Small Antibody Fragments as Alternative Tools in Hemophilia Care

Peter Lenting

RE(Act)-congress, March 2018, Bologna

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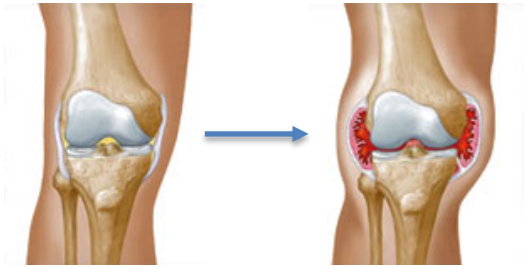
Inflammation - Thrombose

Hemophilia A

Hemophilia A results from a defect in the X-chromosome gene encoding the plasma protein **coagulation factor VIII** (affects 1-2 *per* 10,000 male births)

Severe (<1% FVIII activity):
~ 40% of HemA population
(~ 2200 patients in France)

Spontaneous bleeding in muscles & joints



Moderate & Mild (1-5% & 5-40% activity):
~ 60% of HemA population
(~ 3400 patients in France)

Bleeding provoked by trauma or invasive procedures

tonsillectomy

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Hemophilia A

Severe (<1% FVIII activity):

Life-threathening when untreated

Associated with severe hemarthrose & arthropathy

Moderate & Mild (1-5% & 5-40% activity):

Usually not life-threathening

80% of patients having 2-10% FVIII activity develop serious ankle arthropathy due to subclinical microbleeds

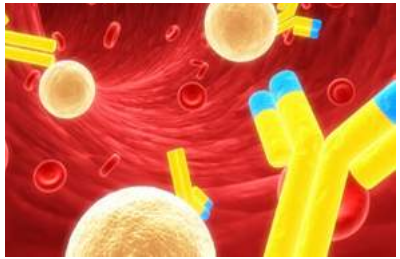
Treatment in Hemophilia A

Treatment of choice: prophylactic replacement therapy using plasma-derived or recombinant FVIII via *IV* infusions

Limitations:

Short half-life
Frequent IV infusions

Costly
100-120 k€/year



Inhibitor development
20-30% severe patients
5-13% mild/moderate
Costs up 3-5 fold

Limited availability
70% of patients do not receive proper treatment

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Evolution of treatment options in Hemophilia A

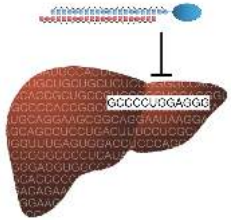
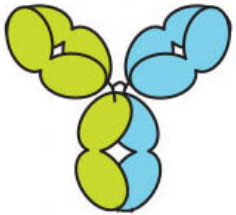
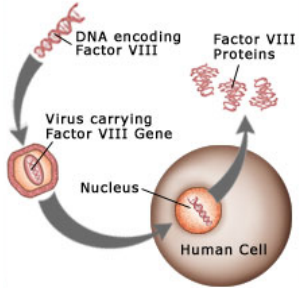
FVIII concentrates

pd-FVIII vs recFVIII

Bypassing agents

FEIBA

FVIIa

Modified FVIII variants	Inhibitors of natural anticoagulants	Bispecific antibody	Improved procoagulant proteins	Gene therapy
				
Pegylated-FVIII FVIII-Fc	- Antithrombin (siRNA) - TFPI (Mab) - Protein C-serpin	Combines FIX(a) & FX(a)	Factor V Factor IX Factor X	

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How to improve hemophilia treatment ?

- Improve half-life to reduce infusion frequency
- Avoid intravenous infusions
- Reduce immunogenity
- Reduce costs

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Single domain antibodies (VHH or nanobodies)

- Single domain antibodies: where do they come from?
- Nanobodies against antithrombin
- Factor VIII – nanobody fusion proteins

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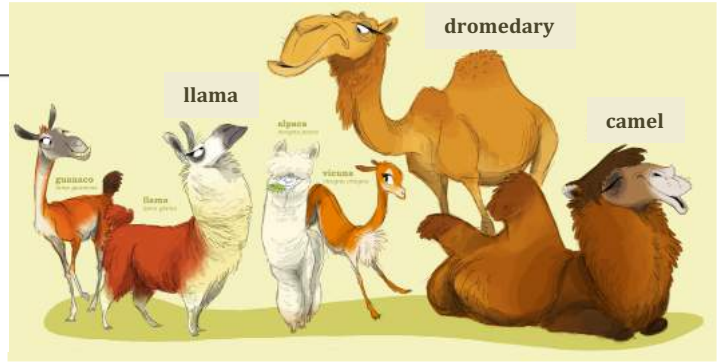
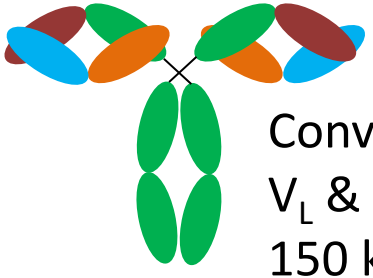
Camelid-derived Heavy chain only-antibodies

LETTERS TO NATURE

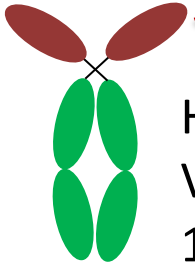
Naturally occurring antibodies devoid of light chains

C. Hamers-Casterman, T. Atarhouch, S. Muyldermans,
G. Robinson*, C. Hamers, E. Bajyana Songa,
N. Bendahman & R. Hamers†





Conventional IgG
V_L & V_H
150 kDa



Heavy chain-only
V_H
100 kDa

VHH (nanobody)
13-14 kDa

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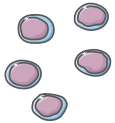
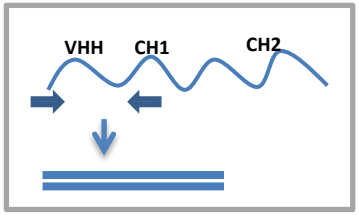
Generation of nanobodies:

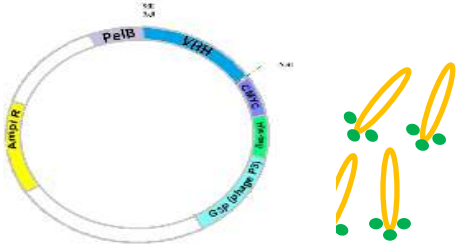
Immunize llama



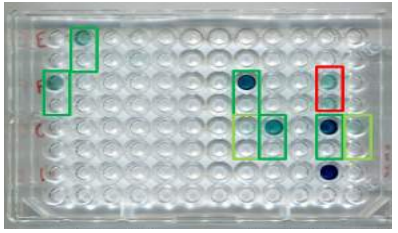
Isolate mRNA from B-lymphocytes



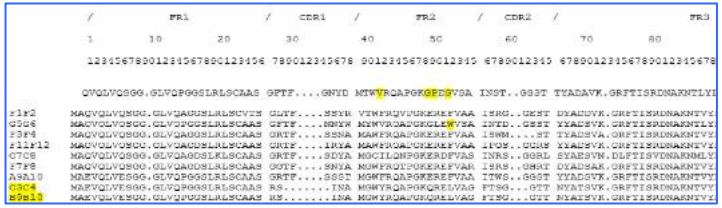
Amplify VHH mRNA



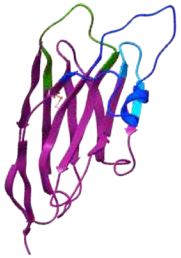
Clone in pHEN6-phagemid to generate phage-library



Screen for antigen-binding phages



Check sequence and expressability



Enjoy your nanobody

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Advantages nanobodies over conventional IgG

