INNOVATIVE THERAPIES and future prospects for haemophilia treatment

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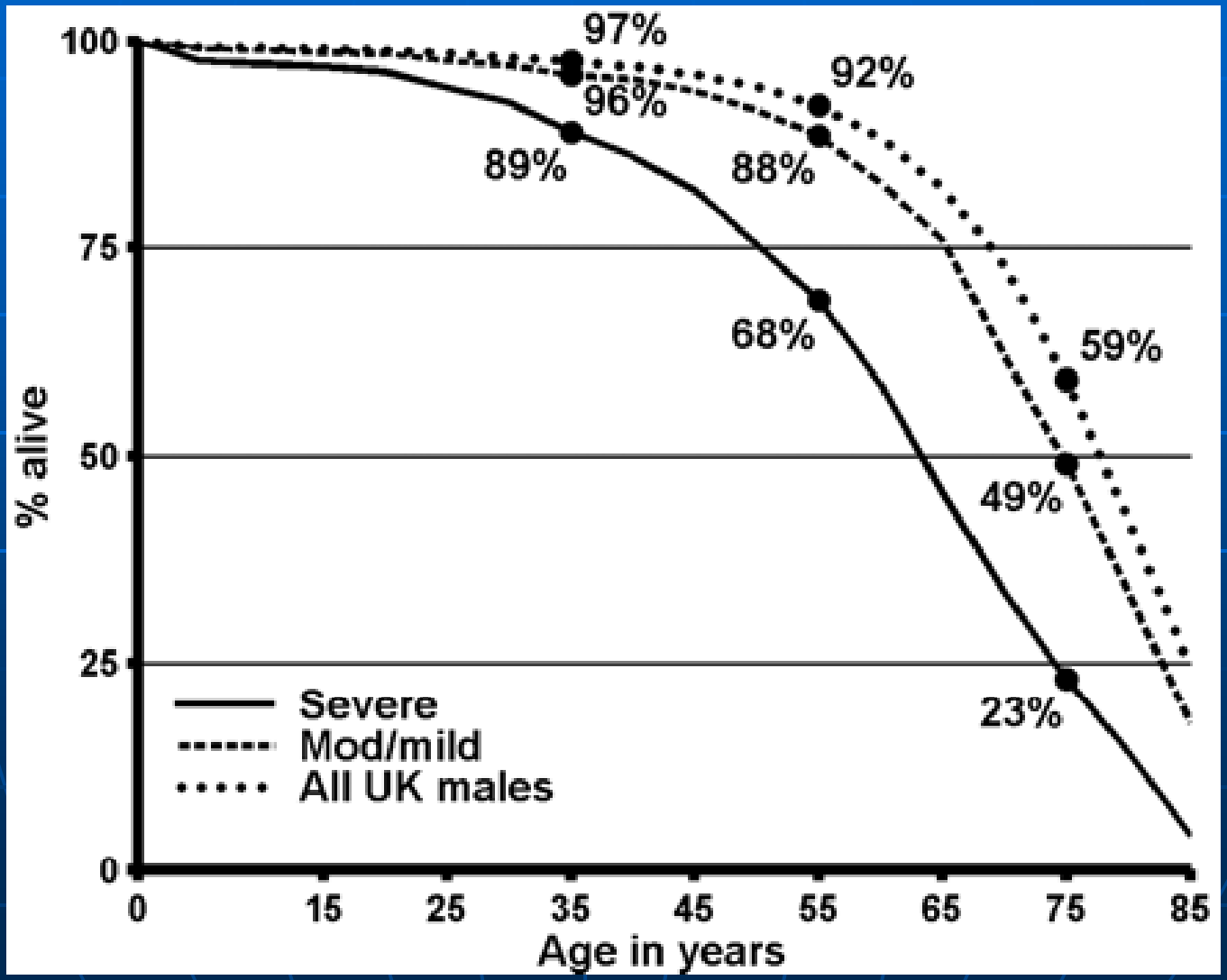
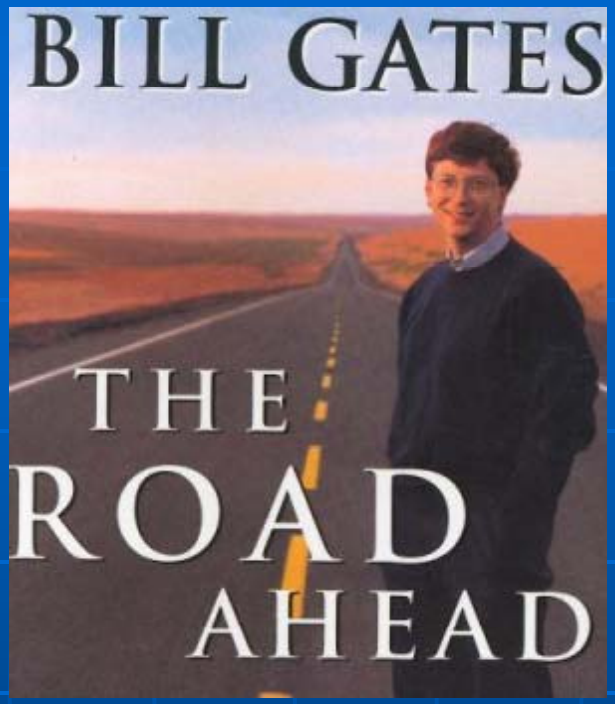
Life expectancy and haemophilia:

Darby SC et al. Blood 110: 815-825 (2007)



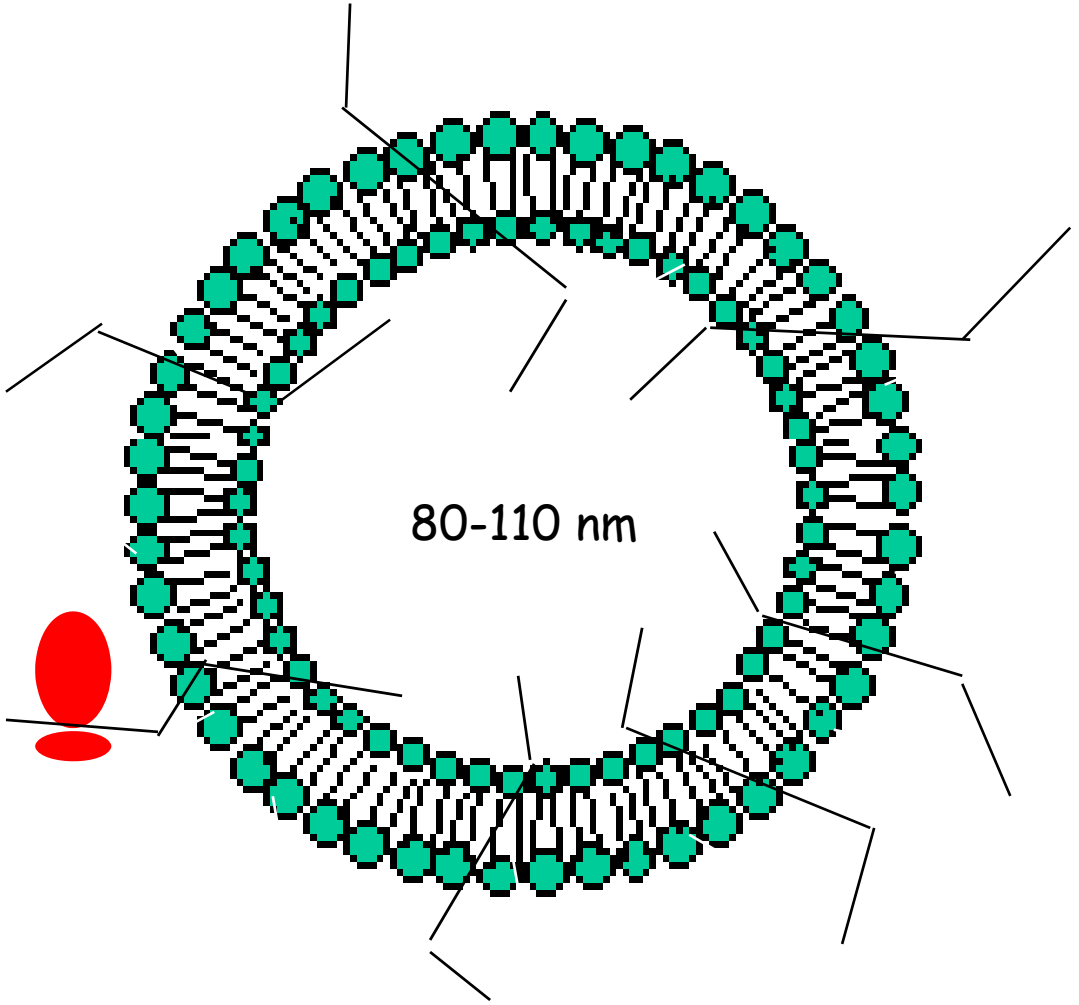


- Starting late can be an advantage
- Avoid mistakes and misfortunes of others
  - Save on cost of development too
- "Gap between have and have-not nations will diminish"

Can we improve on nature?

- Aim to produce modified recombinant proteins with enhanced properties:
  - Prolonged half-life
  - Reduced immunogenicity
  - Improved factor VIII expression in cell lines
  - Hybrid molecules (e.g. human/porcine) for patients with inhibitors
  - Oral administration

Recombinant factor VIII and pegylated liposome:



Early clinical studies:

Spira J, Plyushch OP, Andreeva TA, Khametova RN
Blood 108: 3668-3673 (2006)

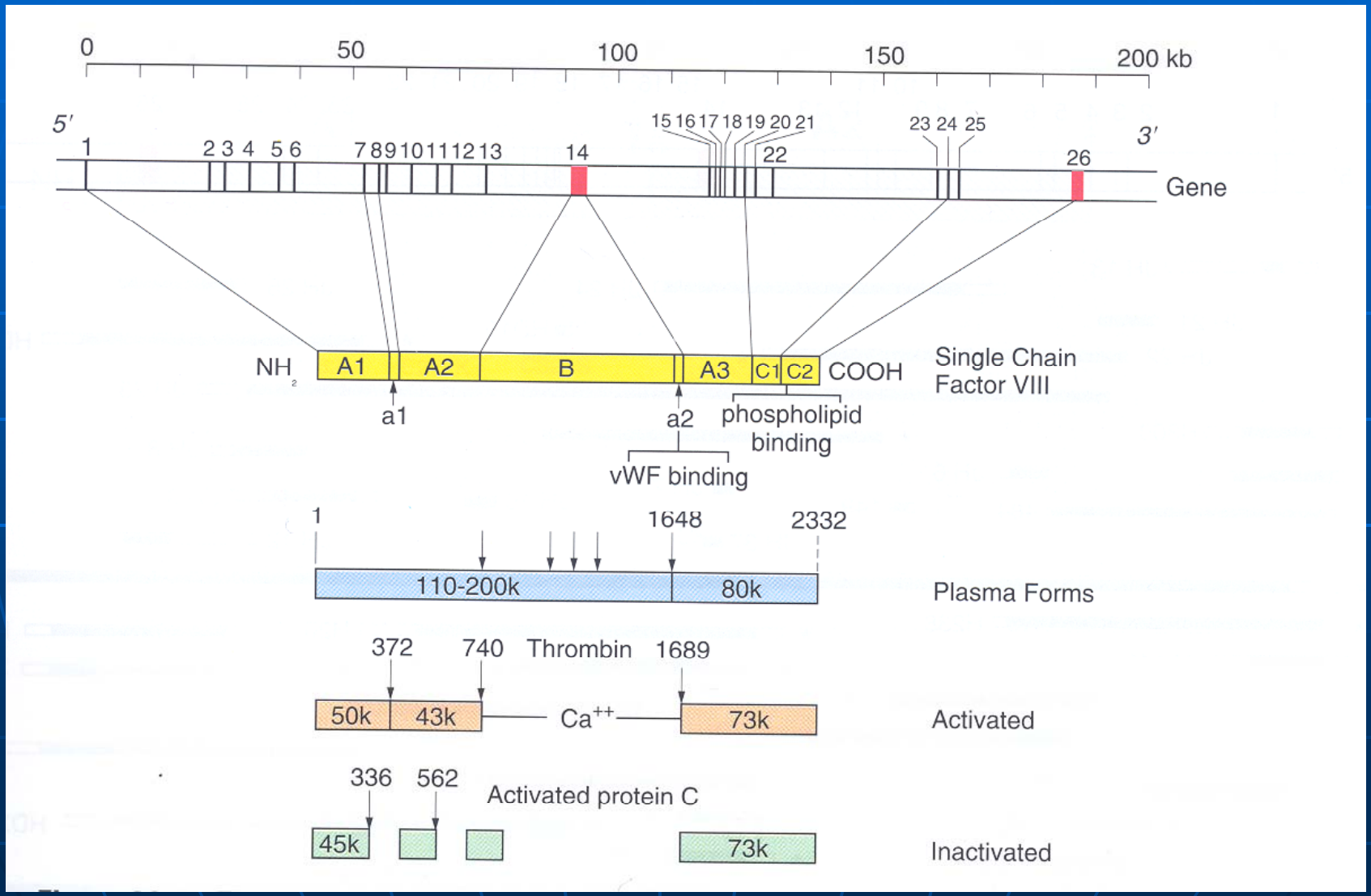
Table 3. Days without a bleeding episode following prophylactic infusions

Treatment	No. of bleeding-free days					
	Mean	Median	Minimum	Maximum	SD	95% CI
rFVIII 35 IU/kg						
Standard	7.2	7.0	5	11	1.7	6.1-8.3
PEGLip	13.3	13.5	6.0	20.5*	4.8	10.1-16.5
rFVIII 25 IU/kg						
Standard		6.5	3	8	1.7	4.8-7.0
PEGLip		11.0	5.5*	16	2.9	9.0-12.7

For patients tr[illegible]with 35 IU/kg rFVIII, n = 11 for each group; with 25 IU/kg rFVIII, n = 12 for each group.

*Mean of 2 infusions.

