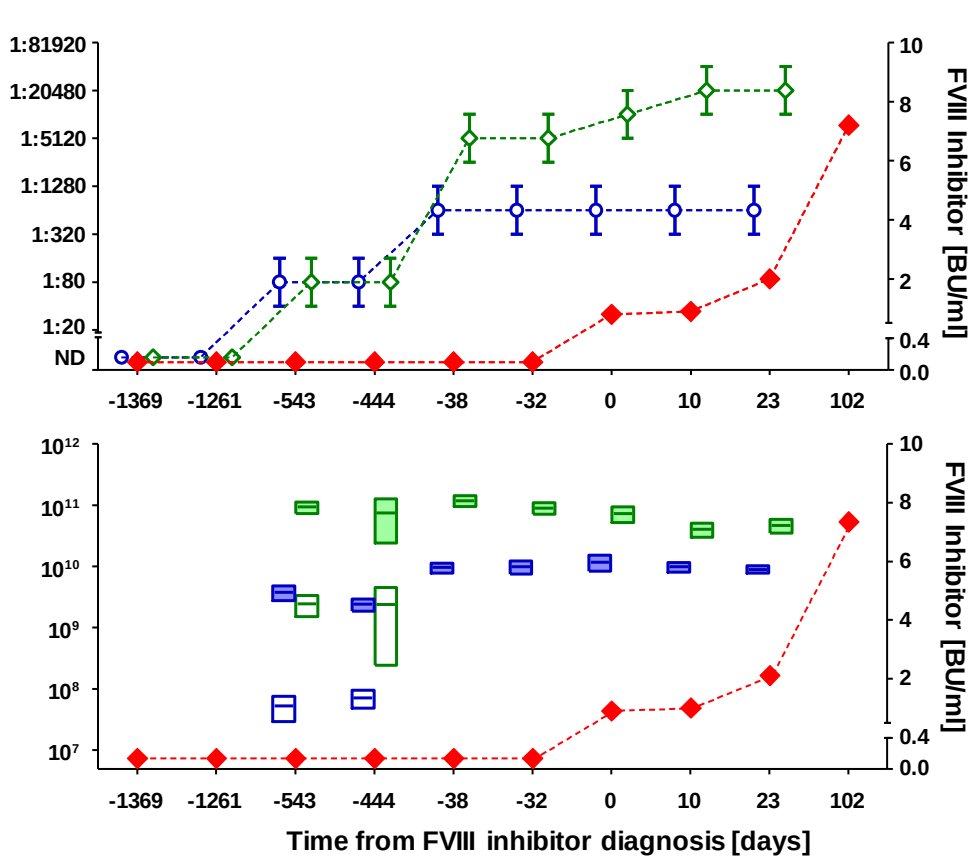
Healthy individual



Severe hemophilia A patient



FVIII inhibitor

FVIII-binding IgG1

FVIII-binding IgG4

Hofbauer et al.
Blood 2015

Longitudinal monitoring of FVIII-binding antibodies
Summary & working hypothesis

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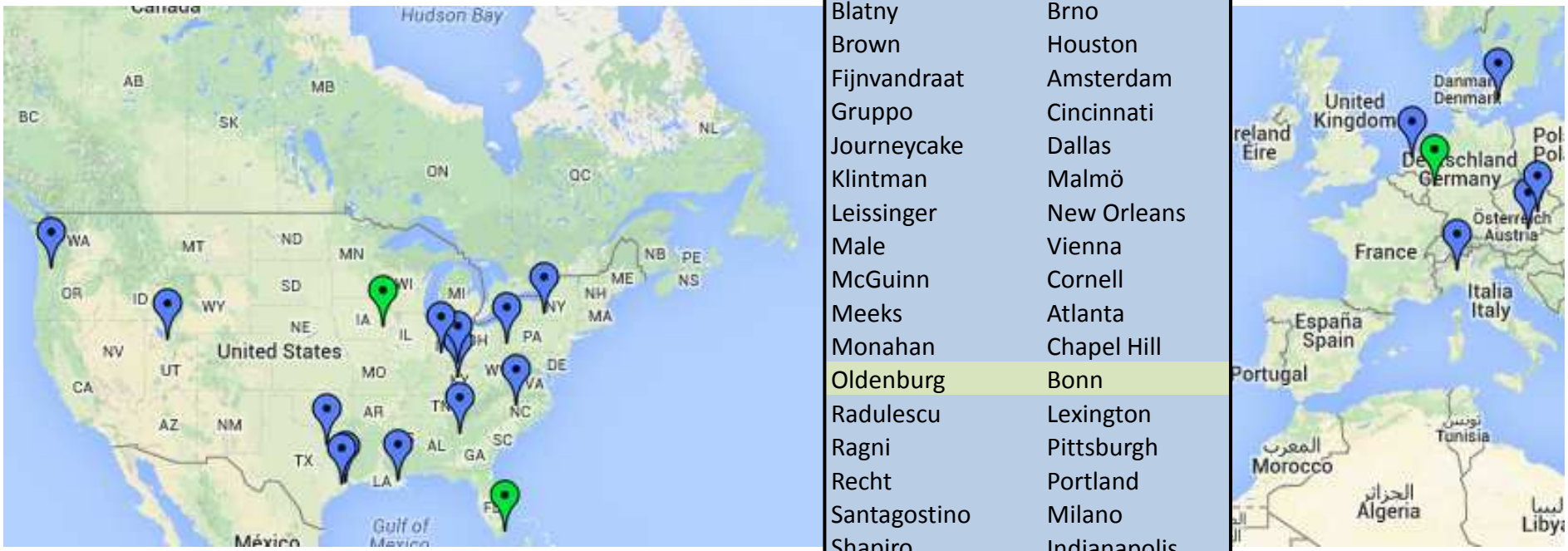
- High-affinity FVIII-specific IgG1 and IgG4 antibodies were detectable up to 543 days prior to the first diagnosis of FVIII neutralizing antibodies
- For comparison – FVIII-specific antibodies seen in some healthy individuals and in some patients without FVIII neutralizing antibodies are of low affinity
- So far, FVIII-specific IgG4 has only been observed in patients with evolving or established FVIII neutralizing antibodies

Working Hypothesis:

High-affinity FVIII-specific IgG4 and IgG1 are early biomarkers of evolving FVIII neutralizing antibody responses in patients with severe hemophilia A

Hemophilia Inhibitor PUP Study (HIPS)

Current status: 21 centers agreed, 18 IRB approvals, 18 centers qualified
9 patients enrolled, 2 patients completed the study
without FVIII neutralizing antibody development



Investigator Name	City
Blatny	Brno
Brown	Houston
Fijnvandraat	Amsterdam
Gruppo	Cincinnati
Journeycake	Dallas
Klintman	Malmö
Leissinger	New Orleans
Male	Vienna
McGuinn	Cornell
Meeks	Atlanta
Monahan	Chapel Hill
Oldenburg	Bonn
Radulescu	Lexington
Ragni	Pittsburgh
Recht	Portland
Santagostino	Milano
Shapiro	Indianapolis