- Efficient production in micro-organisms, allowing low-cost production
- Excellent stability at room temperature, permitting long shelf-life and ready-to-use soluble formulations
- High solubility, compatible with high-dose formulations for subcutaneous applications
- Different epitope repertoire
- Their small size facilitates bio-engineering and their high expression levels make them excellent candidates for use in gene-therapeutic approaches

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Single domain antibodies (VHH or nanobodies)

- Single domain antibodies: where do they come from
- **Nanobodies against antithrombin**
- **Factor VIII – nanobody fusion proteins**

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Haemostasis involves an equilibrium between accelerators and brakes

Pro-coagulant

FVIII, FIX, FX, ……

Anti-coagulant

Antithrombin, TFPI, Protein C, ……

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One of the accelerators is missing in hemophilia , which results in a bleeding tendency

Pro-coagulant	*Anti-coagulant*
~~FVIII~~, FIX, FX, ……	Antithrombin, TFPI, Protein C, ……

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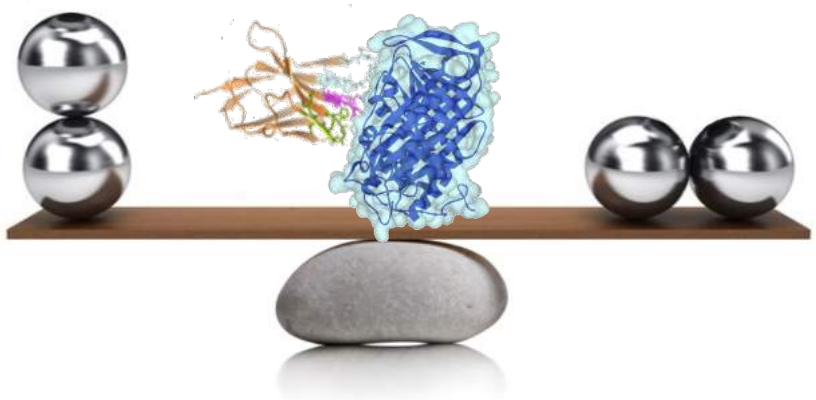
Inhibition of an anti-coagulant may restore hemostatic equilibrium

Pro-coagulant

~~FVIII~~, FIX, FX, ……

Anti-coagulant

Antith~~rombin~~, TFPI, Protein C, ……



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Isolation of anti-Antithrombin nanobodies

Phage display against human antithrombin:
7 unique sequences, with variable cross-species specificity

	Human	Simian	Mouse	Rat	Canine	Rabbit	Porcine	Bovine
KB-AT-01	+	++	++	+	-	+	-	-
KB-AT-02	++	++	++	++	++	++	++	++
KB-AT-03	++	++	++	+	+	-	-	-
KB-AT-04	+	+	++	+	+	-	+	-
KB-AT-05	+	+	+	-	-	-	-	-
KB-AT-06	+	++	+	+	+	++	+	-
KB-AT-07	+	++	+	-	-	-		-

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Analysis of nanobodies

- Neutralizing activity of nanobodies
- Correction of thrombin generation in hemophilic plasma
- Half-life determination
- In vivo efficacy in tail vein transection assays

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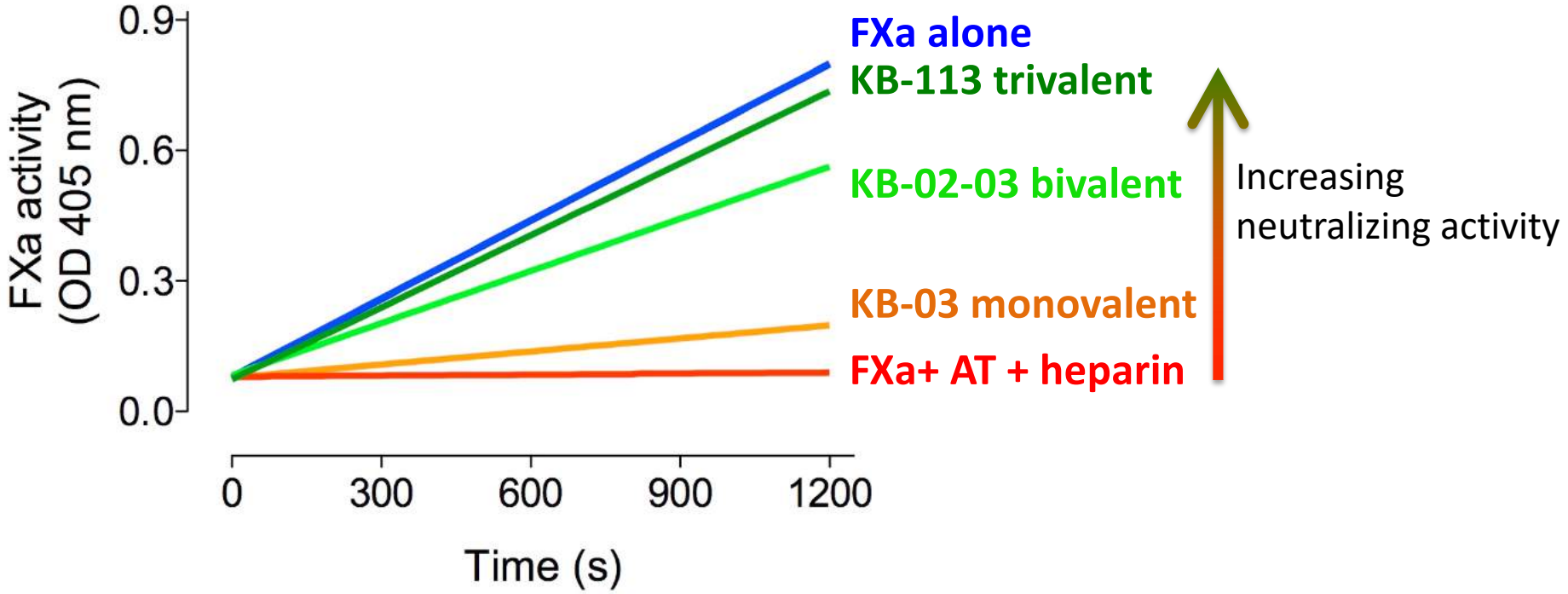
Chromogenic activity:

Neutralizing capacity can be tuned by combining different nanobodies



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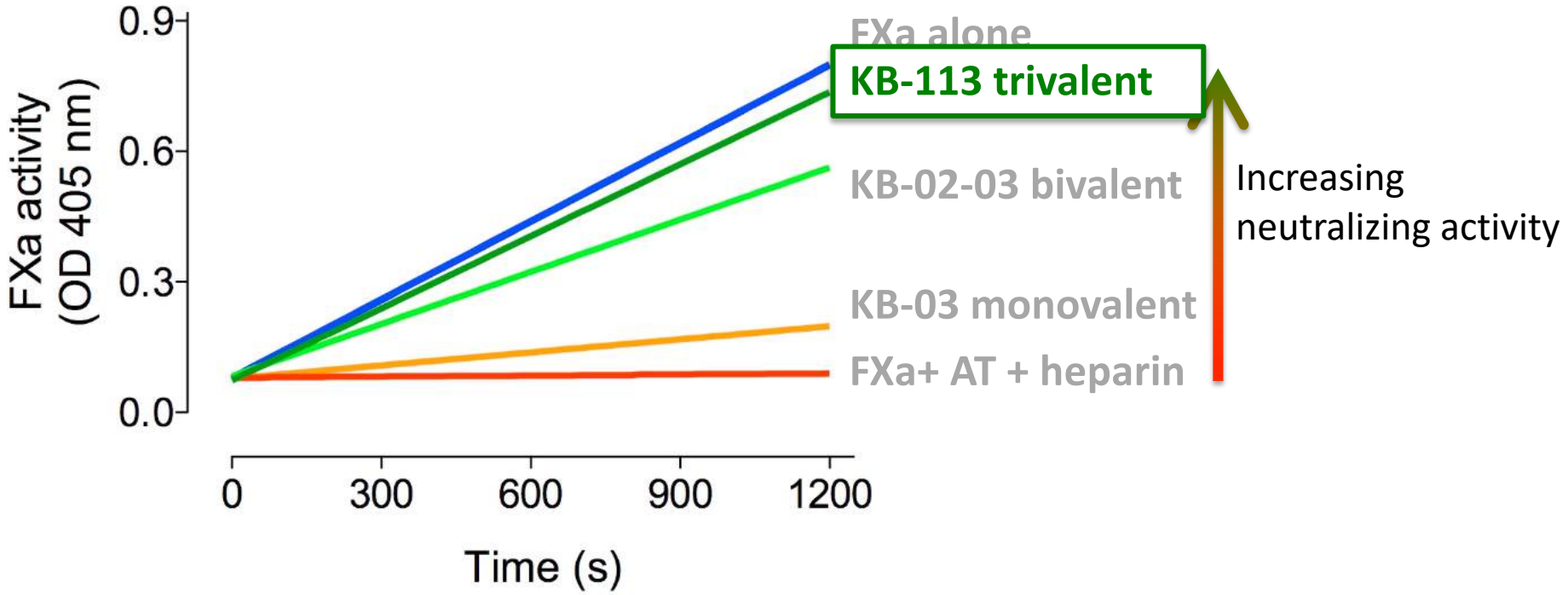

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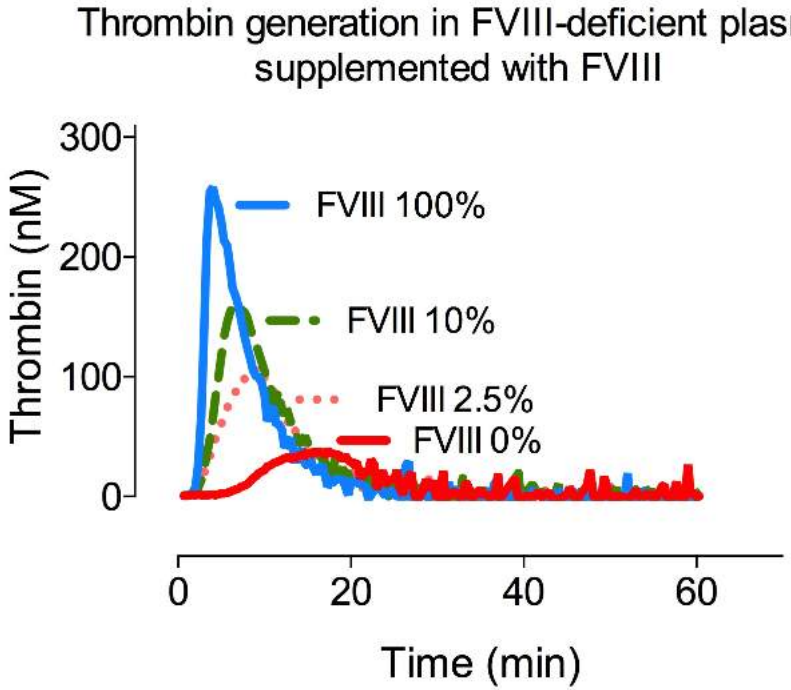
Thrombin generation test

Continuous monitoring of thrombin generation in plasma



Parameters:

- Total amount of thrombin generated (ETP)
- Lag-time
- Peak height
- Time-to-peak

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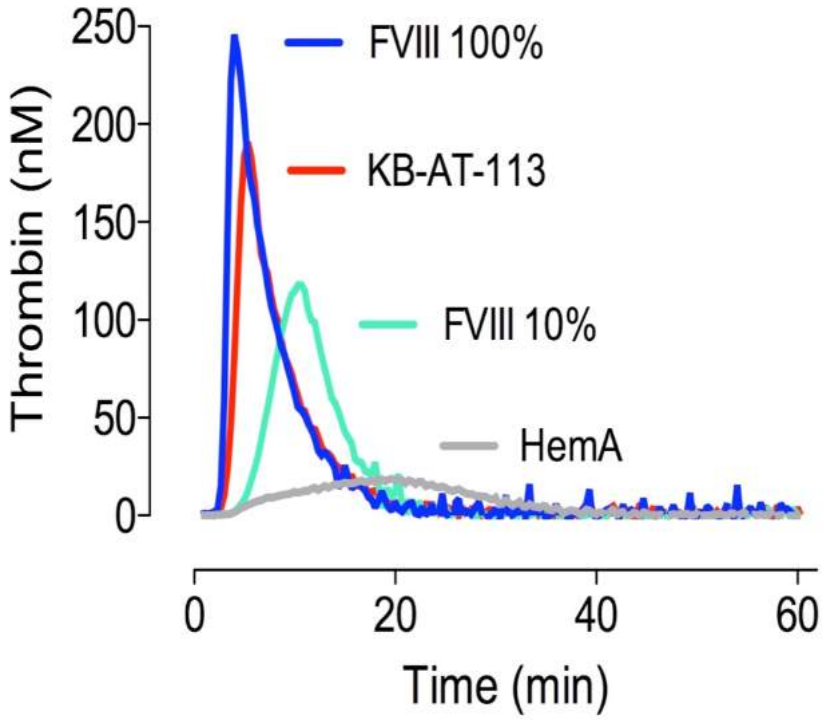
Thrombin generation test:

KB-AT-113 corrects thrombin generation in a similar fashion as FVIII



	Peak (nM)	tt-peak (min)	ETP (nM)
FVIII 0%	24	20	107
FVIII 10%	115	12	1236
FVIII 100%	248	4.2	1814
KB-AT-113	185	4.8	1428

Correction of ETP, and similar tt-peak

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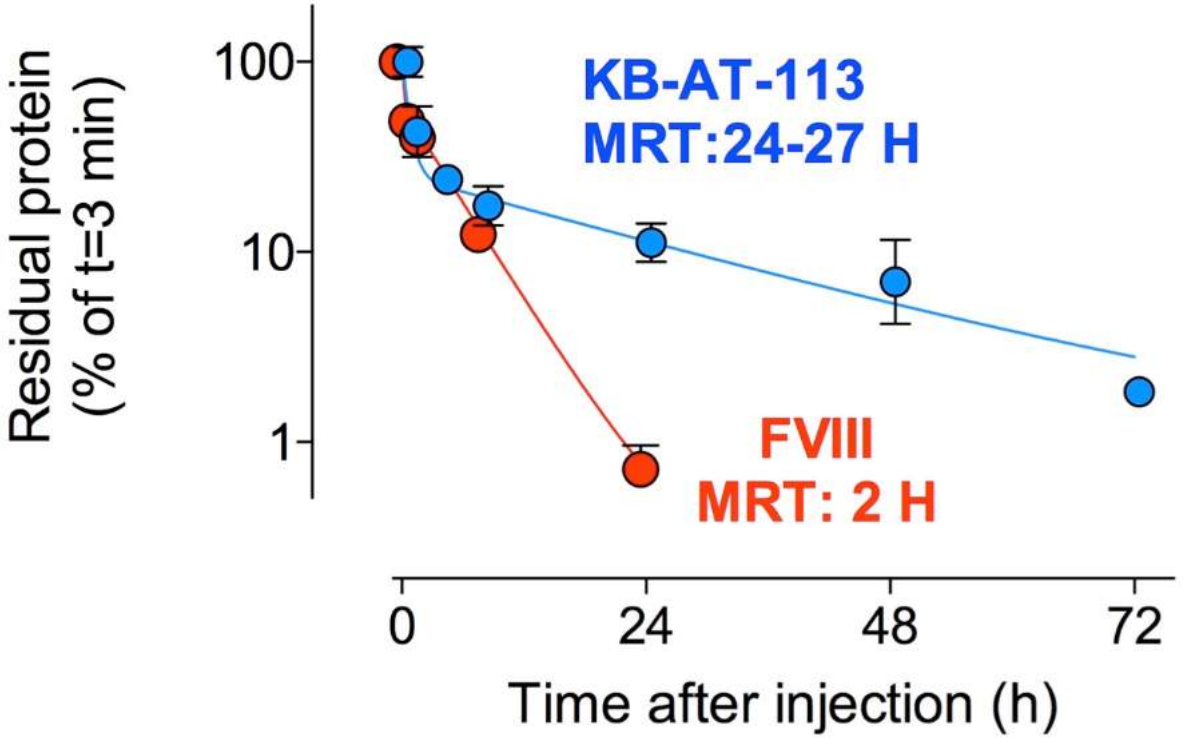
Half-life determination