The factor VIII gene:



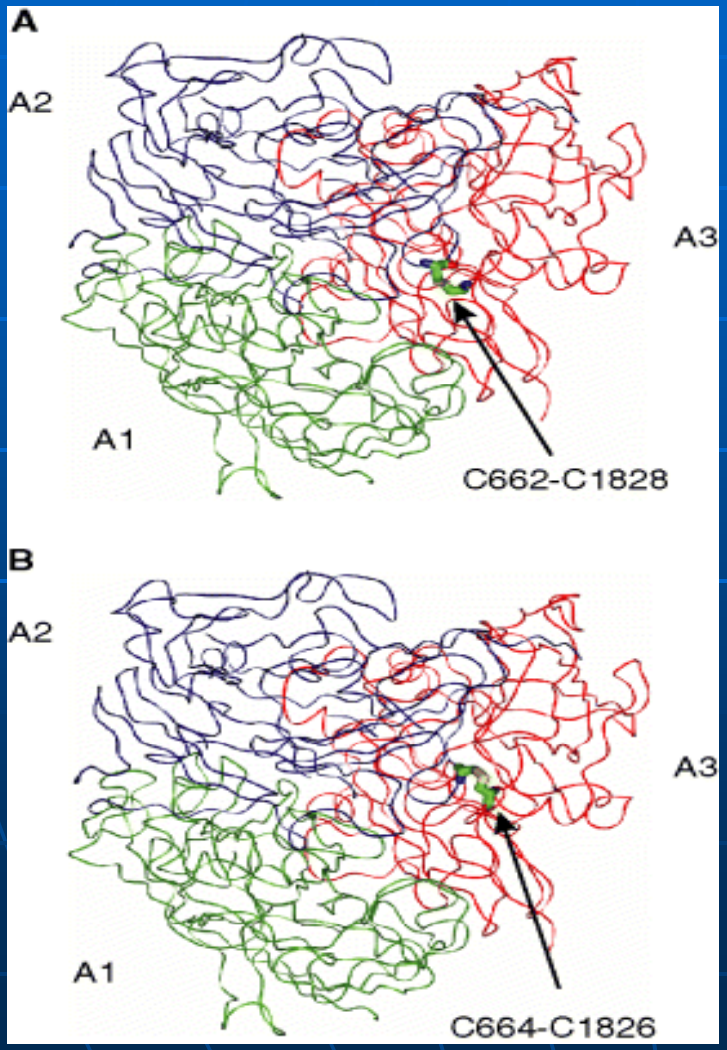
Making a factor VIII molecule resistant to inactivation:

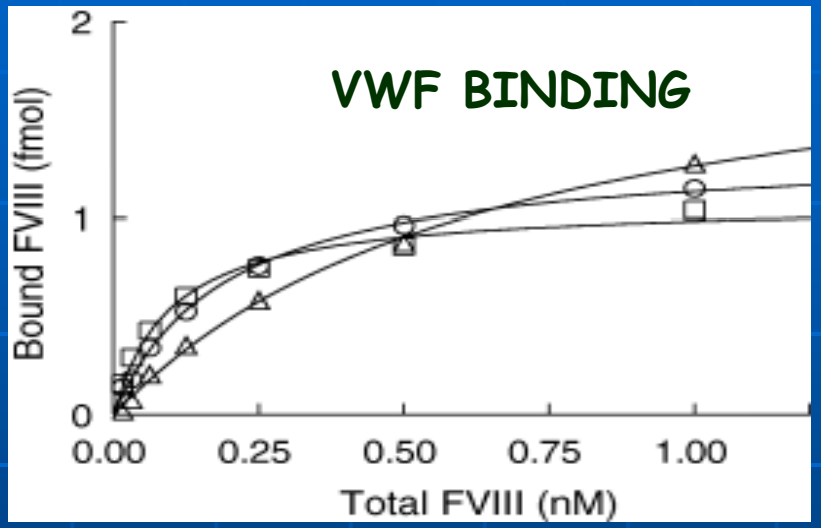
Gale AJ et al. J Thromb Haemostasis 4: 1315-1322 (2006)

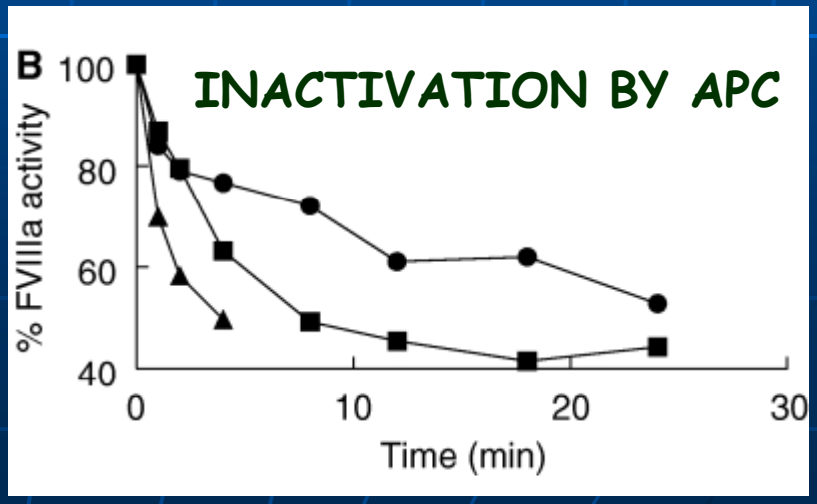
- Normal factor VIII is unstable after activation by thrombin due to spontaneous dissociation of A2 domain
- Creation of disulphide bonds at C664- C1826 and C664-C1826 improves factor VIII stability
- Hinders normal rapid dissociation of A2 domain
- Both variants bind well well to VWF
- Variants inactivated by APC but more slowly

Making a factor VIII molecule resistant to inactivation:

Gale AJ et al. J Thromb Haemostasis 4: 1315-1322 (2006)







Wild-type FVIIIa▲; C662-C1828 FVIIIa ■ ;C664-C1826 FVIIIa●

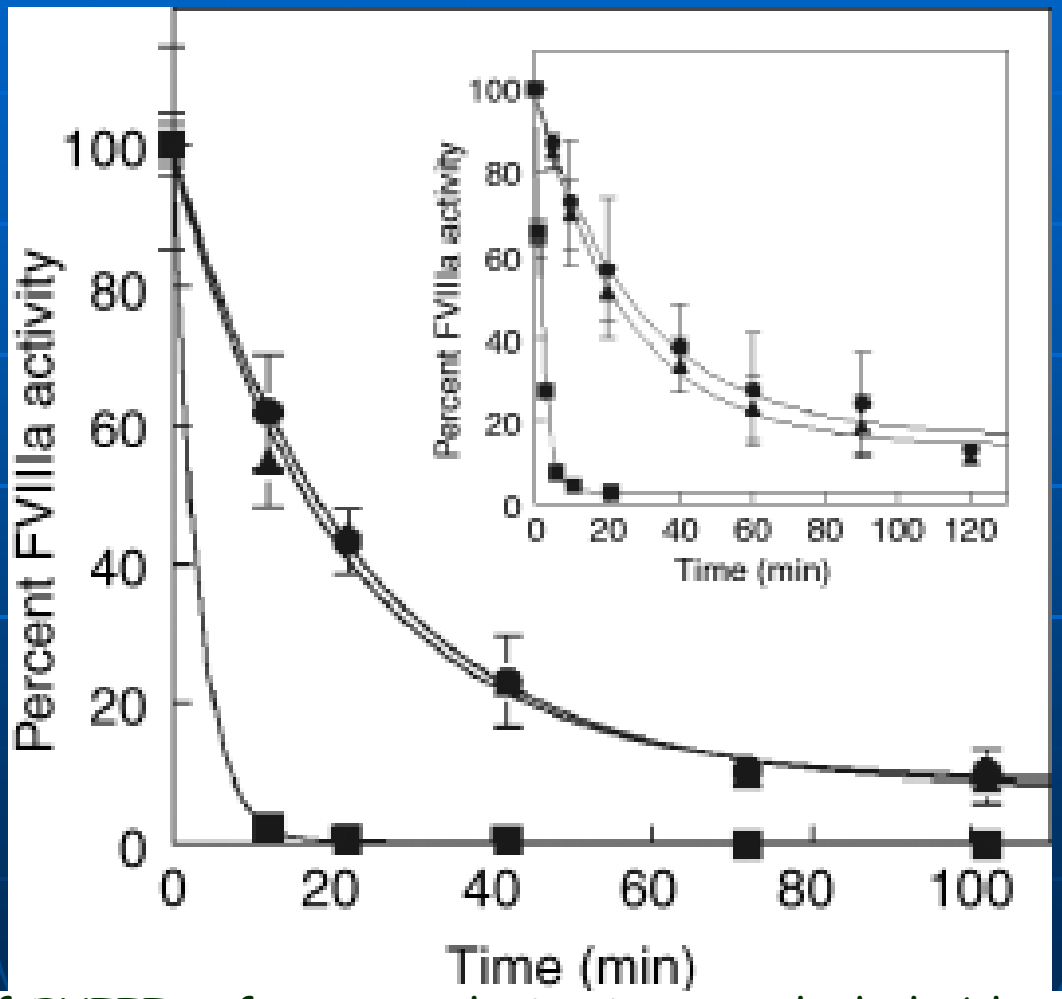
Making a factor VIII molecule resistant to inactivation:

Radtke K-P et al. J Thromb Haemostasis 5: 102-108 (2007)

- Both variants exhibit significantly extended duration of action *in vitro*
- Variants had approximately 5-fold increased half-lives relative to normal FVIII during clot generation
- These genetically-engineered variants also have improved potency:
  - Variants required only 10% as much FVIII to achieve comparable clot-formation rates and clot firmness
  - Stabilised FVIII variants also generate more thrombin than normal FVIII

Making a factor VIII molecule resistant to inactivation:

Radtke K-P et al. J Thromb Haemostasis 5: 102-108 (2007)

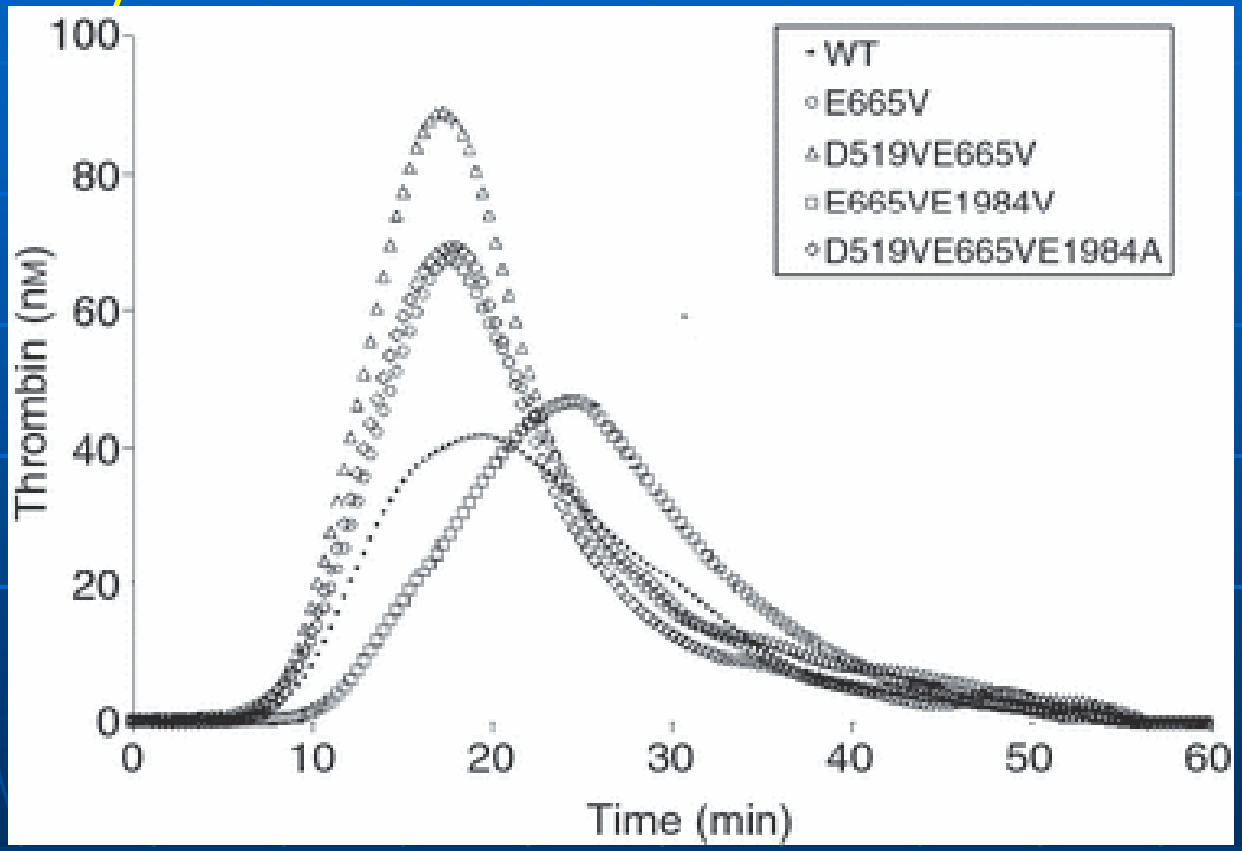


Inactivation of FVIIIa after coagulation in normal whole blood serum.

Wild-type FVIIIa ■ ; C662-C1828 FVIIIa ●;C664-C1826 FVIIIa ▲

Mutations in A2 domain can result in increased FVIII stability:

Wakabayashi H et al. J. Thromb. Haemostasis 7: 438-444 (2008)



Thrombin generation assay. Thrombin generation assays were performed in the presence of 0.3 nmol L−1 factor VIII proteins, 0.5 pmol L−1 rTF, and 4 μmol L−1 PSPCPE vesicles.