Staber	Iowa City
Yaish	Salt Lake City
Yee	Houston
Corrales-Medina	Miami

Laboratory qualification completed

Laboratory qualification ongoing

Outline

- **Introduction**
  - Severe Hemophilia A & FVIII-specific antibody responses
  - Model for immune regulation of FVIII-specific antibody responses
- **Affinity assessment of FVIII-specific antibodies**
  - Methodological approach & Method validation
  - Affinity of FVIII-specific antibodies in healthy individuals and severe hemophilia A patients
- **Longitudinal monitoring of FVIII-binding antibodies**
  - Proof of principle data
  - Hemophilia Inhibitor PUP Study
- **Take home messages**

Take home messages

- Neutralizing antibodies against FVIII are the major treatment complication of current hemophilia A care
- FVIII-specific antibodies found in severe hemophilia A patients without neutralizing antibodies and healthy individuals are of low and medium apparent affinity
- FVIII-specific antibodies in severe hemophilia A patients with neutralizing antibodies are predominately of high apparent affinity
- Apparent affinity of anti-FVIII antibodies is a potential new biomarker helping to differentiate between pathogenic high affinity, neutralizing antibodies and low affinity, non-neutralizing antibodies

Acknowledgements

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- **HIPS immunology lab team**
  - Shawn Whelan (2009-2012)
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- Friedrich Scheiflinger