KB-AT-113 has >5-fold increased half-life compared to FVIII in mice

FVIII: 200 kDa

KB-AT-113: 45 kDa



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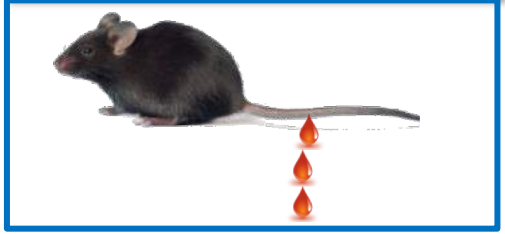
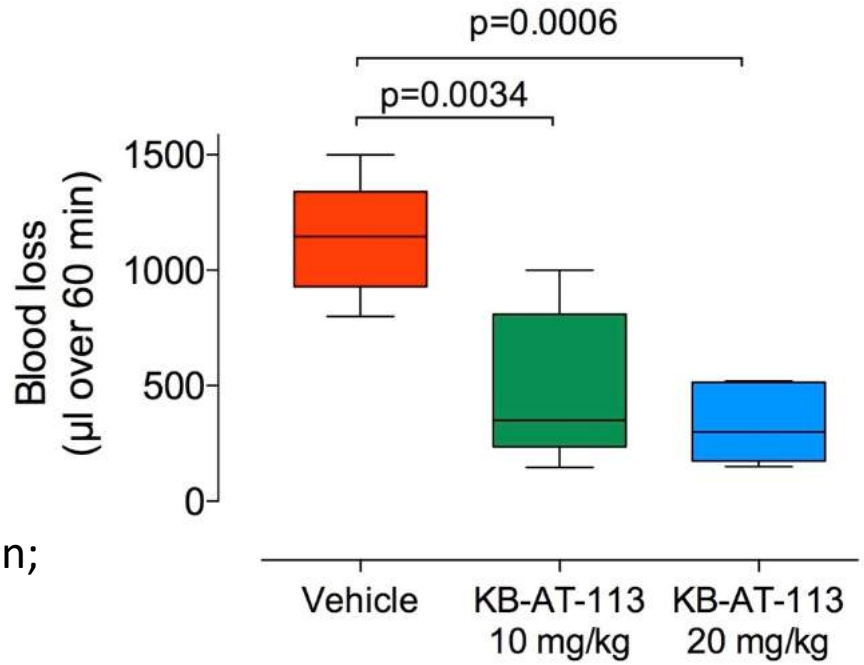
In vivo efficacy in tail vein transection assays

KB-AT-113 significantly reduces blood loss in FVIII-deficient mice

Tail vein-transection assay



Standardized incision of lateral vein;
Depth 0.7 mm



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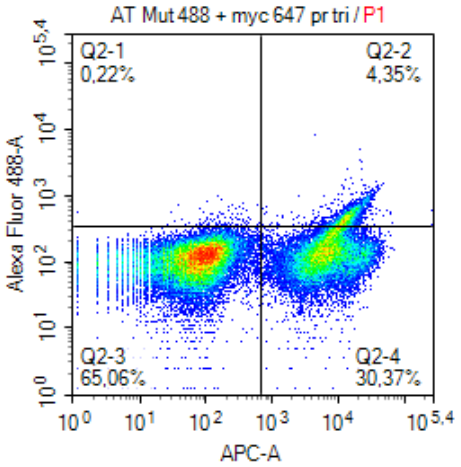
Perspectives

- **Optimization of candidate nanobodies (stability, solubility)**

Yeast-display for nanobody-optimisation



- **Testing of subcutaneous application**

- **Explore AAV-mediated expression**

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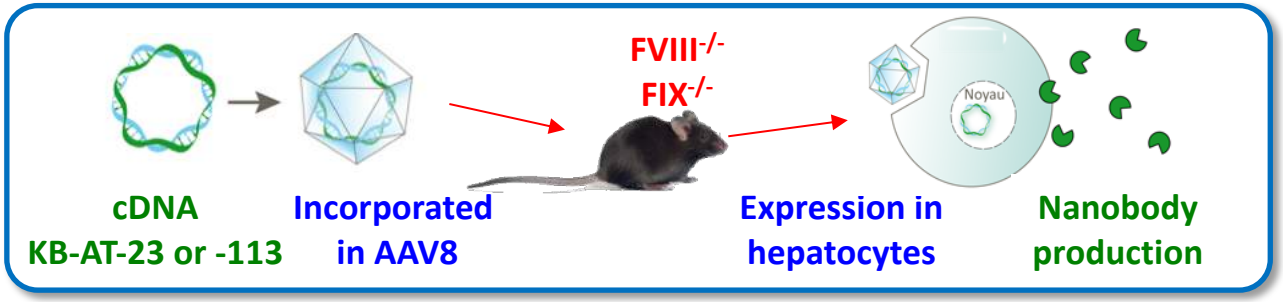
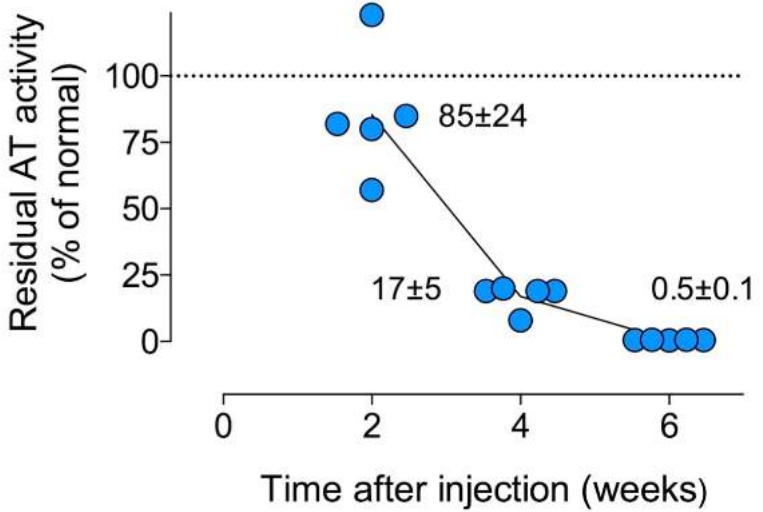
Nanobody expression via gene therapy using AAV8-virus: elimination of antithrombin activity

SMARD - HaemoCare

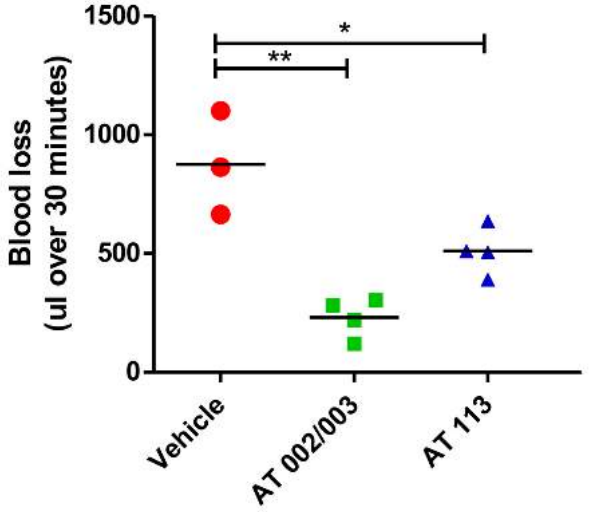
Federico Mingozzi &
Elena Barbon



Efficient loss of antithrombin activity



Efficient reduction of blood loss



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How to improve hemophilia treatment ?