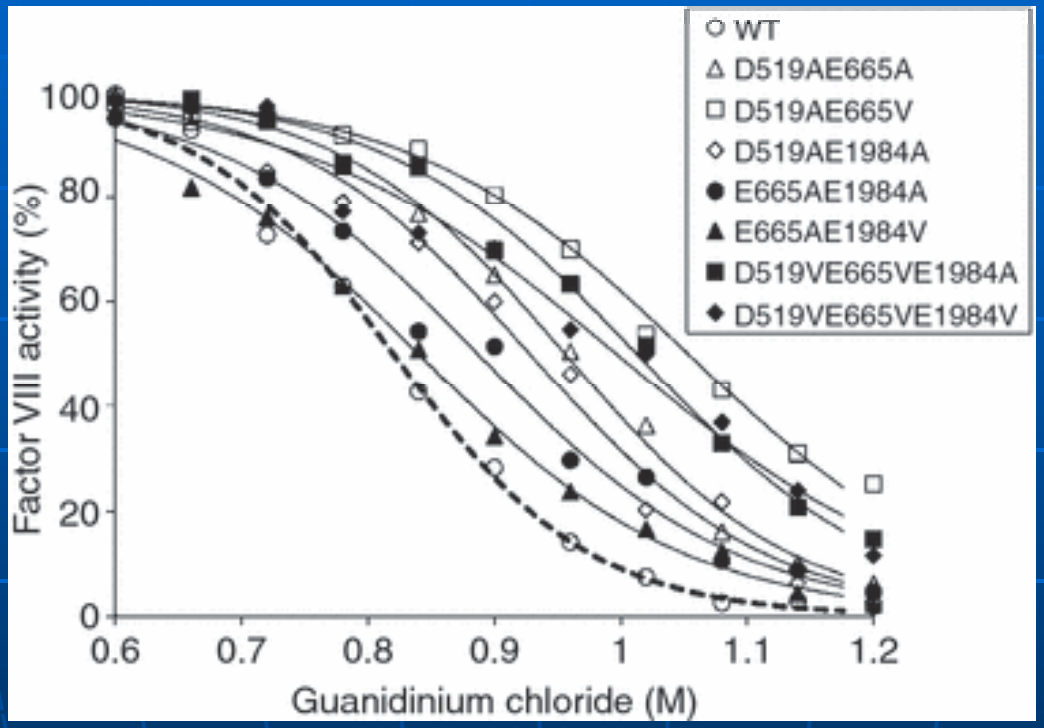
Mutations in A2 domain can result in increased FVIII stability:

Wakabayashi H et al. J. Thromb. Haemostasis 7: 438-444 (2008)

- Mutations induced at three sites:
  - Asp(D)519; Glu(E)665; Glu(E)1984
  - Replaced with either Ala(A) or Valine (V)
- Reduced charge and/or increased hydrophobicity at A2/A1 and A2/A3 domain interfaces
- In vitro testing of heat stability and thrombin generation performed
- Increased duration of action and stability
- Better results with combination of 2 mutation: e.g. D519 and either E665 or E1984

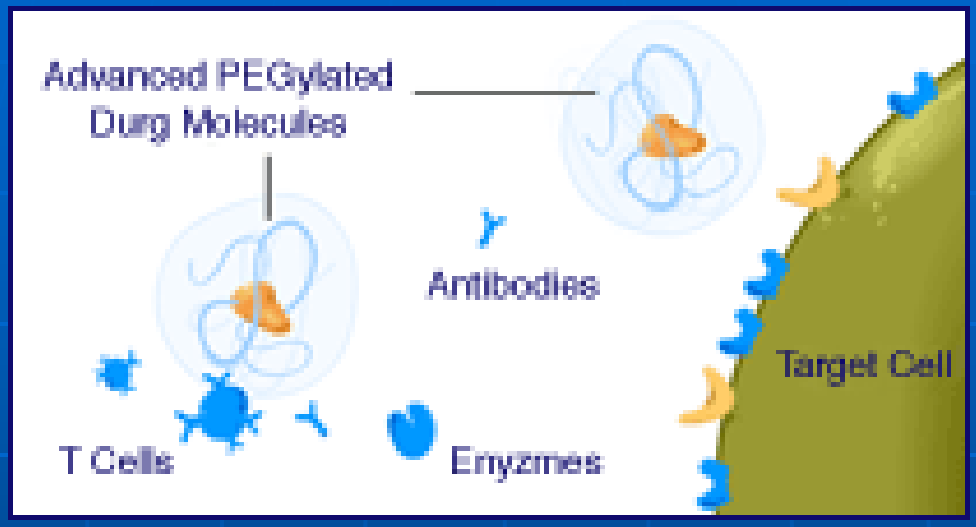
Mutations in A2 domain can result in increased FVIII stability:

Wakabayashi H et al. J. Thromb. Haemostasis 7: 438-444 (2008)



Activity inhibition of WT and selected factor VIII variants by guanidinium. Factor VIII (50 nmol L–1) in 0.6–1.2 M guanidinium chloride was incubated for 2 h at 23 °C, diluted 1/50 and factor VIII activity was measured by factor Xa generation.

Pegylation:



When attached to a drug, polyethylene glycol (PEG) polymer chains can sustain bioavailability by protecting the drug molecules from immune responses and other clearance mechanisms. In an aqueous medium, the long, chain-like PEG molecule is heavily hydrated and in rapid motion. This motion causes the PEG to sweep out a large volume and prevent the interference of other molecules.

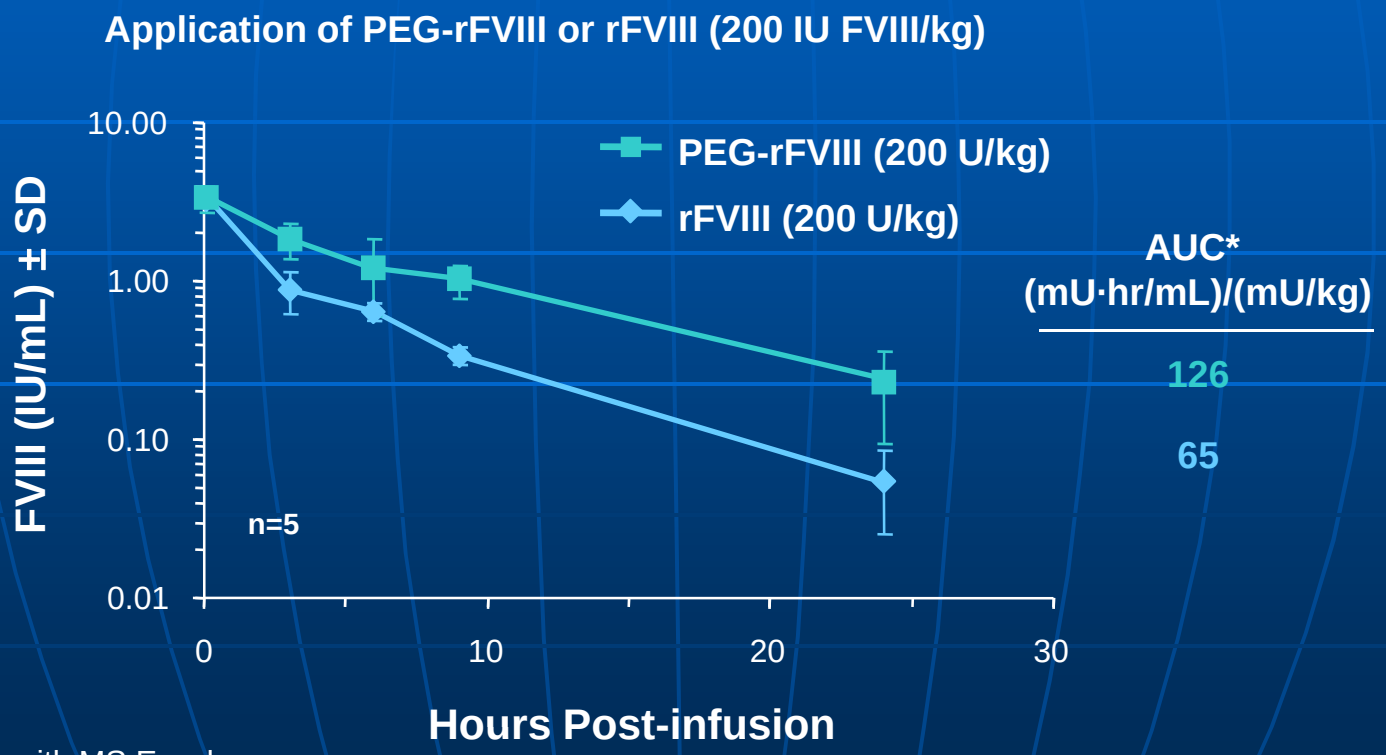
Pegylation of FVIII:

Turecek PL et al. ASH 2007 Abstract 3147

- Full-length rFVIII subjected to pegylation
- PEG-FVIII retained about 30-40% of activity
- SDS-PAGE and immunoblot similar to normal rFVIII
- Function retained *in vitro*: activated and inactivated by thrombin
- Pharmacokinetics in haemophilic mice:
  - given 200 iu/kg PEG-FVIII or rFVIII
  - blood samples taken up to 24 hours
  - $T\frac{1}{2}$ 4.9 hrs with PEG-rFVIII vs. 1.9 with rFVIII
  - AUC approximately doubled with PEG-rFVIII

PEGylated rFVIII in haemophilia A knock-out mice:

half-life increased ~ 2-fold



*Calculation with MS Excel.
AUC = area under the curve.

Pegylated coagulation factors:

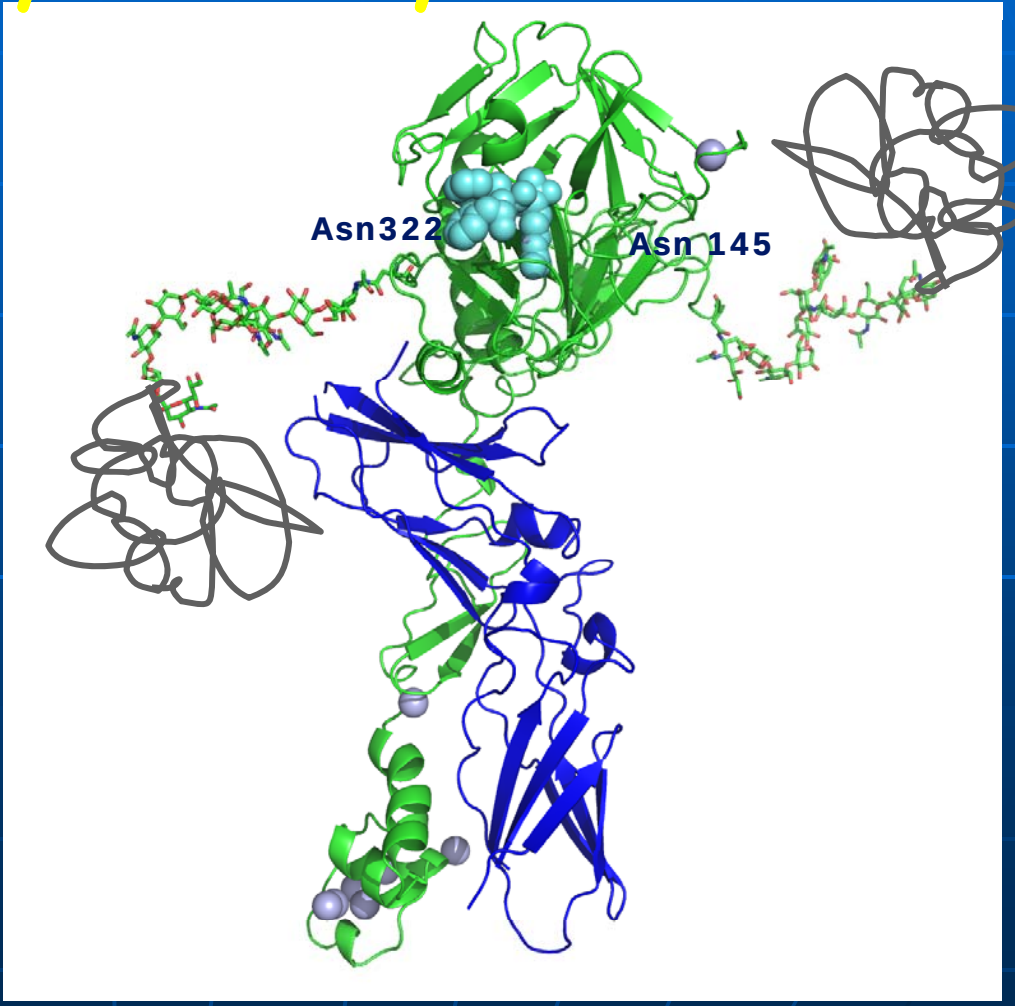
- "rFVIII can be …modified with PEG whilst at least partly retaining its major functions, but at the same time prolonging its survival in the circulation"
- Baxter also using to pegylation to develop factor IX and von Willebrand factor molecules with prolonged action

Long acting rFVIIa by site specific glycoPEGylation:

Application of a selective enzymatic technology which does not effect the protein backbone

glycoPEGylation:

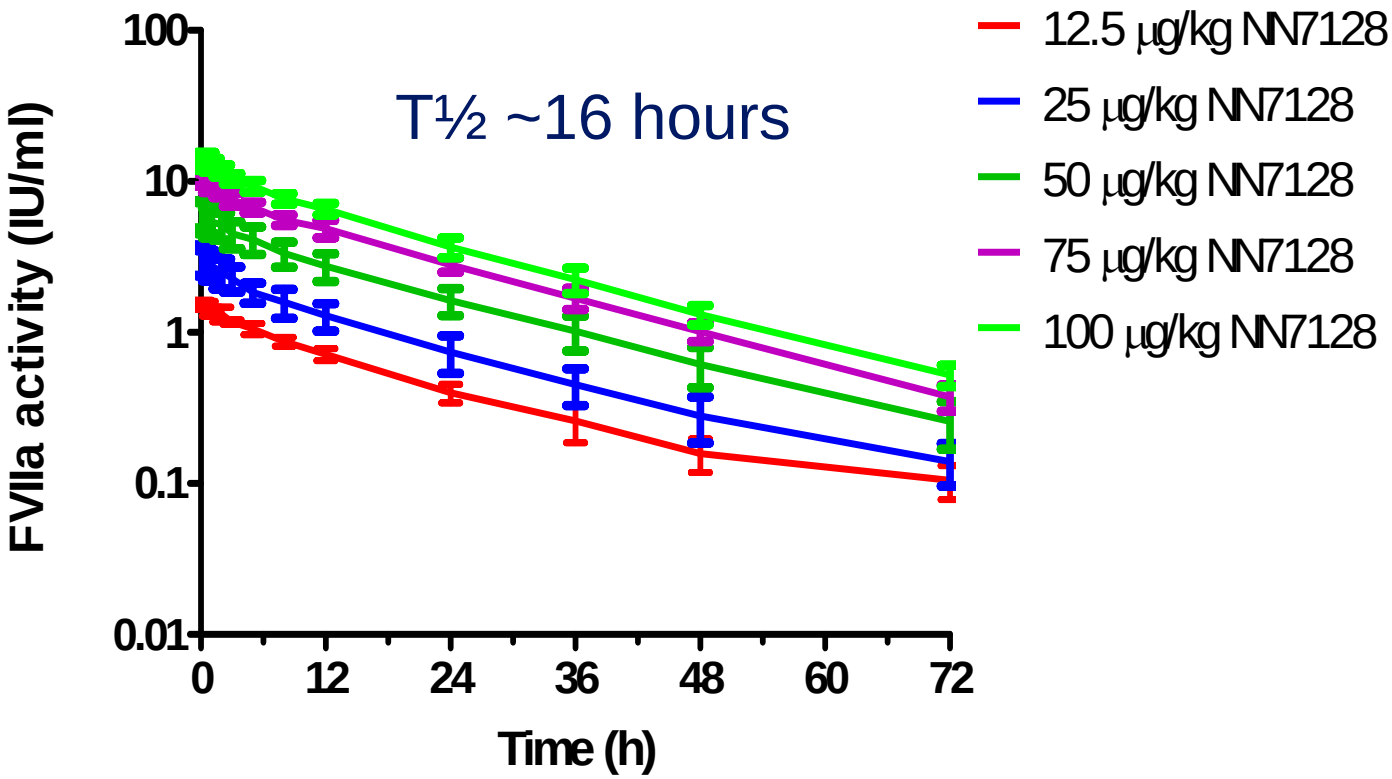
Specific pegylation at tips of the natural glycan structures found in rFVIIa



Stennicke et al, JTH suppl. 2007

Long Acting rFVIIa in healthy volunteers:

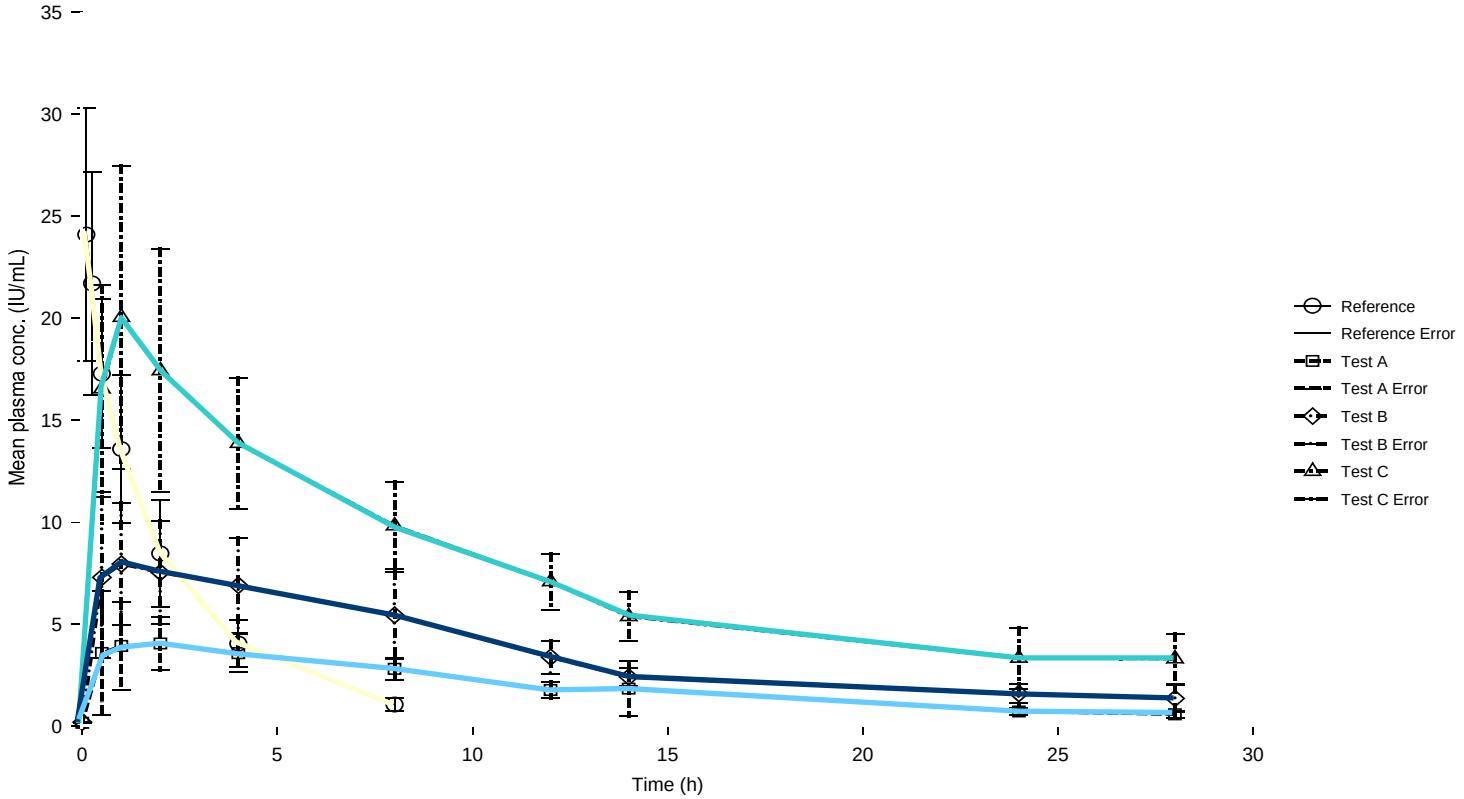
Mean plots of 12.5, 25, 50, 75 and 100 µg/kg



GlycoPEGylated rFVIIa:

- In vitro studies:
  - Effective interaction with TF and FX
  - TFPI and AT effectively inhibits GlycoPEGylated FVIIa-TF complexes
  - Full effect measured by thrombin generation and clot formation in whole blood
- Animal models:
  - Significantly prolonged functional plasma half-life in mice, rabbits, dogs, pigs, and monkeys
  - Reduction of bleeding in haemophilic mouse model: tail bleed
  - Extended duration of action in rabbit venous stasis model

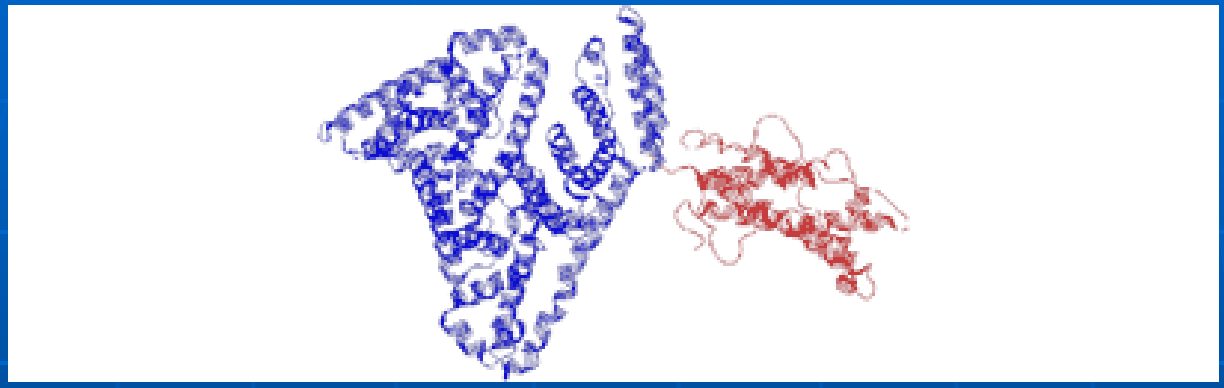
Subcutaneous administration of NovoSeven:



Mean FVIIa activity versus time profiles following i.v. administration of reference (90 µg/kg rFVIIa/NovoSeven®) and s.c. administration of NN7720 at 180 µg/kg (Test A), 360 µg/kg (Test B) and 720 µg/kg (Test C) to pigs

Albumin fusion (Albafuse™):

www.novozymes.com



- Marketed as "the natural alternative to pegylation"
- Genetic engineering to fuse albumin with other molecules
- "Albuferon" ready to be marketed
- Recombinant FVIIa-albumin fusion protein developed:
  - half-life extended 6 to 9 fold in rats and rabbits
  - 30 amino-acid spacer to avoid steric interference from albumin
  - function in vivo and in vitro comparable to rVIIa (NovoSeven)

    Schulte S et al. ASH 2007 (abstract 3142)

    Schulte S et al. WFH 2008 (abstract 08 FP 04)

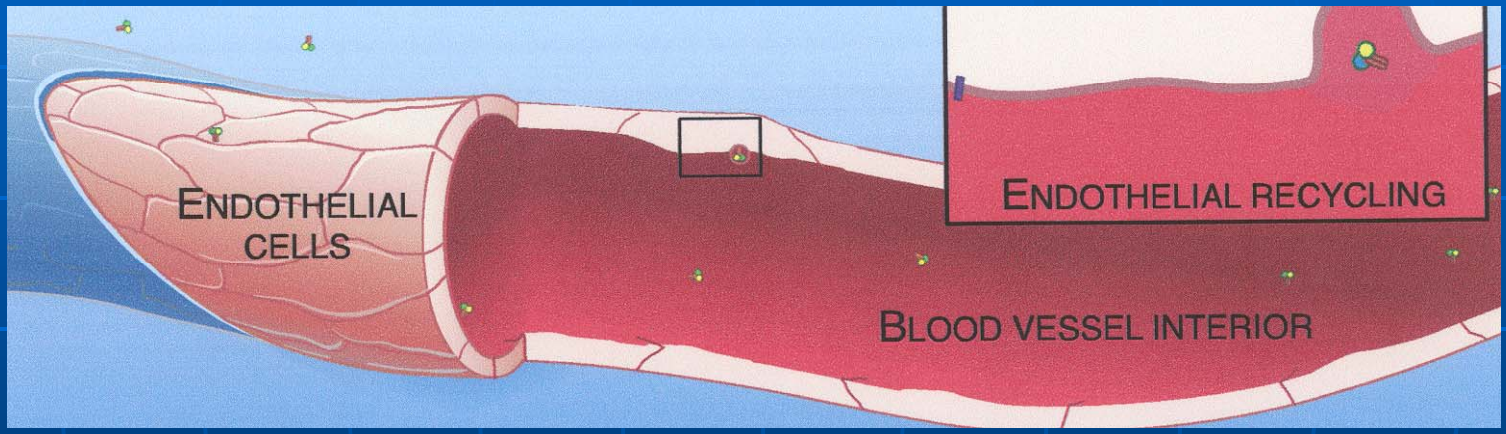
Long-life factor IX:

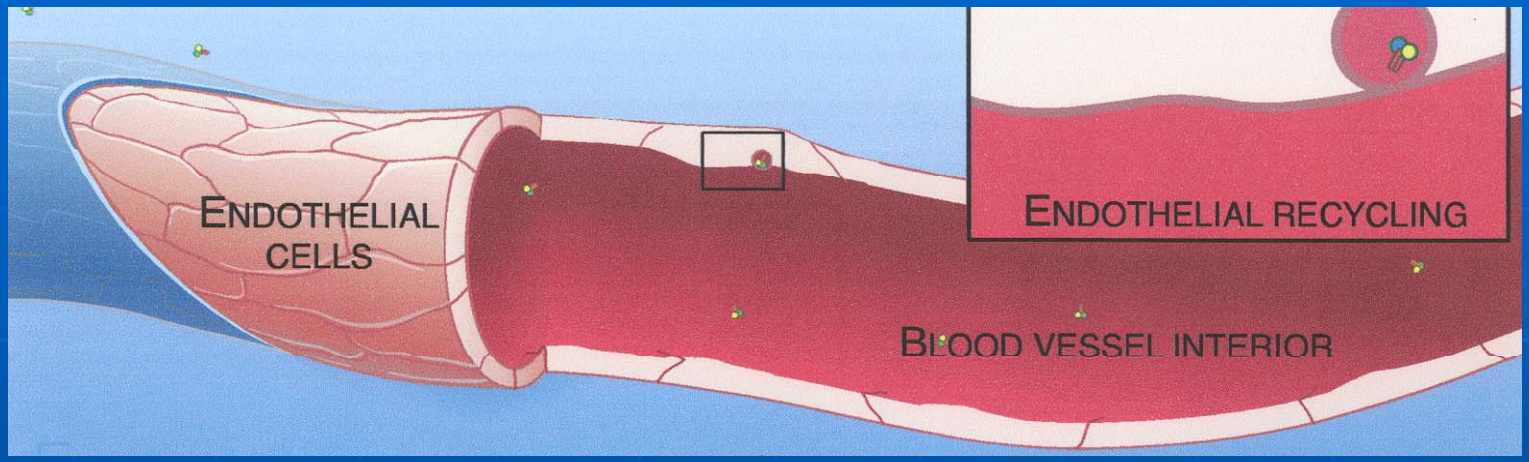
www.syntnx.com/synfusion.php

- Biovitrum and Syntonix working together to develop factor IX product with enhanced half-life
- SynFusion™ drugs consist of a novel Fc-fusion construct that links a single copy of the drug to the Fc region on an antibody
- Optimized to bind to Fc receptors (FcRn) in the endothelial cells that line the blood vessels, "recycling" the drug to increase its circulating half-life.

Endocytosis and FcRn:

- IgG antibodies have a very long half-life in the human body
- FcRn mediates this longevity by preventing degradation. IgG taken up into endothelial cells will either be bound by FcRn and released back into the circulation, or if they are not bound they will be degraded
- This ability of FcRn to bind and release functional IgG's back into the bloodstream acts like recycling (endocytosis), allowing IgG antibodies to outlive other antibodies.