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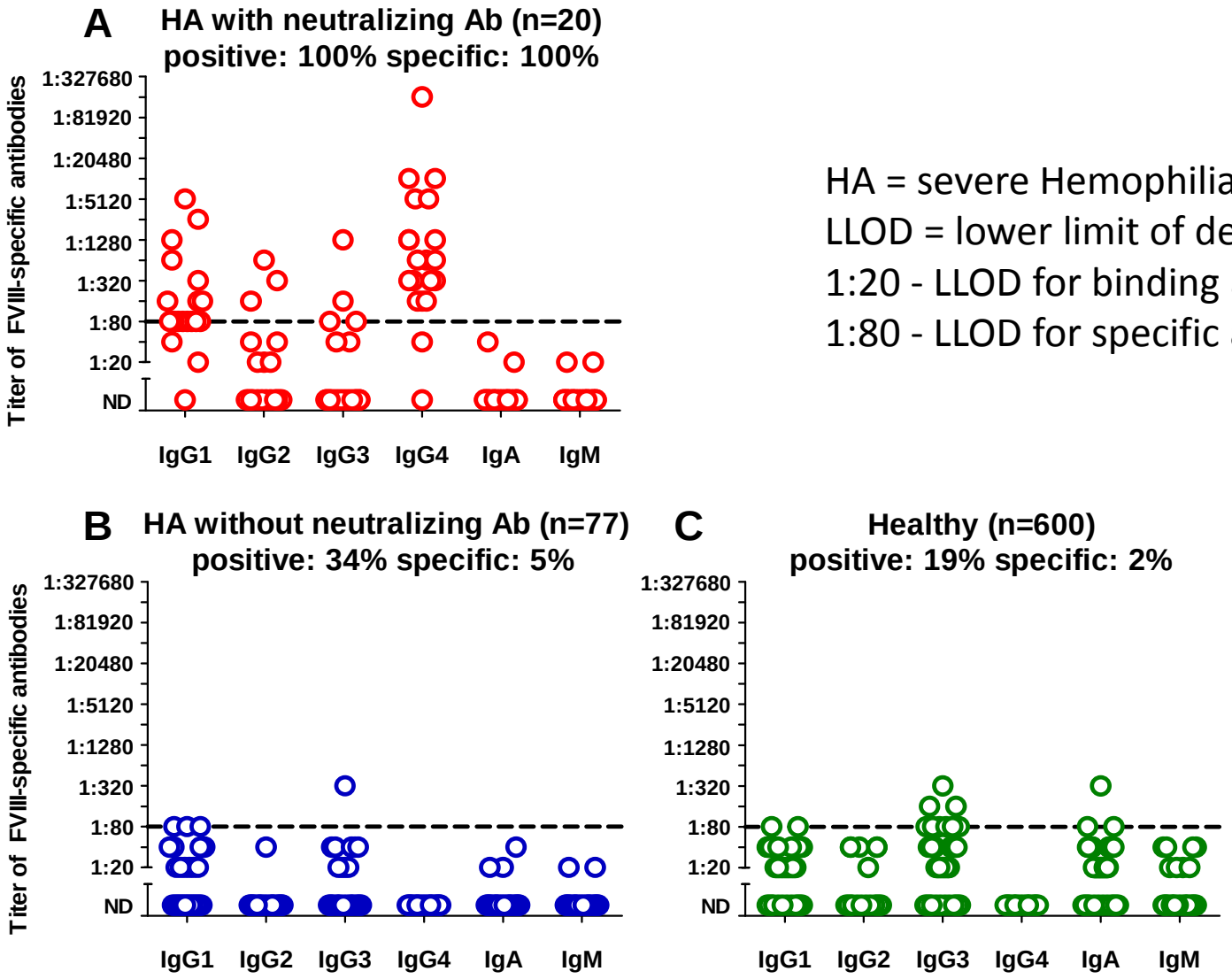
- **UNIVERSITY OF TEXAS HOUSTON**
  - Deborah Brown (HIPS PI)
- **UNIVERSITY OF MILAN**
  - Elena Santagostino (HIPS PI)
- **All participating patients and their families**