Anti-antithrombin nanobodies

- Improve half-life to reduce infusion frequency ✔
- Avoid intravenous infusions ✔
- Reduce immunogenity ✔
- Reduce costs ✔

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Single domain antibodies (VHH or nanobodies)

- Single domain antibodies: where do they come from
- Nanobodies against antithrombin
- **Factor VIII – nanobody fusion proteins**

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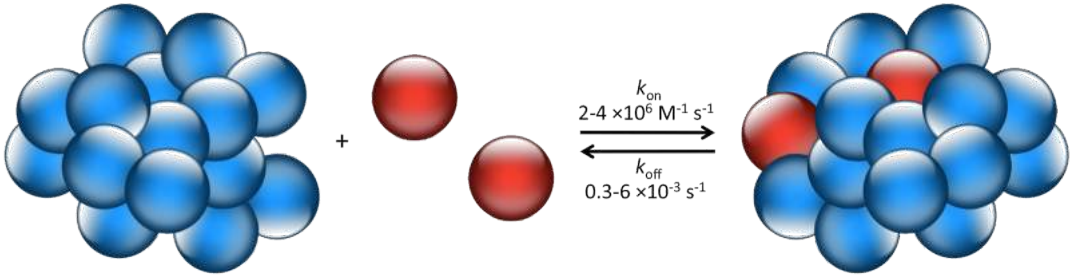
FVIII circulates in complex with von Willebrand factor (VWF)

- VWF protects FVIII from premature clearance
- VWF determines the half-life of FVIII
- VWF modulates immunogenicity of FVIII by affecting uptake by antigen-presenting cells
- Not all FVIII is in complex with VWF



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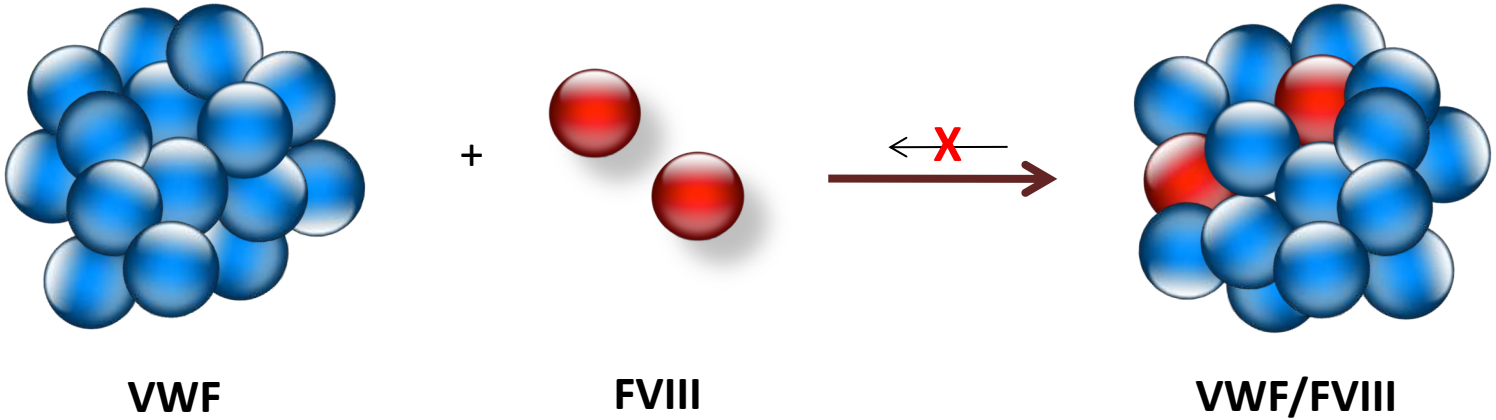
What happens if we create a hyper-stable FVIII-VWF complex?



Investigate effect on:
- Half-life
- Inhibitor formation

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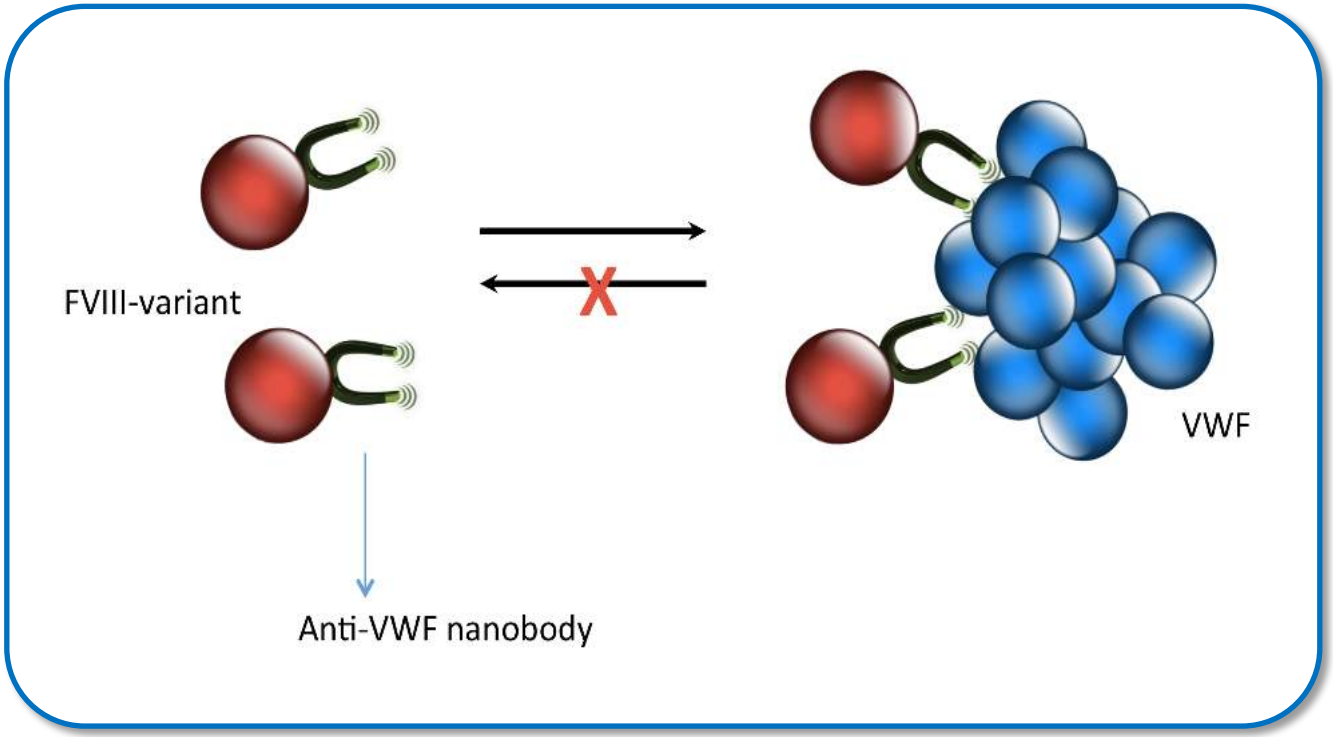
Approach: reduce dissociation of the FVIII – VWF complex via an anti-VWF nanobody