ENDOTHELIAL CELLS
ENDOTHELIAL RECYCLING
BLOOD VESSEL INTERIOR


ENDOTHELIAL CELLS
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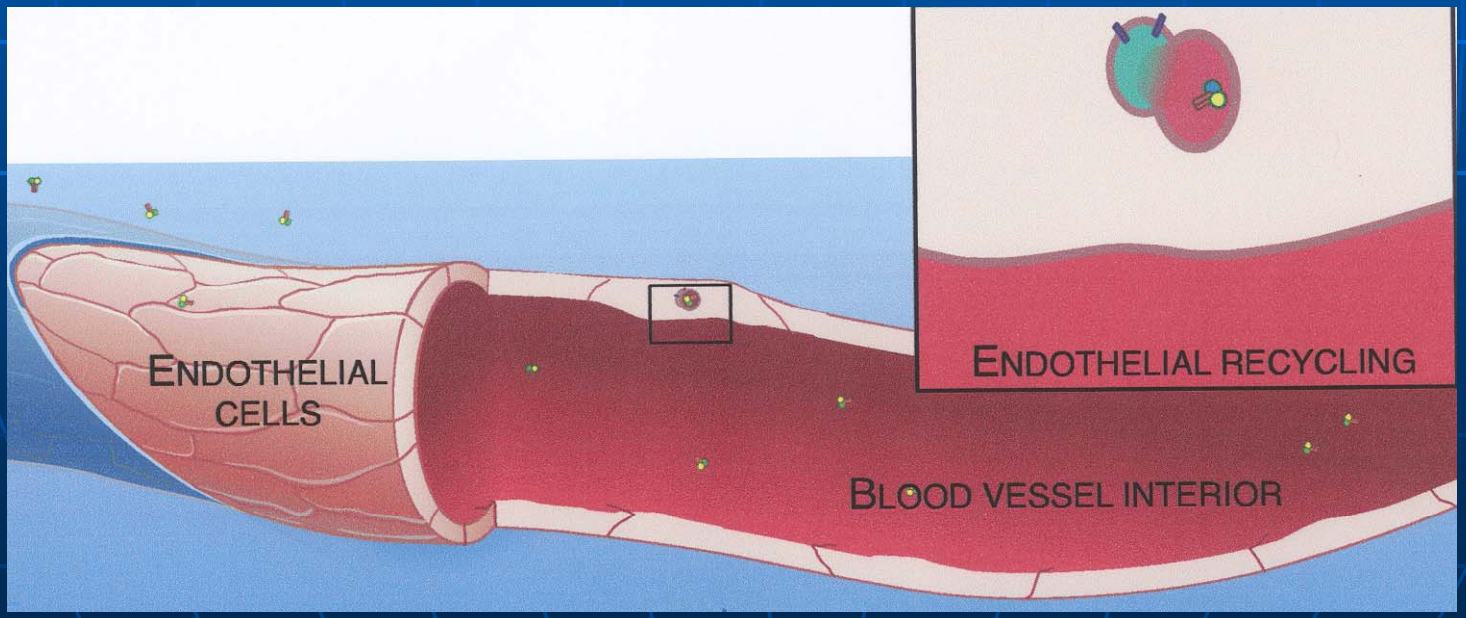
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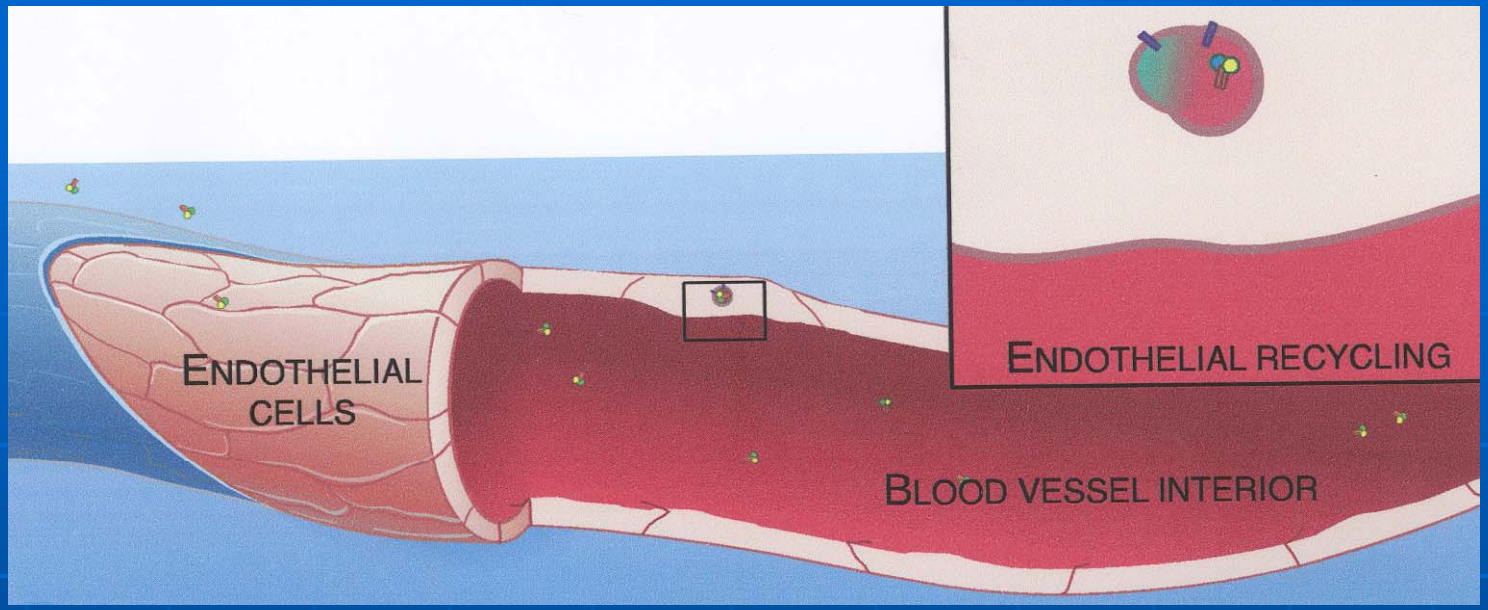
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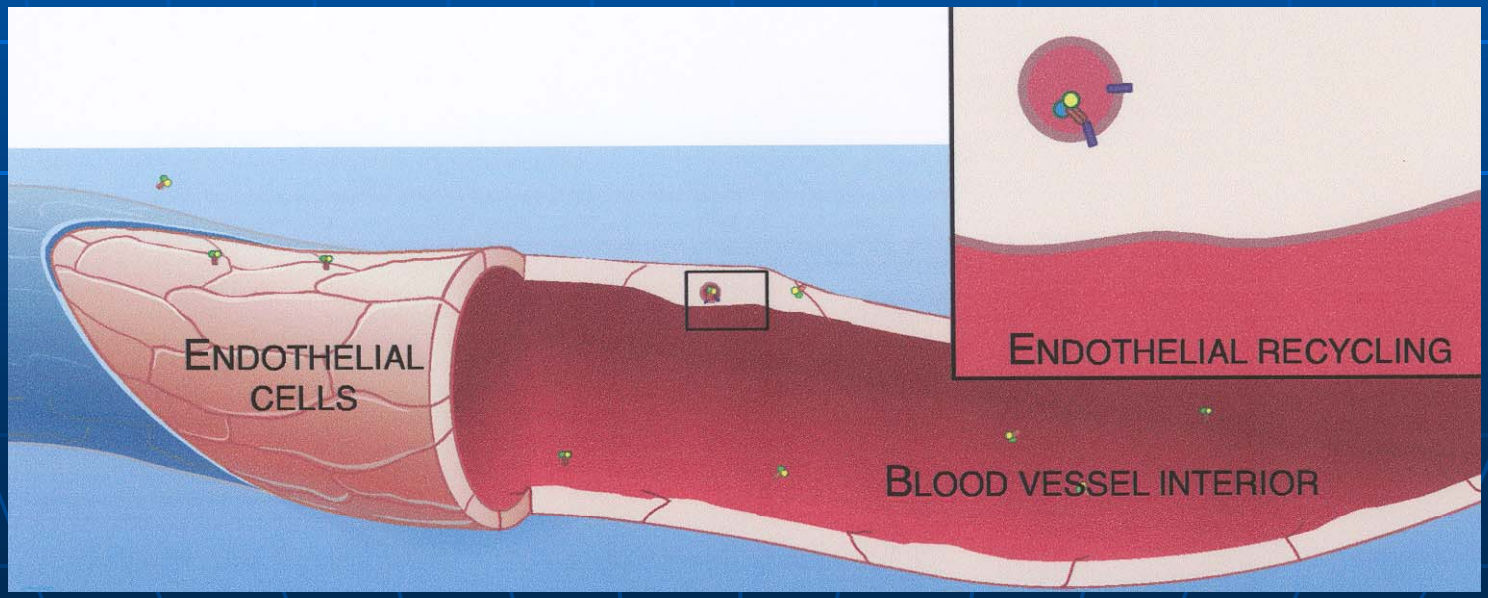
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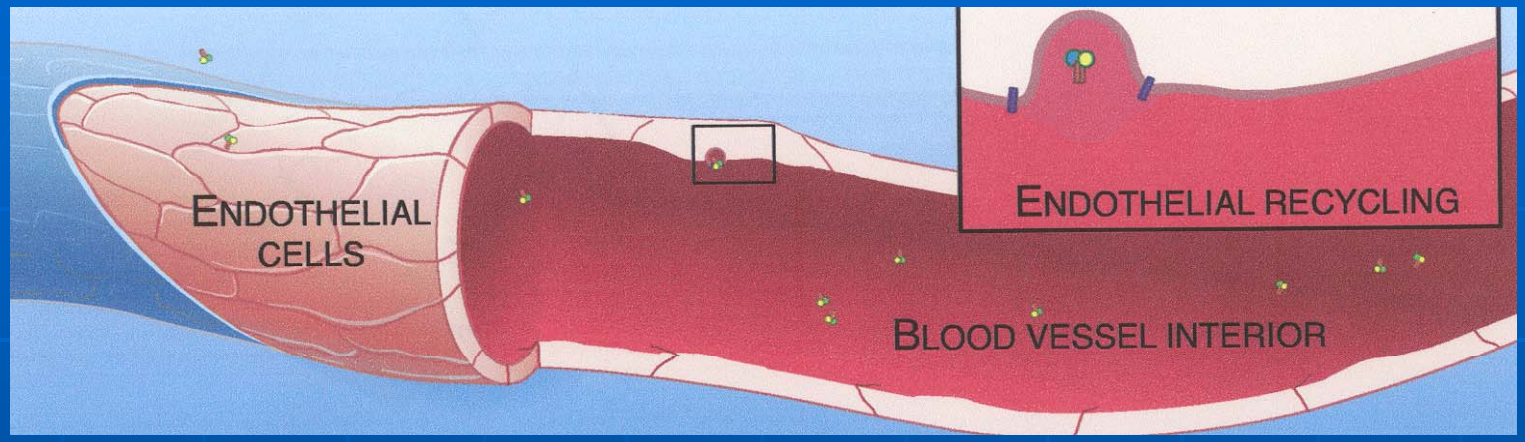
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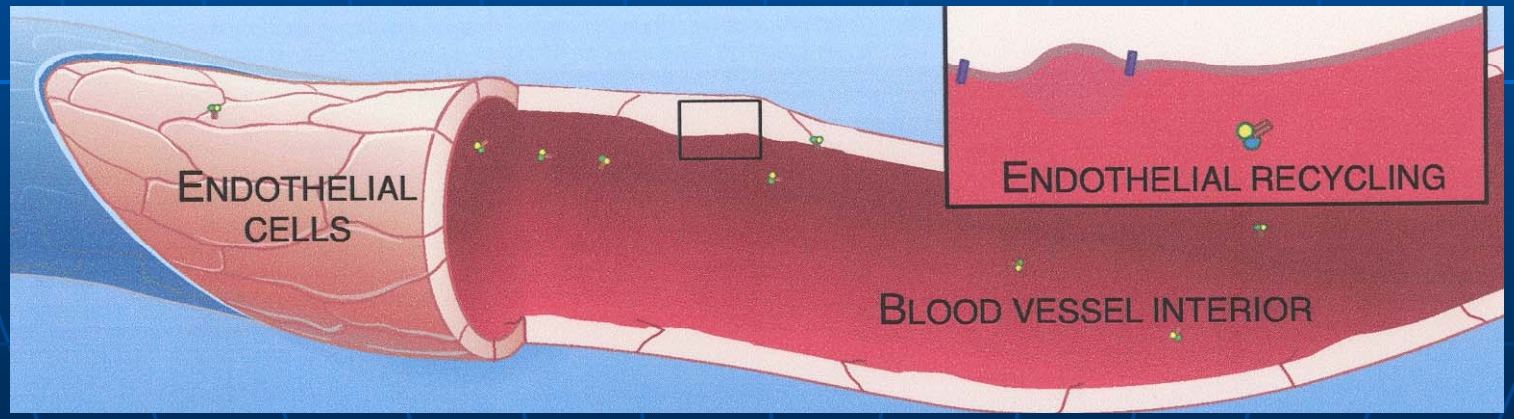
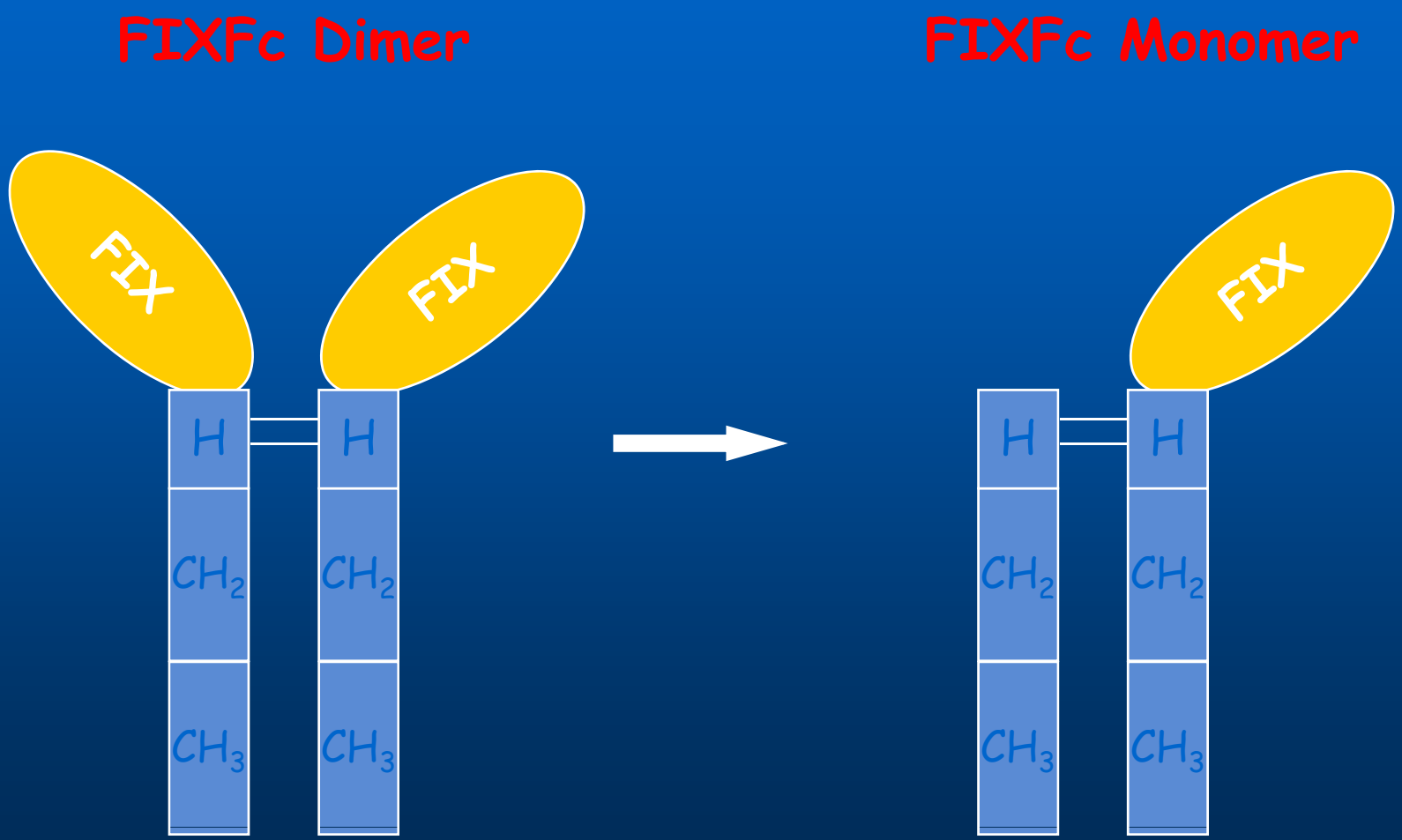
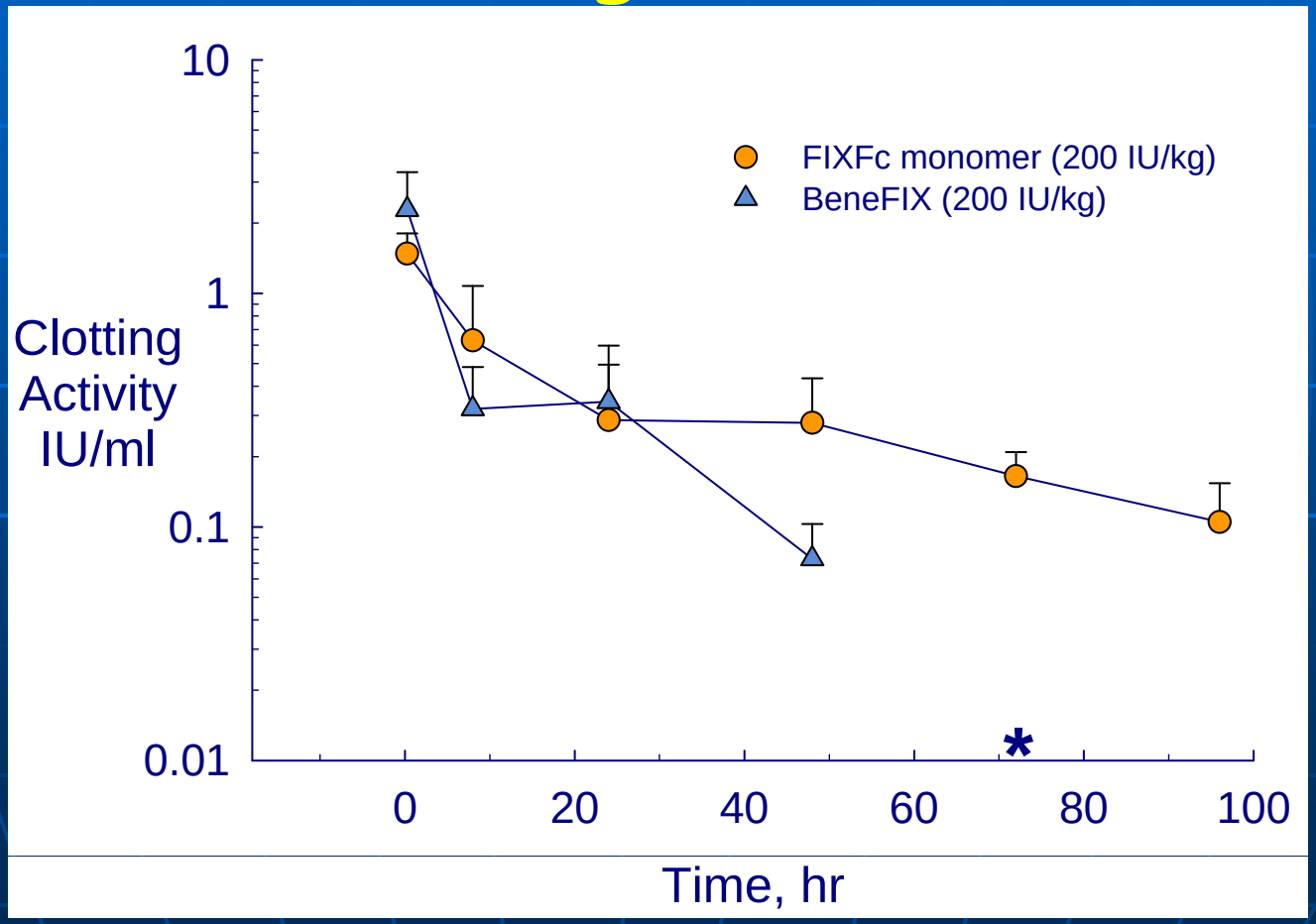