THANK YOU FOR YOUR ATTENTION

EIP
European Immunogenicity Platform

BACKUP

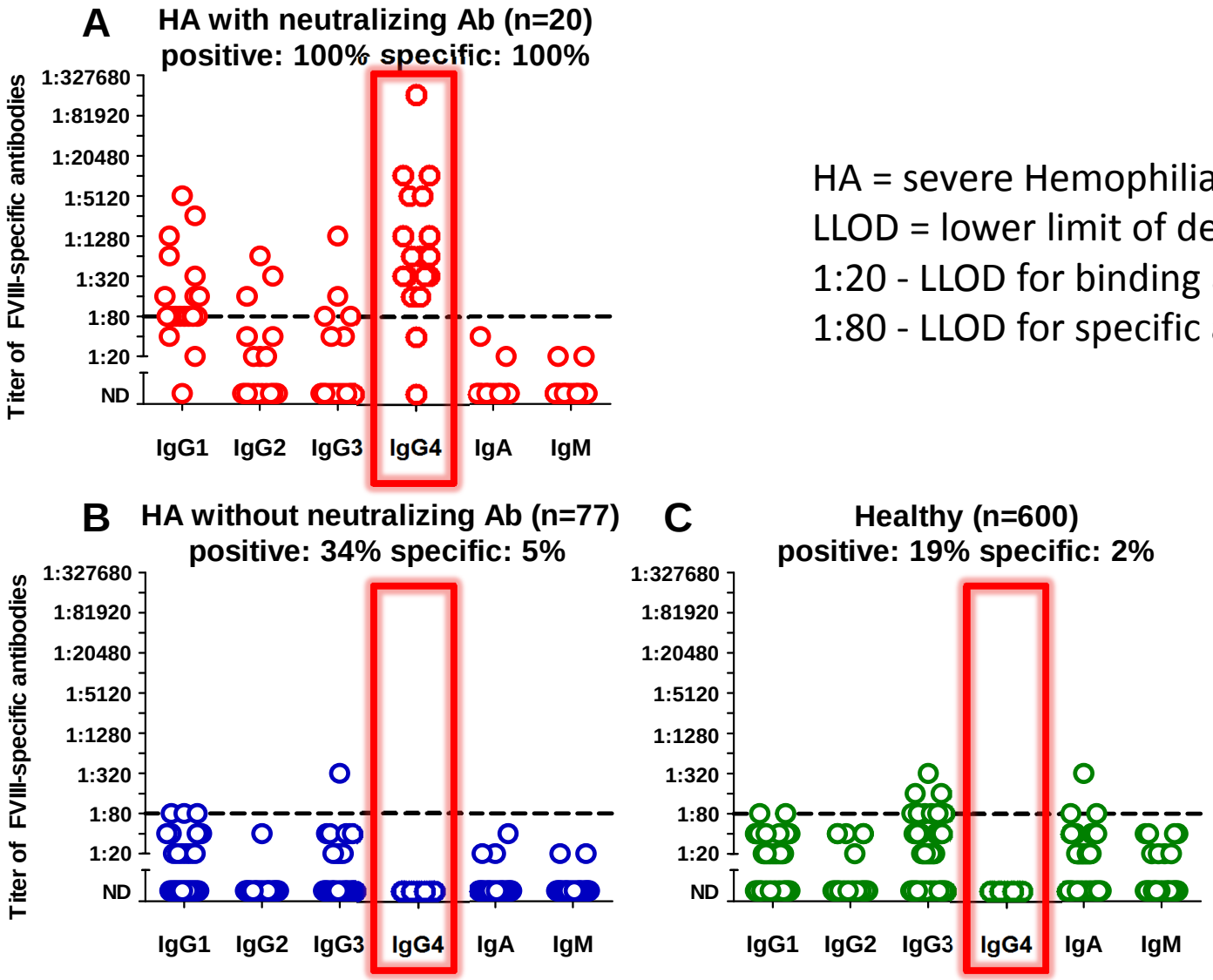
Distinct patterns for Ig isotypes and IgG subclasses of FVIII-binding antibodies in healthy individuals and in severe hemophilia A patients



HA = severe Hemophilia A
LLOD = lower limit of detection
1:20 - LLOD for binding antibody
1:80 - LLOD for specific antibodies