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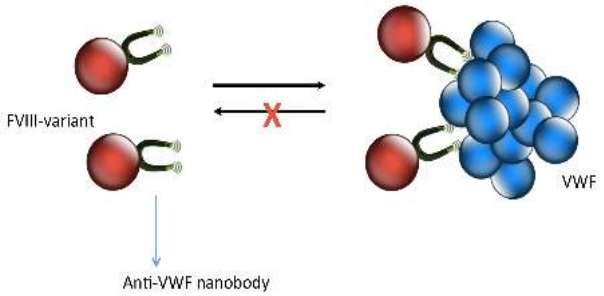
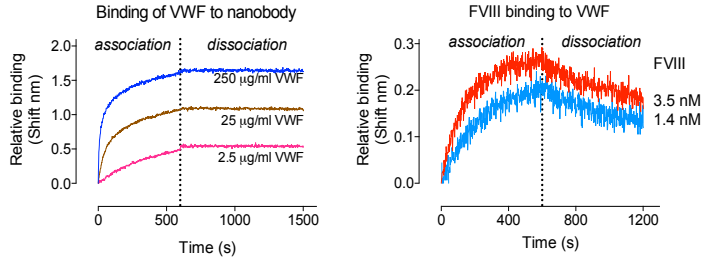
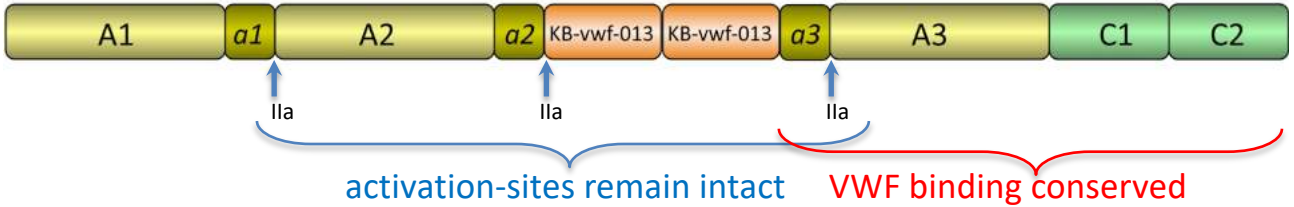
Introduce anti-VWF nanobody in FVIII molecule to prevent dissociation



Select nanobodies targeting VWF



100-fold reduced dissociation rate constant VWF-nanobody vs VWF-FVIII



VWF-binding nanobodies will be released upon activation (like B-domain)

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Analysis of FVIII-nanobody fusion protein

- Expression as recombinant protein in BHK-cells
- Binding to VWF
- Activity (in vitro and in vivo)
- Half-life
- Immunogenicity

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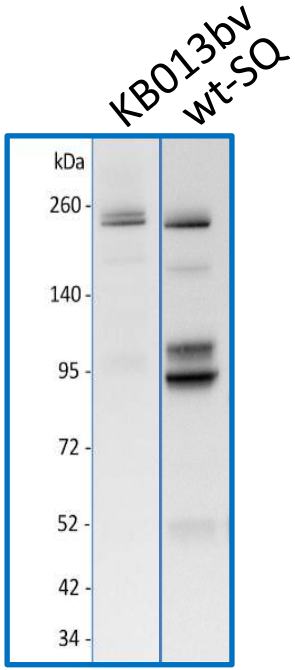
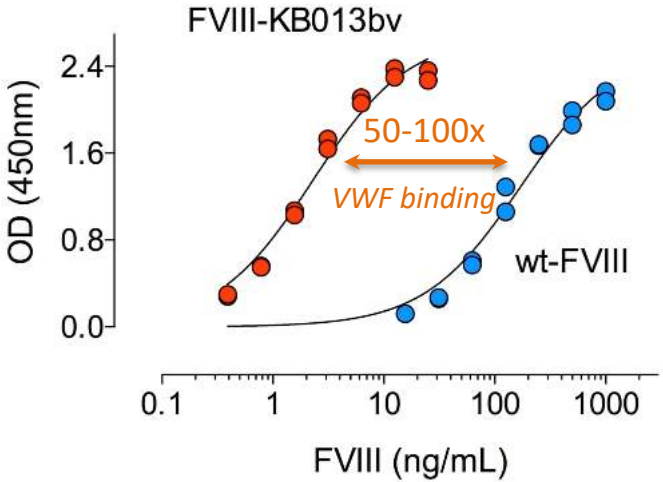
FVIII-KB013bv is a single-chain molecule having substantially increased affinity for VWF

Single-chain protein



Increased VWF binding



Full cofactor activity

Assay	*Activity/antigen ratio*
chromogenic	1.1 ± 0.3 (n=5)
1-stage clotting	1.0 ± 0.3 (n=5)

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FVIII-KB013bv has a prolonged half-life

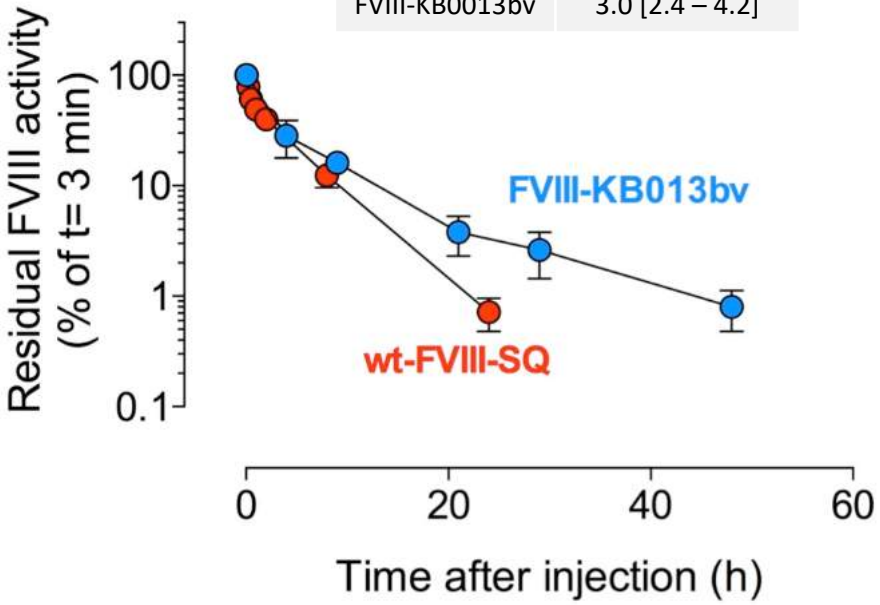
In vivo survival of FVIII-KB013bv

FVIII

F8-deficient

Measure residual FVIII activity

MRT (h [95%CI]*)*	
WT-FVIII-SQ	1.6 [1.3 – 2.1]
FVIII-KB0013bv	3.0 [2.4 – 4.2]



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Analysis of FVIII-KB013bv activity in vivo

In vivo efficacy of FVIII-KB013bv

FVIII (500 U/kg)

F8-deficient

24 h

Tail-clip
Bleeding assay

For tail clip-assay, 0.2 U/ml FVIII are needed to arrest bleeding

When given a dose of 500 U/kg:
- wt-FVIII levels are calculated to be 0.07 U/ml after 24H
  → one would expect bleeding tendency

- FVIII-KB013bv levels are calculated to be 0.27 U/ml after 24H
  → bleeding tendency should be corrected