ENDOTHELIAL CELLS
ENDOTHELIAL RECYCLING
BLOOD VESSEL INTERIOR

Structure of Fc Fusion Monomers: SynFusion™



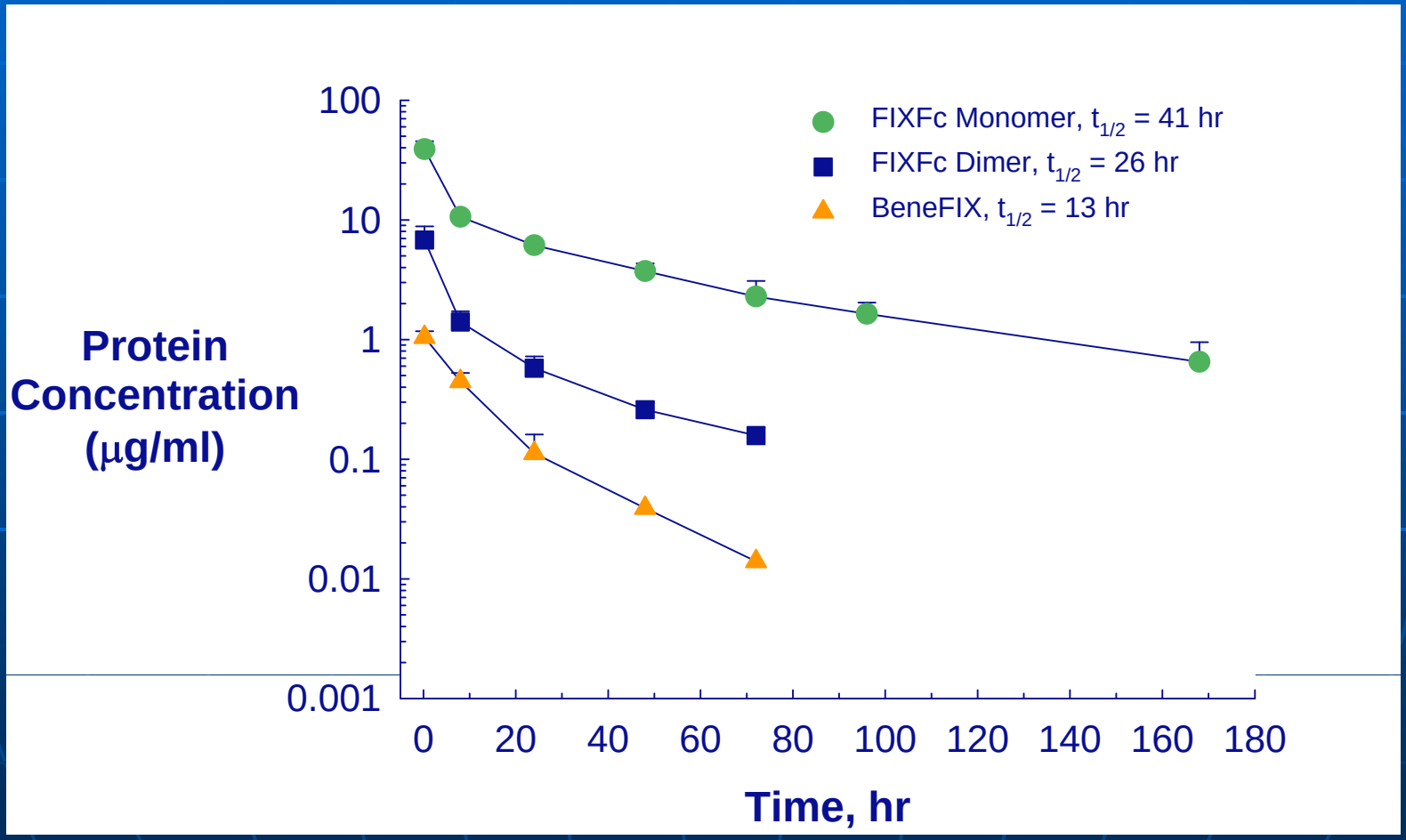
Activity of FIXFc Monomer v. BeneFIX in FIX-Deficient Mice After Single IV Dose:



*BeneFIX activity undetectable after 48 hr

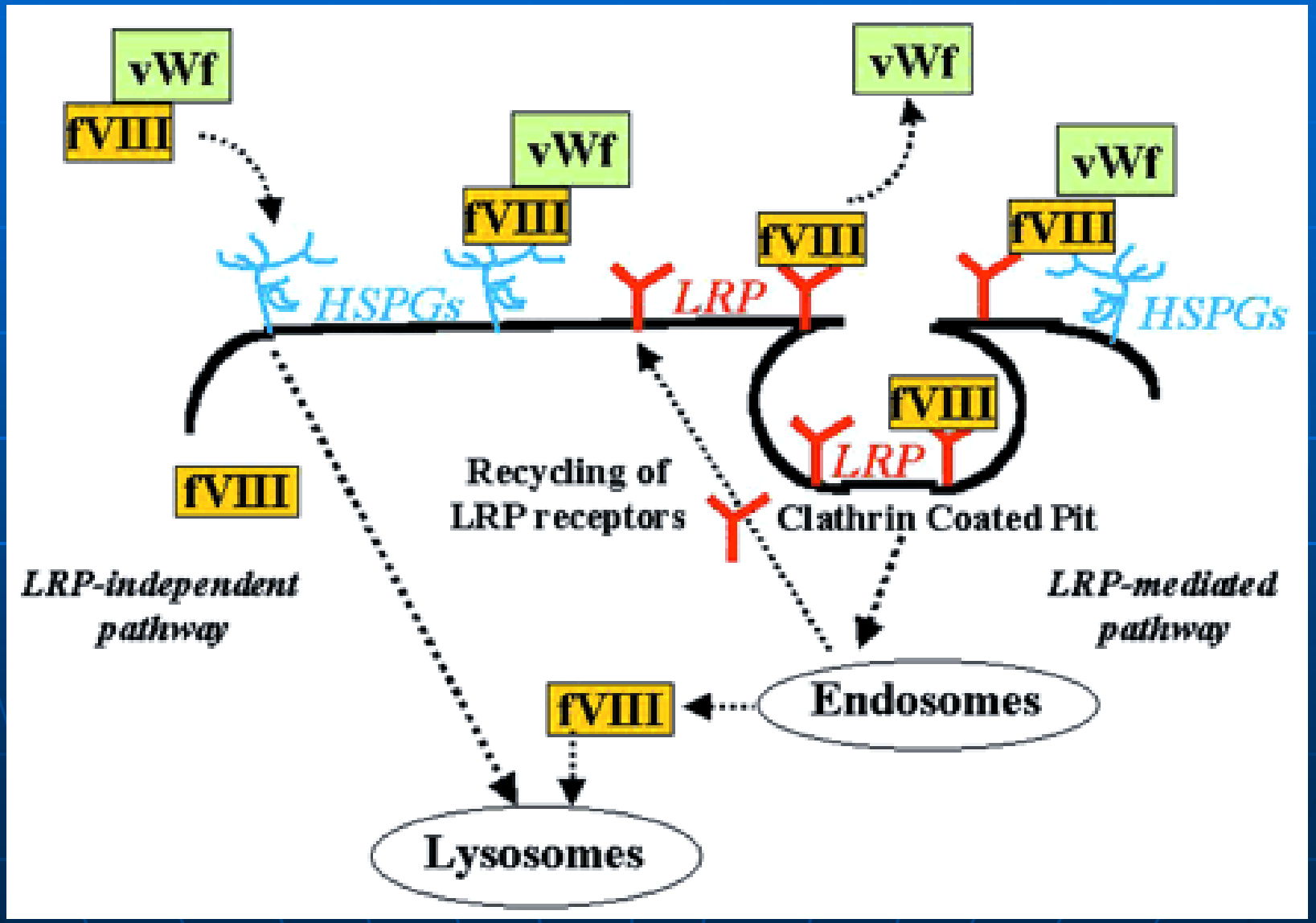
FIXFc Monomer $t_{1/2}$ = 47

Intravenous Pharmacokinetics in FIX-deficient mice:



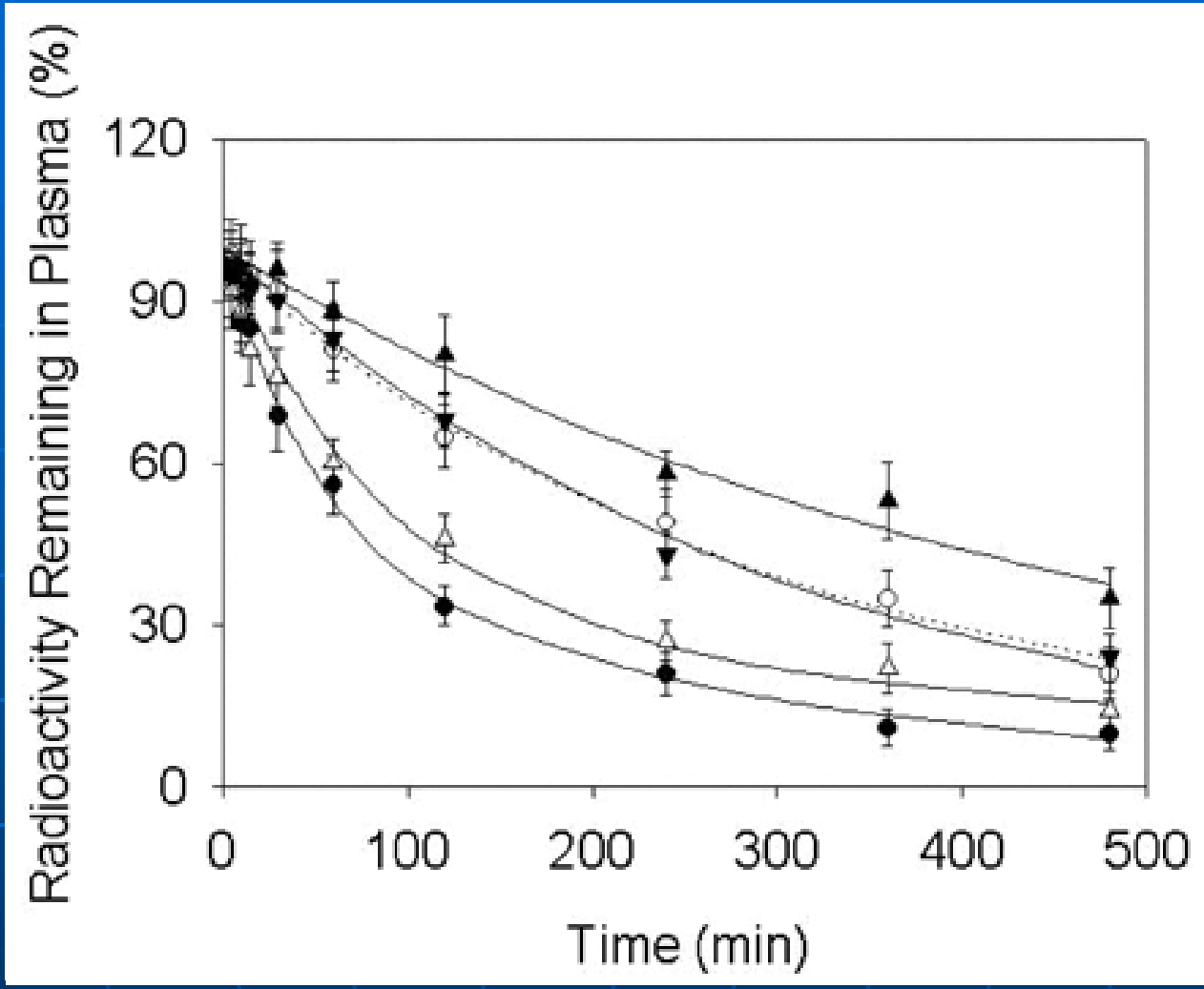
March 2008: FDA approved phase I/IIa study PTPs with haemophilia B (to be conducted in 2 centres in USA)

How is factor VIII eliminated from the blood?



How is factor VIII eliminated from the blood?