Whelan et al.
Blood 2013

IgG4 was exclusively found in patients with neutralizing antibodies



HA = severe Hemophilia A
LLOD = lower limit of detection
1:20 - LLOD for binding antibody
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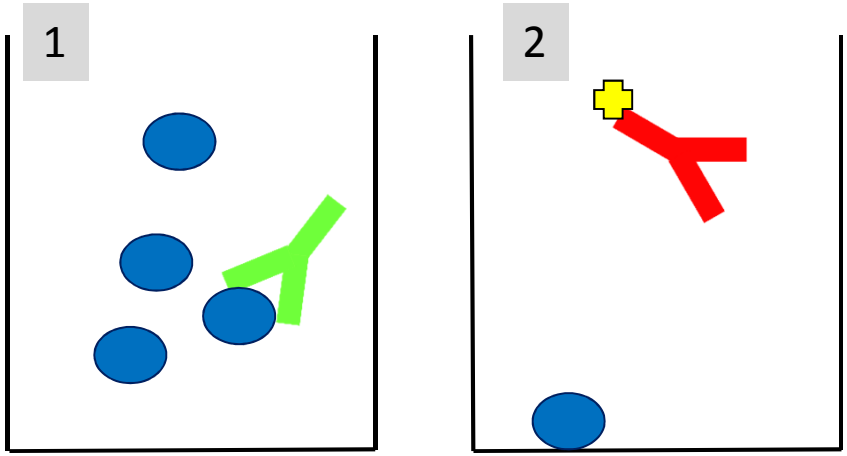
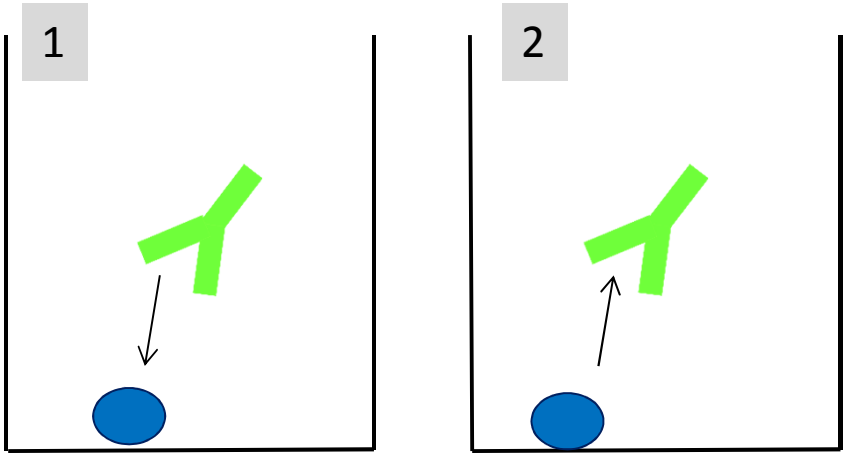
Whelan et al.
Blood 2013

Competition-based vs. direct binding affinity assessment

1) Competition



2) Direct binding



+	Concentration of antibodies is not critical
+	Specificity via secondary antibody
+	High sensitivity
+	High throughput and low cost
-	Determination of average affinity constant

-	Concentration of antibodies is critical
-	Bias via unspecific binding
-	Low sensitivity
-	Low throughput and high cost
+	Characterization of binding kinetics (association and dissociation)

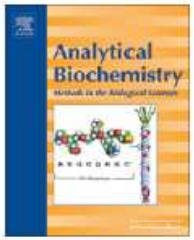
A recent biacore publication

Analytical Biochemistry 429 (2012) 58–69

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio



Biacore surface matrix effects on the binding kinetics and affinity of an antigen/antibody complex

Andrew W. Drake [1], Margaret L. Tang, Giuseppe A. Papalia [2], Gregory Landes [3], Mary Haak-Frendscho [1], Scott L. Klakamp [*,4]

Takeda San Francisco, 285 E. Grand Ave. South San Francisco, CA 94080, USA

Determined affinity indirect proportional to negative charge density on Biacore Sensor

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Table 1
Ag/mAb comparison results.