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FVIII-KB013bv is fully active in vivo

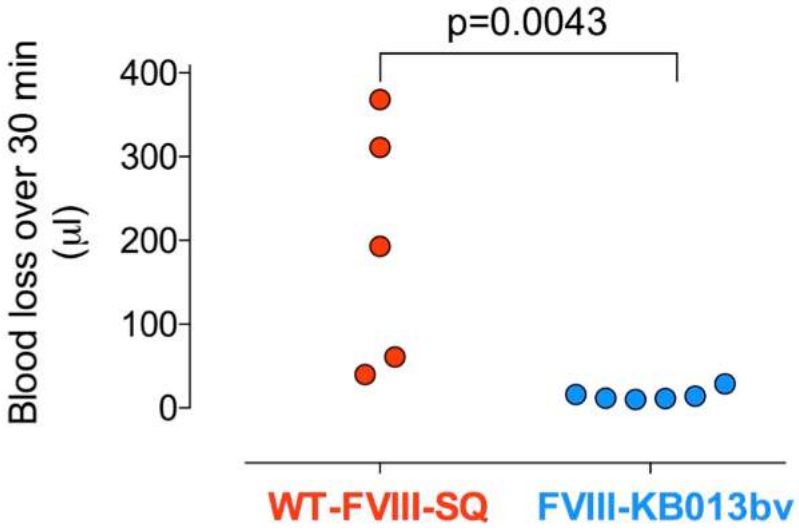
In vivo efficacy of FVIII-KB013bv

FVIII (500 U/kg)

F8-deficient

24 h

Tail-clip
Bleeding assay



For tail clip-assay, 0.2 U/ml FVIII are needed to arrest bleeding

When given a dose of 500 U/kg:
- wt-FVIII levels are calculated to be 0.07 U/ml after 24H
  → one would expect bleeding tendency

- FVIII-KB013bv levels are calculated to be 0.27 U/ml after 24H
  → bleeding tendency should be corrected

Correction of bleeding tendency confirms full activity and prolonged half-life of FVIII-KB013bv

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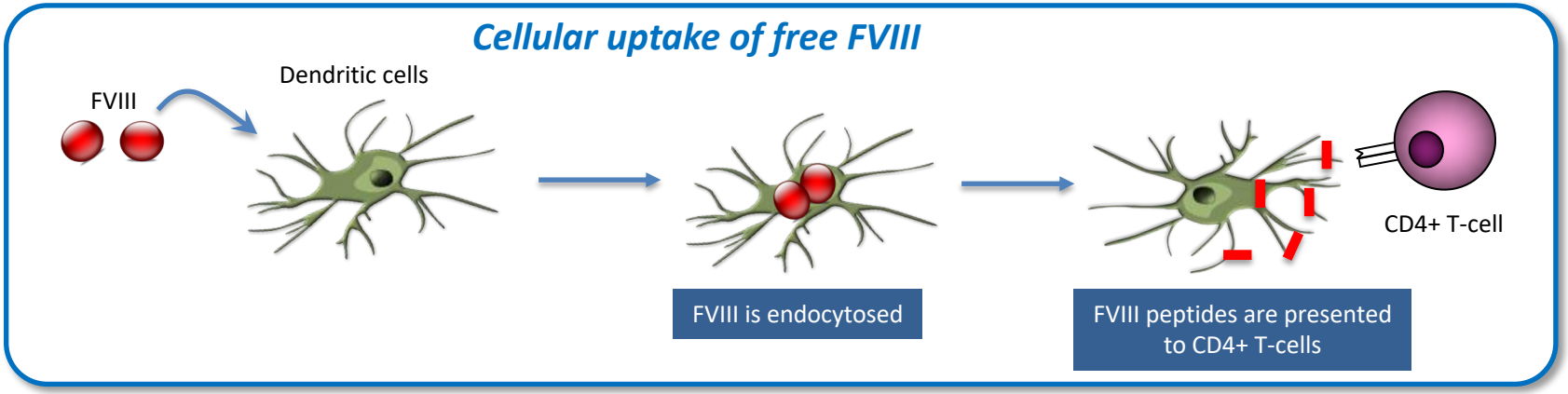
FVIII-KB013bv: any effect on immunogenicity?



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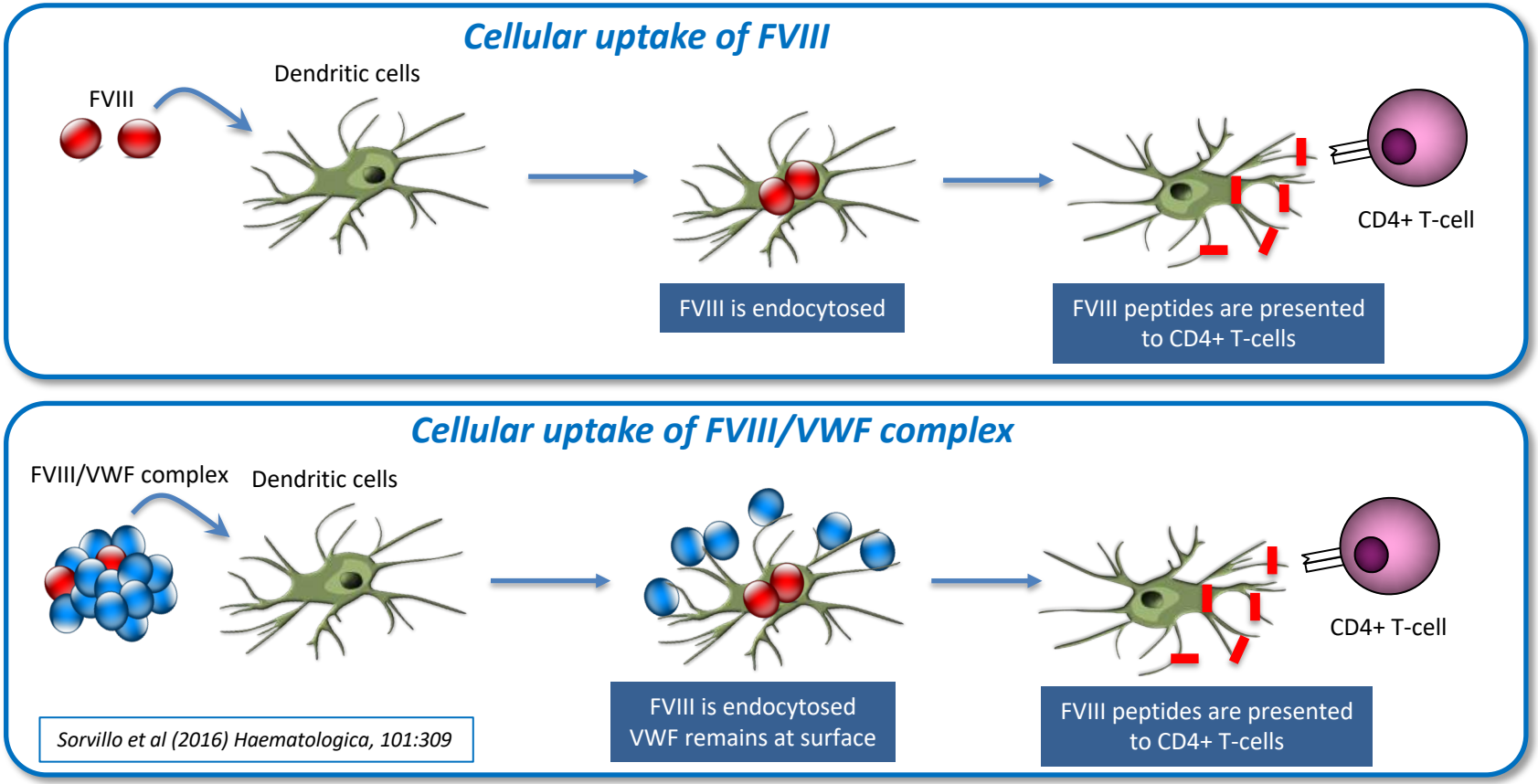
FVIII/VWF complex: FVIII but not VWF enters DCs.