- FVIII binds to hepatic receptor with broad specificity
  - also found in other tissues (incl. placenta, lung and brain)
- Principal receptor is low-density lipoprotein receptor related protein (LRP):
  - main binding sites in A2 and A3 domains
- RAP (receptor associated protein) is a competitive antagonist, blocking uptake of factor VIII
- Pharmacological blockade of LRP offers prospect of prolongation of half-life of factor VIII



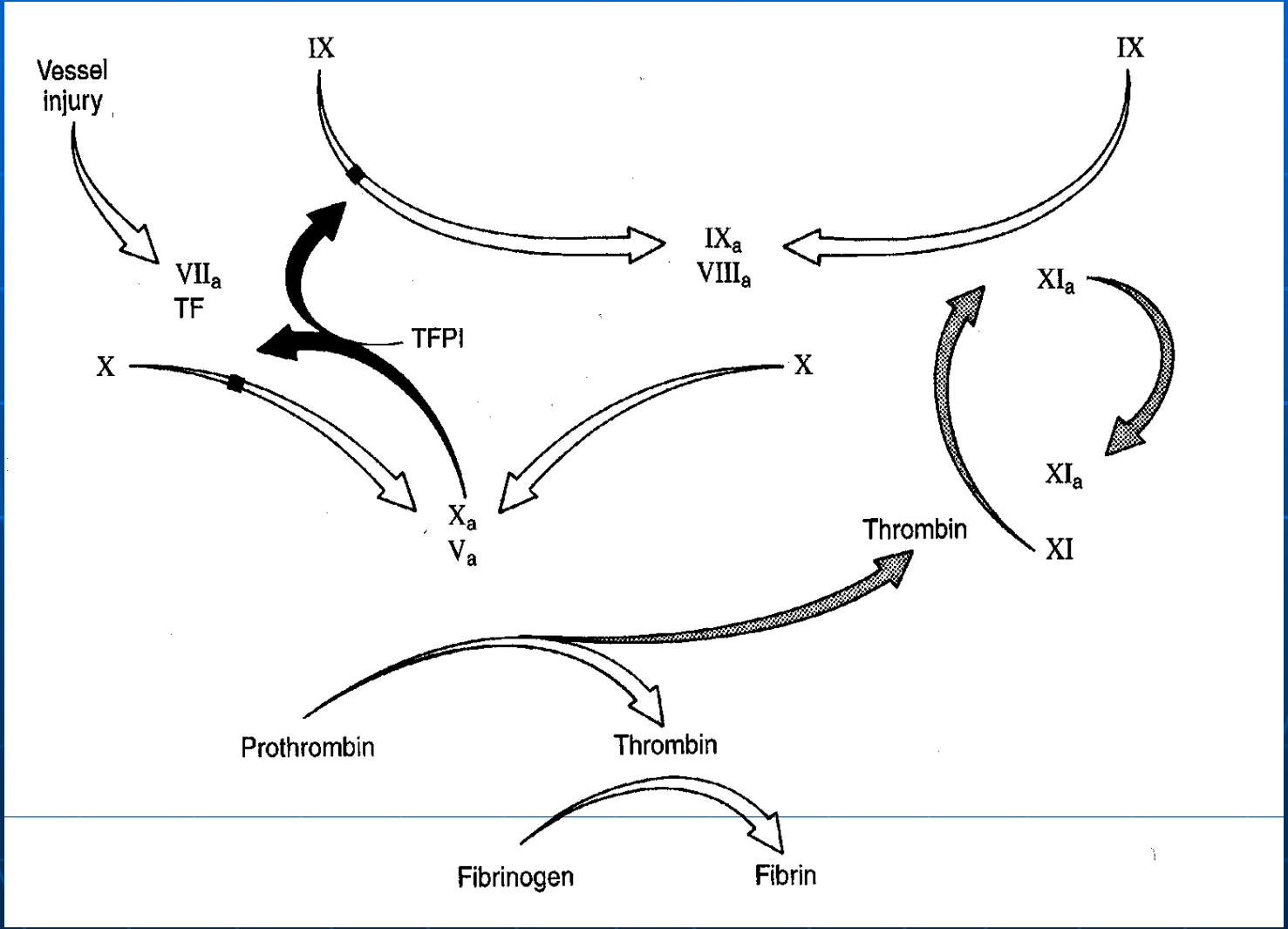
Inhibitory effect of RAP on clearance of labelled factor VIII:

50μM RAP white triangle; 150 μM white circle;

250 μM black triangle; black circle=no RAP

The revised scheme of blood coagulation:

Broze G: Thrombosis & Haemostasis 70: 72-74 (1993)



Tissue Factor Pathway Inhibitor (TFPI):

- Serine protease inhibitor protein
- Encoded on chromosome 2q
- Associated with lipoproteins in plasma
- ≈10% located in platelets
- Heparin enhances activity
- TFPI inhibits TF-FVIIa complex
- Recombinant version (tifacogin) under evaluation as antithrombotic agent in sepsis

Matyal R et al. Int Anesthesiol Clin 43: 135-144 (2005)

Inhibition of TFPI and haemophilia:

Liu T et al. Thromb. Haemost. 95: 68-76 (2006)

- Fucoidan is a non-anticoagulant sulphated polysaccharide
- Inhibits TFPI
- Accelerates clotting time of human hemophilic plasma in vitro in dilute prothrombin time assays
- Does not reduce normal plasma APTT times, implying specificity for extrinsic pathway control
- Increases survival of haemophilic mice following bleeding challenge
- Possible role as adjunctive therapy in haemophilia?

Inhibition of TFPI and haemophilia:

Liu T et al. Thromb. Haemost. 95: 68-76 (2006)

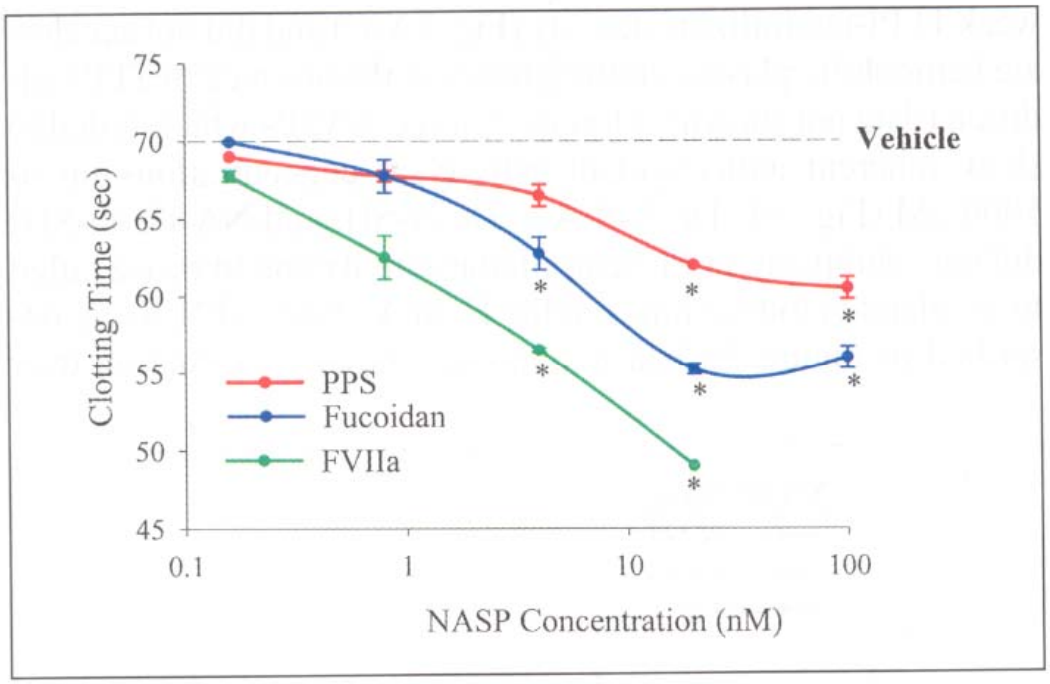


Figure 3: NASP acceleration of hemophilia A plasma clotting times. Dilute prothrombin time coagulation assays of unmodified (i.e. no TFPI supplementation) hemophilia A plasma. Evaluation of dose-response for procoagulant effect of positive control (factor VIIa), fucoidan, or PPS. Vehicle control clotting time = 69 sec. Results are representative of duplicate determinations from two studies of each format. Data points are mean ± SD. * $p \leq 0.05$ by Students t test.

Inhibition of TFPI and haemophilia:

Liu T et al. Thromb. Haemost. 95: 68-76 (2006)

Table 2: Improved hemostasis in PPS-treated hemophilic mice. Mice were randomized and dosed subcutaneously with indicated agent twice daily for 4.5 days followed by tail cut (t = 0).

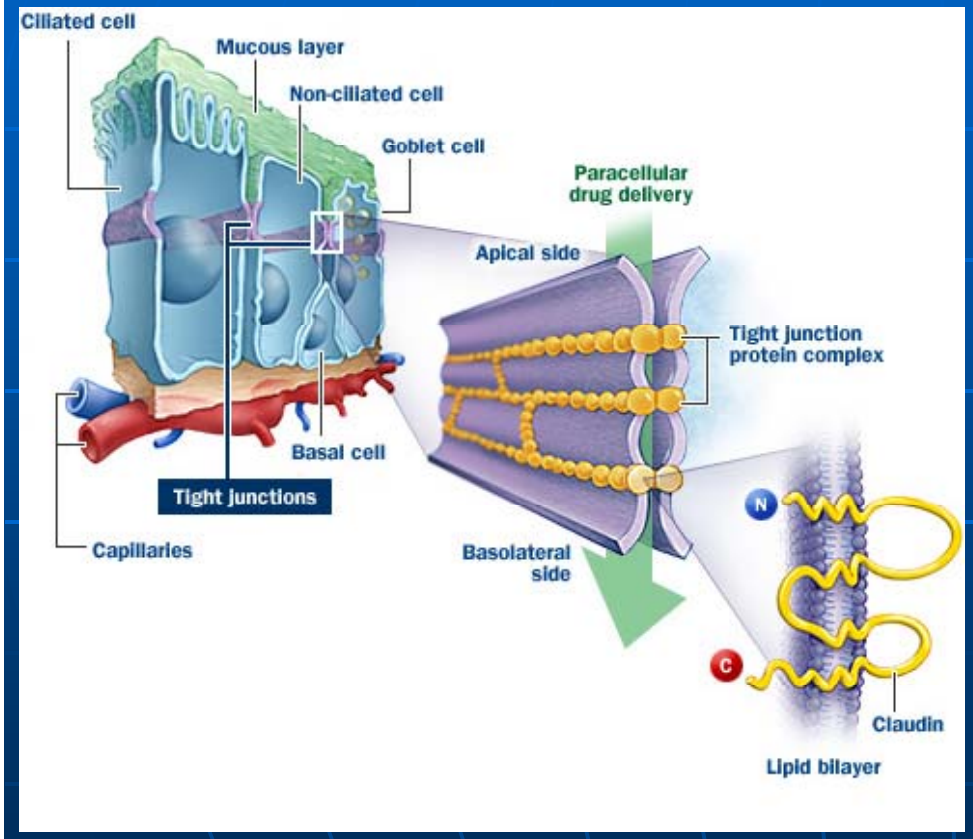
Hemophilia	Treatment Group	n/group	% Survival (20 hr post-cut)
A (FVIII-deficient)	Vehicle control	8	25
	0.02 mg/kg	5	20
	0.06 mg/kg	9	44 #
	0.2 mg/kg	5	40
B (FIX-deficient)	Vehicle control	8	25
	0.06 mg/kg	9	44 #

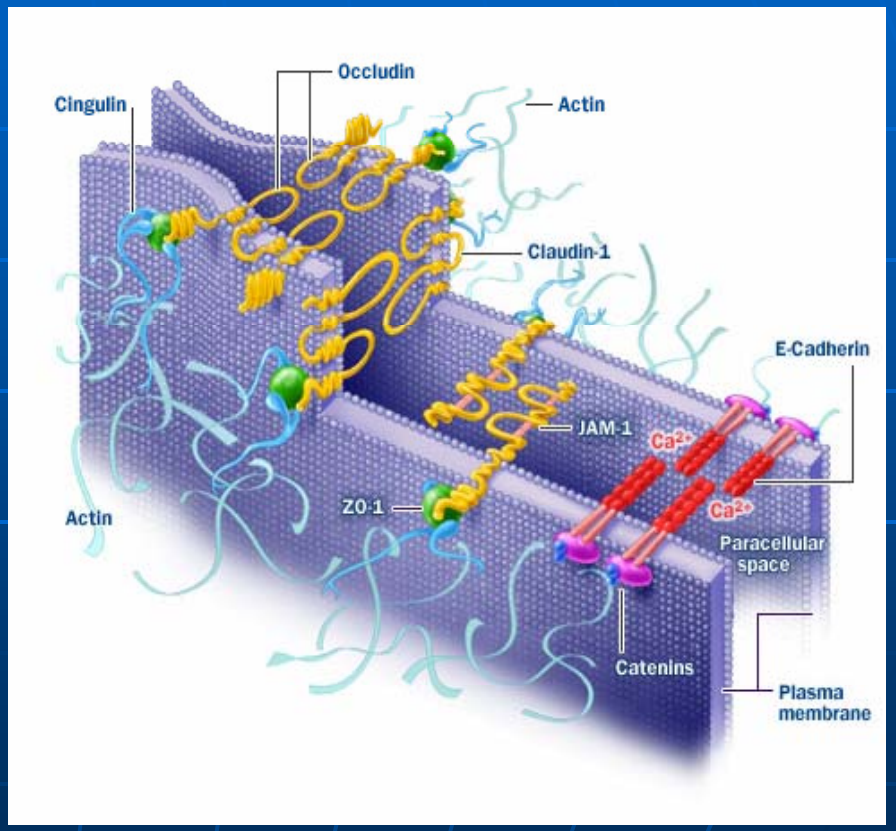
p = 0.07 vs vehicle

Can injections be avoided?
www.nastech.com

- DDAVP nasal spray already available
- "Tight junctions" between adjacent epithelial and endothelial cells impede the transfer of larger molecules and proteins across membrane surfaces
- Opening up these "molecular gates" could allow transfer of large molecules across:
  - nasal mucosa
  - gastrointestinal tract
- Goal is to produce formulations of factor IX for oral use or nasal spray

"Tight junctions": barriers to intercellular transport





Porcine factor VIII for patients with inhibitors:

- Sufficiently similar to human factor VIII to work like natural factor VIII but
- Sufficiently different in structure to avoid neutralisation by inhibitory antibodies
- Previous product derived from porcine plasma (Hyate:C) withdrawn in 1996
- Recombinant porcine factor VIII now manufactured:
  - OB1: recombinant B-domain deleted porcine factor VIII *(Abshire T et al, WFH 2006 Abstract 644 & JTH 4: 2223-9 [2006]).*
  - OB2: under development

- ≈400,000 people with haemophilia globally
- WFH estimates that two thirds still receive no treatment at all
- Many still not even diagnosed

Other possible initiatives:

- Twin track pricing:
  - cheaper products in less affluent countries
  - bulk buying by blocks of affiliated countries
- Donation of surplus plasma from one nation to another for fractionation
- Intellectual property (copyright) law:
  - Manufacture of cheaper copies (rVIIa, recombinant VIII and IX)
- Pre-implantation diagnosis: religious, ethical and legal viewpoints

45p
Friday
December 19
1997
Published in London
and Manchester

NEWSPAPER OF THE YEAR

The Guardian

These lambs are clones and carry the human factor IX. They may save thousands of lives

Polly and some of the other cloned lambs which mark a step along the road to 'pharming' and which scientists hope will help in the treatment of haemophilia

Tim Radford
Science Editor

The factor is a vital treatment for haemophilia B, sometimes called Christmas

fears that it would be possible to clone humans. But the Roslin scientists said from the

Austin

tain that they will actually produce the healing protein in their milk. But another

lamb. Ron James, the head of PPL Therapeutics, said it "allows products to be developed

that would be useful simply because the scientists now know how to make groups of

hepatitis C; as a way of altering milk for people with lactose intolerance; as a way of

"Pharming": using transgenic animals to manufacture recombinant human therapeutic proteins (e.g. coagulation factors, antithrombin, a2-antitrypsin)

Transgenic animals:

- Farmyard animals to which a human gene is inserted using a viral vector
- Desired protein is secreted into milk
- Milk is treated with acid to precipitate unwanted milk proteins (e.g. casein)
- Protein can then be extracted from supernatant (e.g. chromatography)
- Offers possibility of production of large amounts of protein at low cost

Use of transgenic animals:

Velander WH et al, 2004

- Pig is best for functional activity of coagulation molecules
- High reproductive rate
- Total milk yield/sow ≈ 200-300 litres/year
- "Milk from 60 pigs could supply the entire amount of factor IX prophylaxis in the US" (3000 patients 200,000 IU each)
- Animal source is acceptable to most cultures around the world
- No evidence of transmission of viruses with porcine products so far (also not prone to BSE)

February 2007: Intas Biopharmaceuticals in India entered into a joint venture with Progenetics LLC, a US based company that has created transgenic animals producing Factor-IX in milk. As per the agreement Intas will develop the drug from such transgenic animals, and carry out clinical trials and launch the drugs in India and in overseas markets.