Method	n	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (pM)
Biacore				
CM5 (amine)	5	1.13 (0.04) × 10^5	2.23 (0.10) × 10^{-4}	1970 (140)
CM5 (capture)	3	5.81 (0.12) × 10^4	1.67 (0.05) × 10^{-4}	2870 (120)
CM4 (amine)	4	2.87 (0.16) × 10^5	1.91 (0.06) × 10^{-4}	664 (47)
CM4 (aldehyde)	4	3.19 (1.05) × 10^5	1.78 (0.05) × 10^{-4}	580 (219)
CM4 (capture)	3	1.28 (0.30) × 10^5	1.65 (0.14) × 10^{-4}	1290 (260)
C1 (amine)	6	1.02 (0.06) × 10^6	1.91 (0.09) × 10^{-4}	186 (8)
C1 (capture)	3	8.60 (0.47) × 10^5	2.86 (0.44) × 10^{-4}	333 (63)
Solution phase	6	n/a	n/a	91.9 (32.4)
KinExA				
Standard	4 K_D, 4 k_a	3.31 (0.10) × 10^6	7.32 (0.23) × 10^{-5}	22.1 (4.7)
With dextran	6 K_D, 5 k_a	2.45 (0.18) × 10^6	1.47 (0.10) × 10^{-4}	60.1 (27.3)

Note: k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant; n/a, not applicable. The numbers shown in parentheses are the 95% confidence intervals.

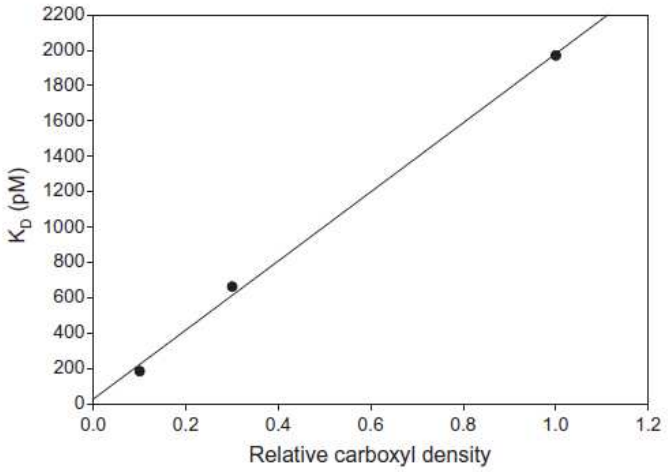
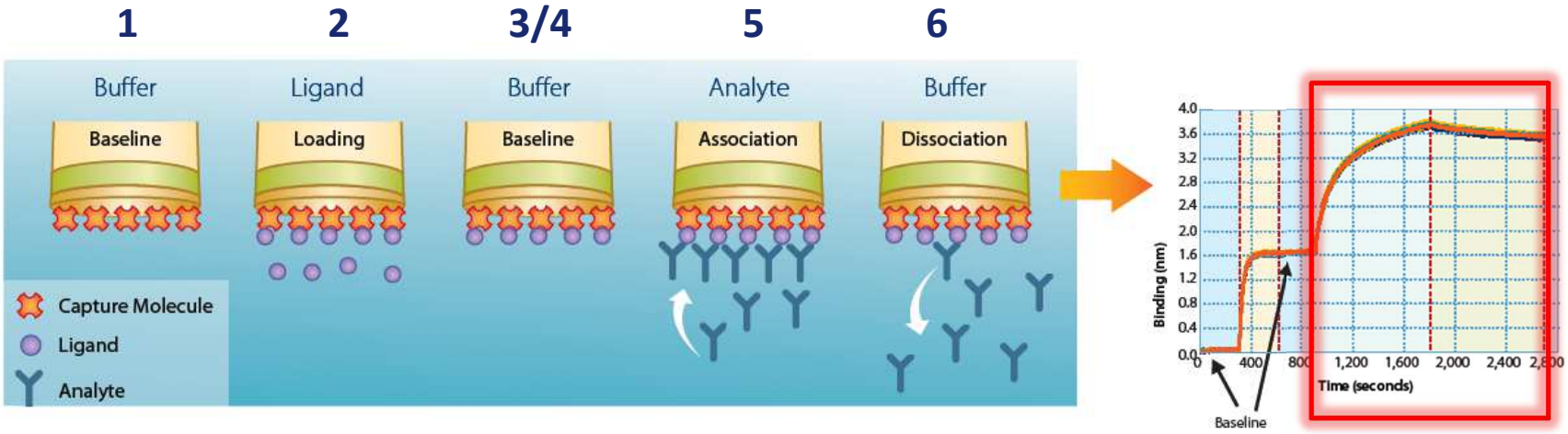


Fig.6. Correlations between the K_D and the relative negative charge of the dextran matrix for Ag binding to amine-coupled mAb on C1, CM4, and CM5 chips.