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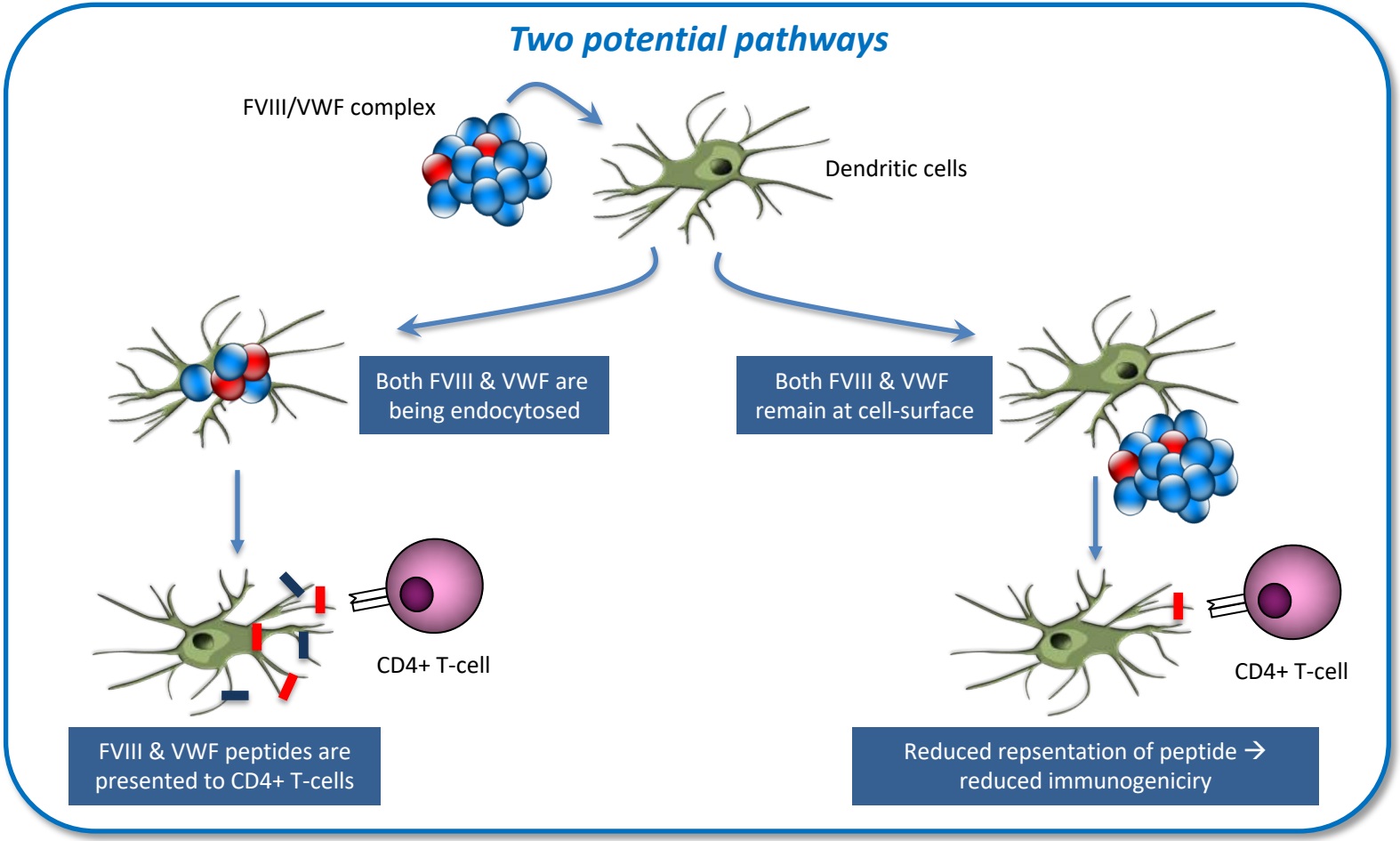
What if FVIII does not dissociate from VWF?



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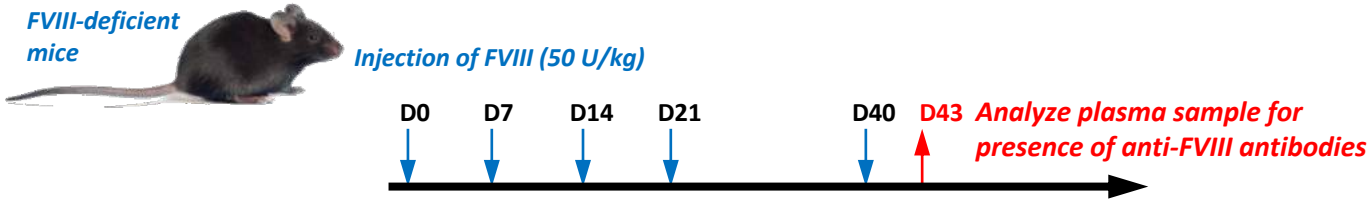
Data on inhibitor development: *wt-FVIII vs FVIII-KB013bv*



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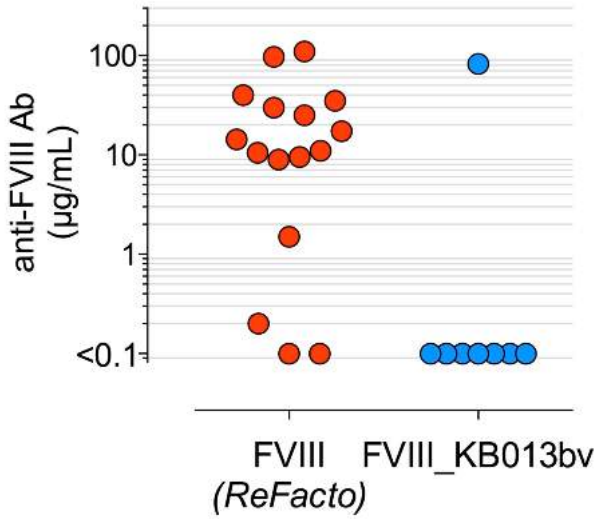
Data on inhibitor development: *wt-FVIII vs FVIII-KB013bv*

FVIII-deficient mice

Injection of FVIII (50 U/kg)

D0 D7 D14 D21 D40 D43 *Analyze plasma sample for presence of anti-FVIII antibodies*



p=0.0056 in Mann-Whitney test

- Fewer mice develop inhibitors
  14/16 mice for wt-FVIII
  1/8 mice for FVIII-KB013bv

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Perspective

- FVIII-nanobody fusion proteins represent a novel approach to modify interactions with its ligands, in particular VWF

- Stabilization of FVIII/VWF interactions:
  - improves the half-life of FVIII
  - reduces the formation of anti-FVIII antibodies

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General conclusion

- Single domain antibodies are efficient tools for the development of novel therapeutic approaches, both regarding protein- and gene-therapy

- The small size of the nanobodies allows straightforward engineering to optimize their application

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Acknowledgements

Cécile Denis
Olivier Christophe
Gabriel Aymé
Vincent Muczynski
Charlotte Kawecki
Caterina Casari
Amel Mohamadi
François Moreau

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Funding:

SMART - HaemoCare

E-Rare CALLS

David Lillicrap (Queen's University)
Federico Mingozzi (Inserm/Genethon)
Dirk Grimm (Heidelberg University)

InsermTransfert

Association française des hémophiles