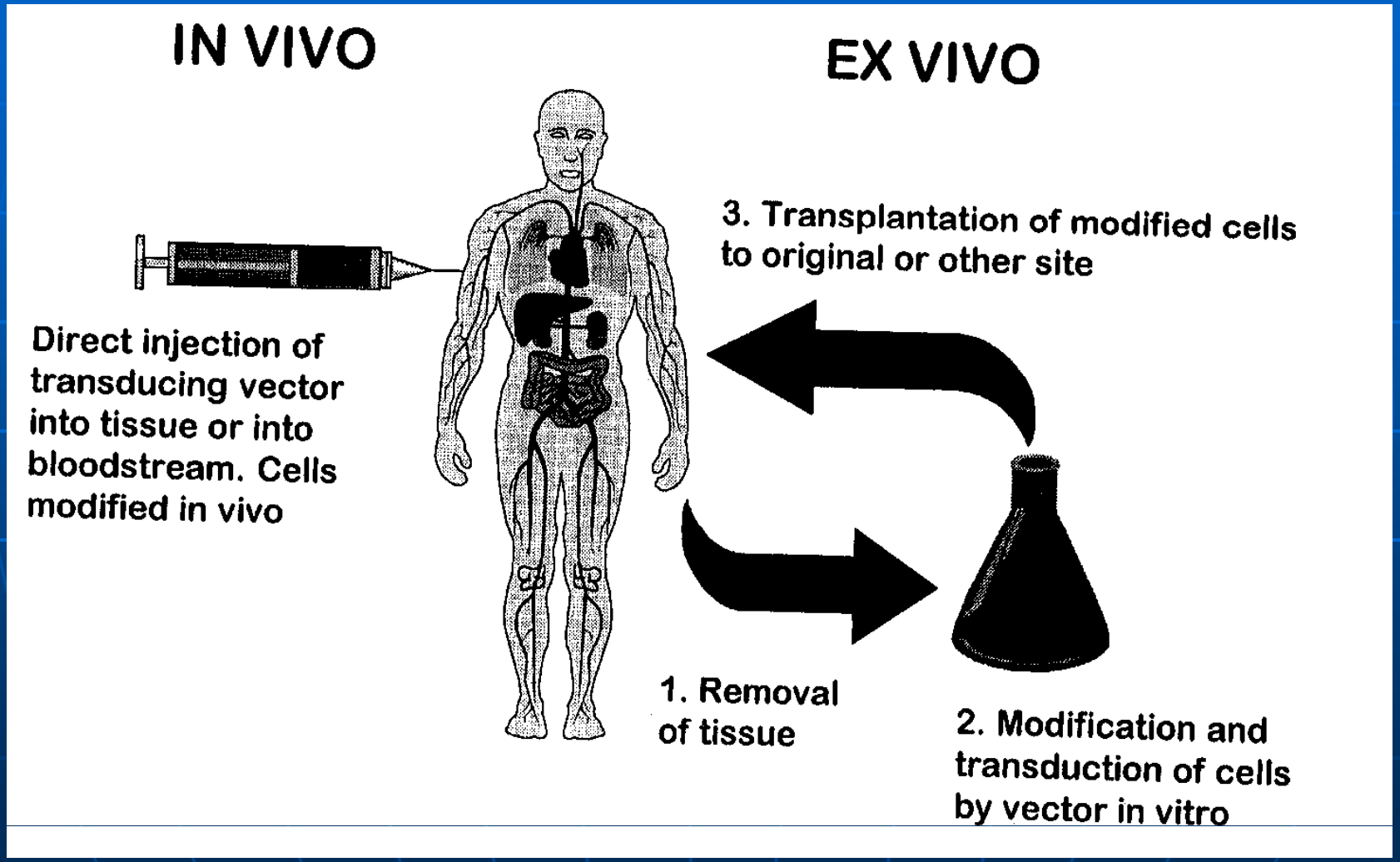
Gene therapy for haemophilia:

New England Journal of Medicine: 344: 1735-1742 (2001)

"There is no doubt in my mind that gene therapy is going to cure haemophilia. It is just a question of when."

Dr. Mark A. Kay

Gene therapy for haemophilia:



Haemophilia is an ideal target disease for scientists:

- Molecular basis well understood
- Patients and their treaters can be readily identified in specialist centres
- Animal model for preliminary work already exists
- Single gene fits in various viral vectors
- Only partial correction of deficiency offers prospect of improving bleeding tendency
- Target tissues can be readily accessed
- Factor level in blood can be monitored easily

Hemophilia Gene Therapy: Clinical Trial Update

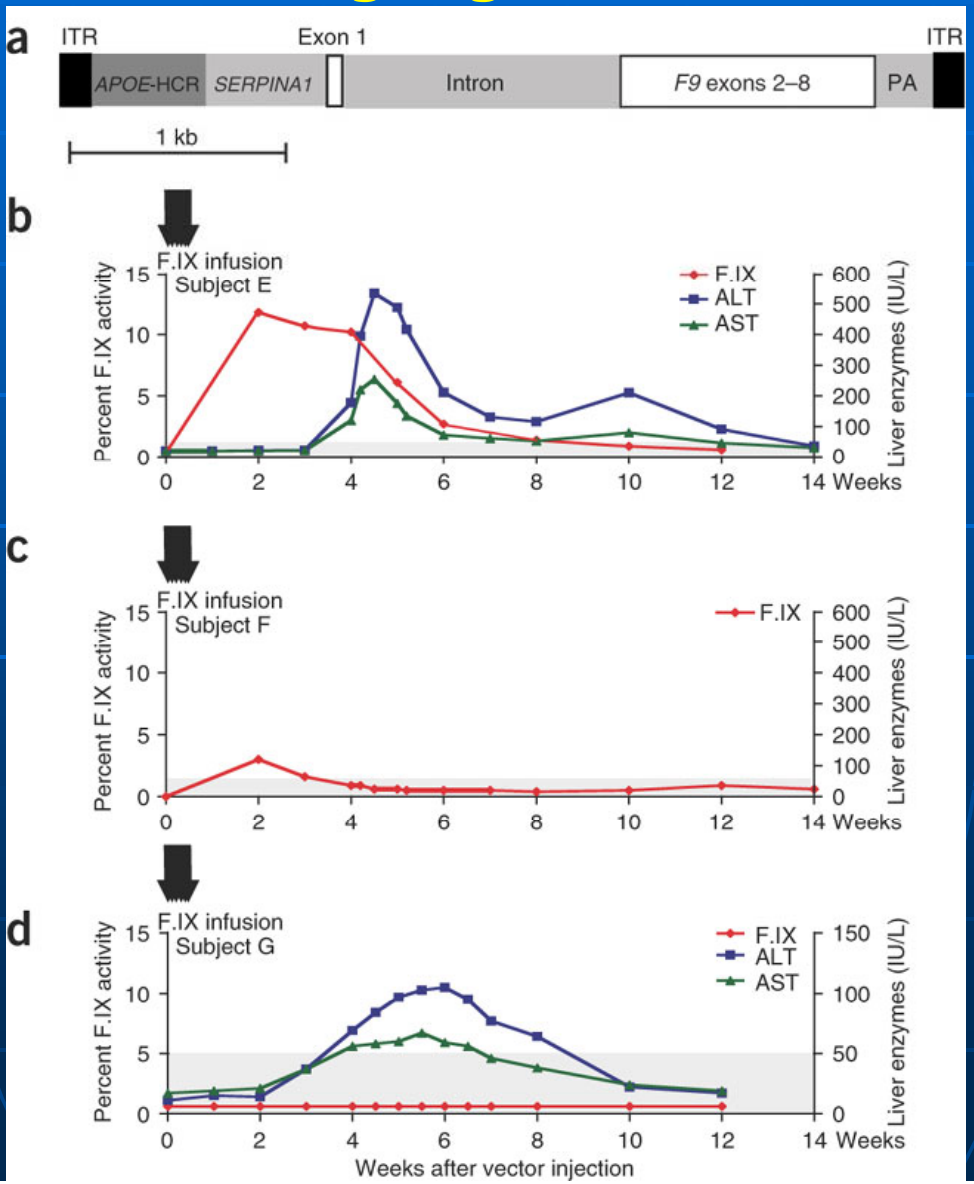
6 trials (3 FVIII: 3 FIX)

43 patients

- Ex vivo gammaretrovirus
- Ex vivo electroporation
- IV gammaretrovirus
- IV adenovirus
- IM/hepatic artery AAV

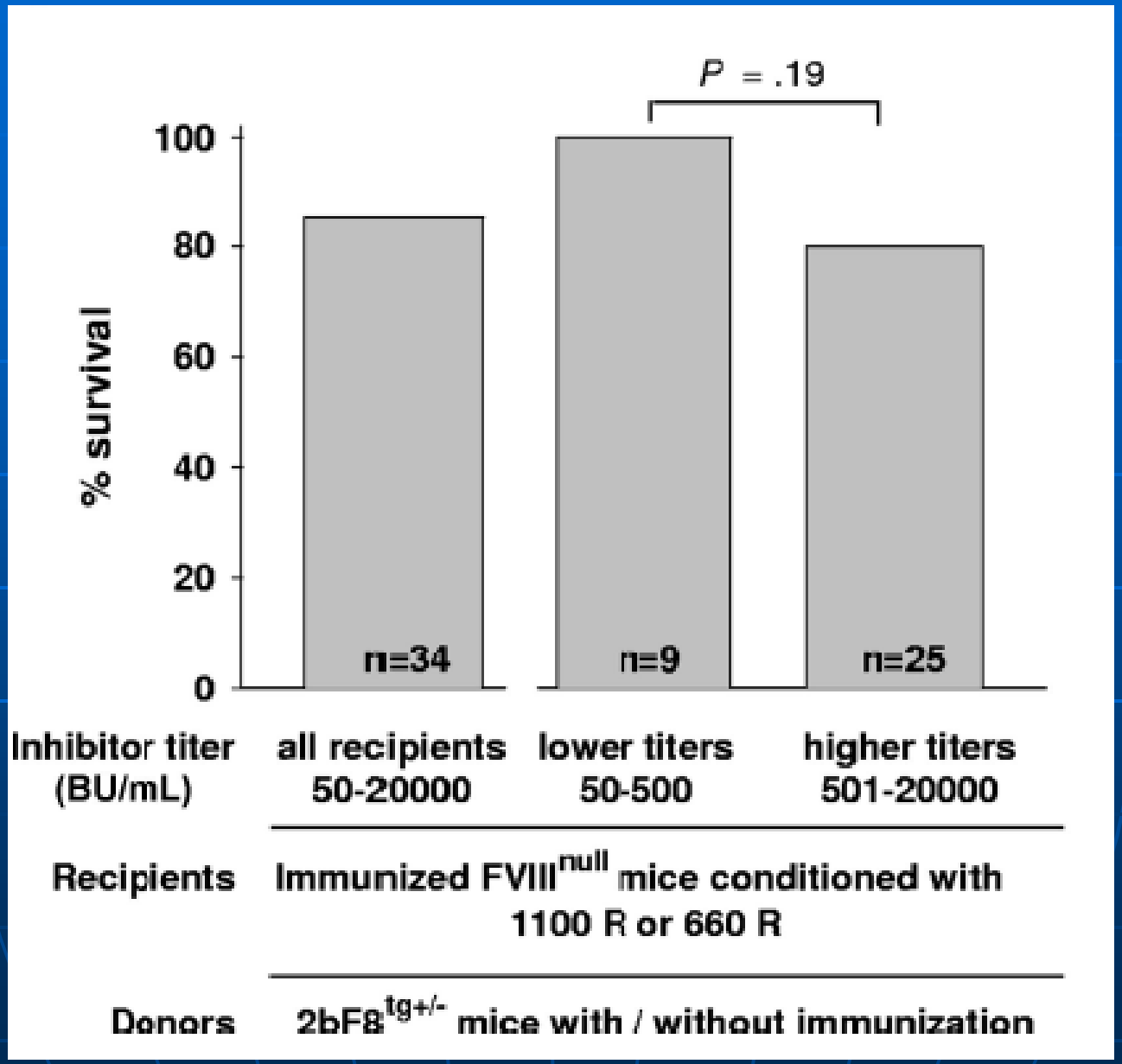
Avigen Liver AAV Trial

2 x 10^{12} vg/kg AAV2 dose



Using platelets to carry FVIII:
Shi Q et al. 112: 2713-27121 (2008)

- Transgenic mice produced which express FVIII only in their platelets
- Bone marrow transfused into $FVIII^{null}$ mice after irradiation
- Recipient mice immunized against FVIII
- Survival rate of recipient mice improved after tail-cutting
- FVIII in platelets protected from neutralization by inhibitory antibodies
- Transportation of FVIII in platelets facilitates delivery to site of injury for immediate effect


P = .19
100
80
60
40
20
0
% survival
n=34
n=9
n=25
Inhibitor titer (BU/mL)
all recipients 50-20000
lower titers 50-500
higher titers 501-20000
Recipients
Immunized FVIII^null mice conditioned with 1100 R or 660 R
Donors
2bF8^tg+/- mice with / without immunization

Conclusions:

- Gene therapy will ultimately offer a permanent cure for haemophilia
- Longer-acting factor concentrates are a much more realistic early goal
- Activity of coagulation factors can be extended by various technologies: e.g.
  - pegylation
  - genetic fusion with albumin
- TFPI inhibitors may also be useful for adjunctive therapy

What stage are we at now?

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Sir Winston Churchill

London, November 10th 1942