Follow-up activity:
C1 measurement in BG (Gerald Schrenk)

Biolayer Interferometry Anti-FVIII kinetic assay steps



Figures courtesy of fortéBIO

- Samples under investigation:

Sample	Info
Anti-FVIII MAb	Human monoclonal IgG1 antibody
Healthy donor - purified antibody	High salt elution from FVIII affinity column
Healthy donor - plasma	IgG1 pos. healthy donor plasma
Patient plasma with neutralizing antibodies	IgG1 & IgG4 pos. severe HA patient

Comparison of anti-FVIII MAb & anti-FVIII purified antibody

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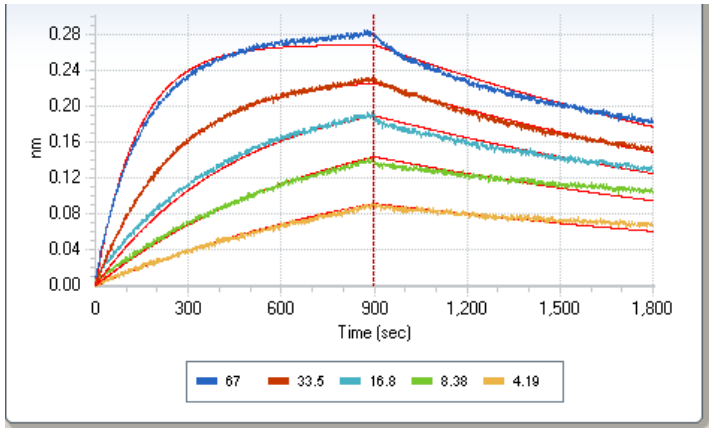
Fitting Model: Global Fit, 1:1 Interaction, R_{max} unlinked by sensor

A

Anti-FVIII MAb

1:2 dilution series in buffer

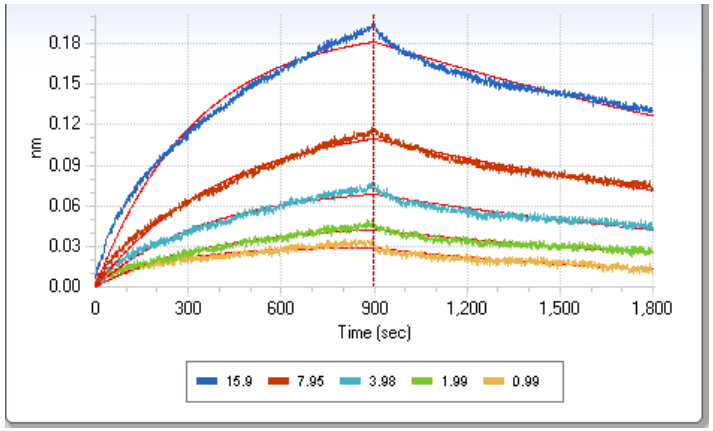


Sample ID	k_a (1/Ms)	k_d (1/s)	**K_D (nM)**
Anti-FVIII MAb	1,04E+05	4,63E-04	**4,5**

B

Healthy donor - purified antibody

1:2 dilution series in buffer



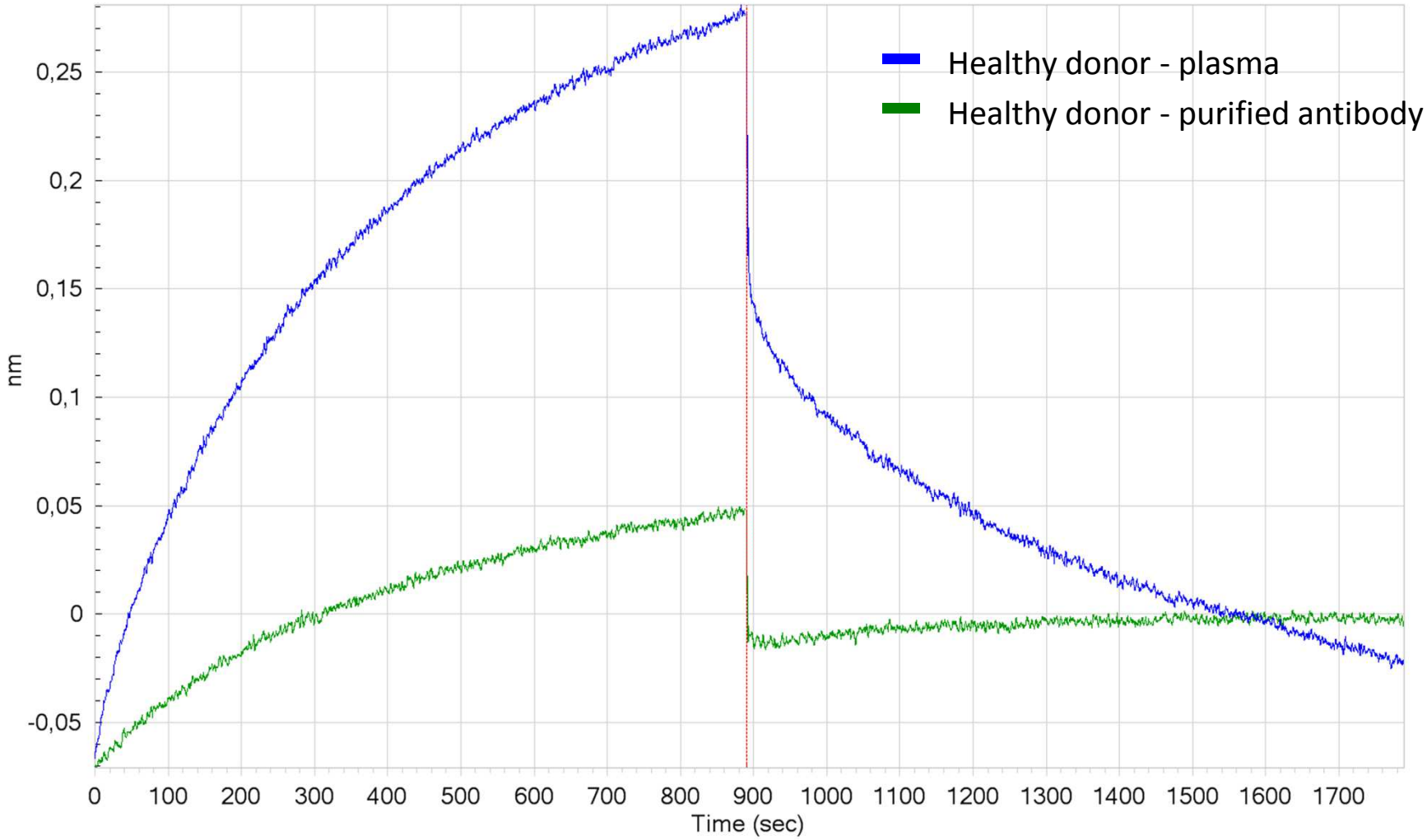
Sample ID	k_a (1/Ms)	k_d (1/s)	**K_D (nM)**
Anti-FVIII pAb1	1,70E+05	4,74E-04	**2,8**

Quantitative comparison of Affinity ELISA vs. BLI

$K_D = 1/K_A$

Sample	Anti-FVIII ELISA Titer	Anti-FVIII Affinity ELISA K_D [nM]	Anti-FVIII Affinity BLI K_D [nM]
Anti-FVIII MAb	n/a	0.2	4.5
Healthy donor purified antibody	n/a	7.8	2.8
Healthy donor plasma	IgG: 1:640	13.0	n/a
Patient plasma with neutralizing antibodies	IgG: 1:640	0.1	n/a

Qualitative comparison of purified antibody & plasma (healthy donor)



Qualitative comparison of anti-FVIII healthy donor plasma and anti-FVIII patient plasma