von Willebrand factor: a hemostatic protein

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Presentation schedule

- ✓ Basic notions about hemostasis
- ✓ Von Willebrand factor: introduction
- ✓ Von Willebrand disease
- ✓ Animal models of von Willebrand disease
- ✓ The use of hydrodynamic injection to generate new murine models

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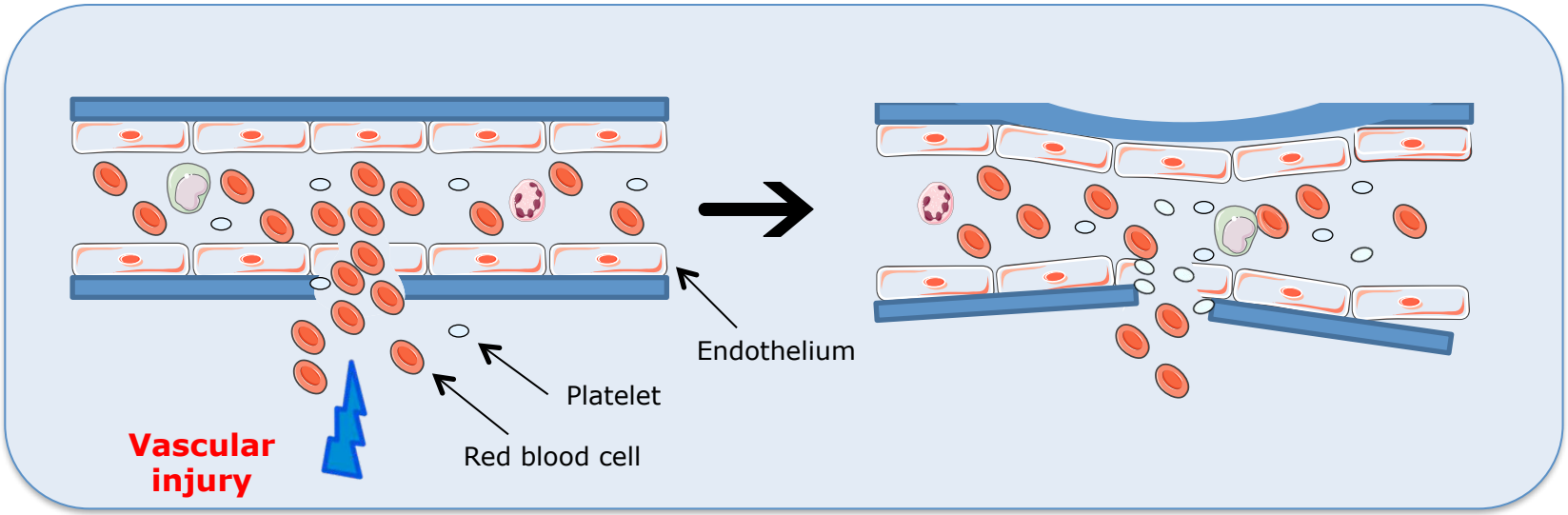
Hemostasis

The major goal of the hemostatic system is to keep the blood in its fluid state in the vascular compartment, while preventing excessive blood loss following vessel injury

<u>Four important steps can be distinguished:</u>

1- Vasoconstriction of the damaged vessel reducing locally the blood flow and limiting the blood loss



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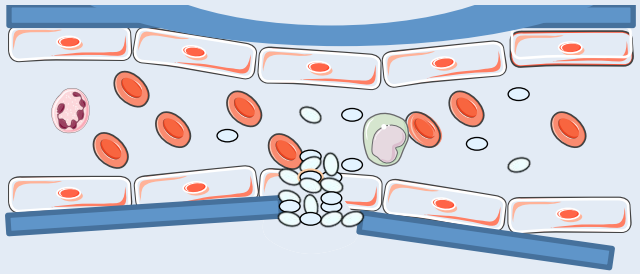
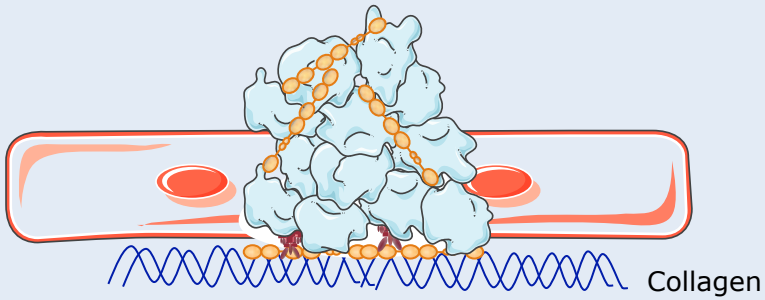
2- Primary hemostasis initiated by platelet adhesion to exposed subendothelial components and leading to platelet plug formation





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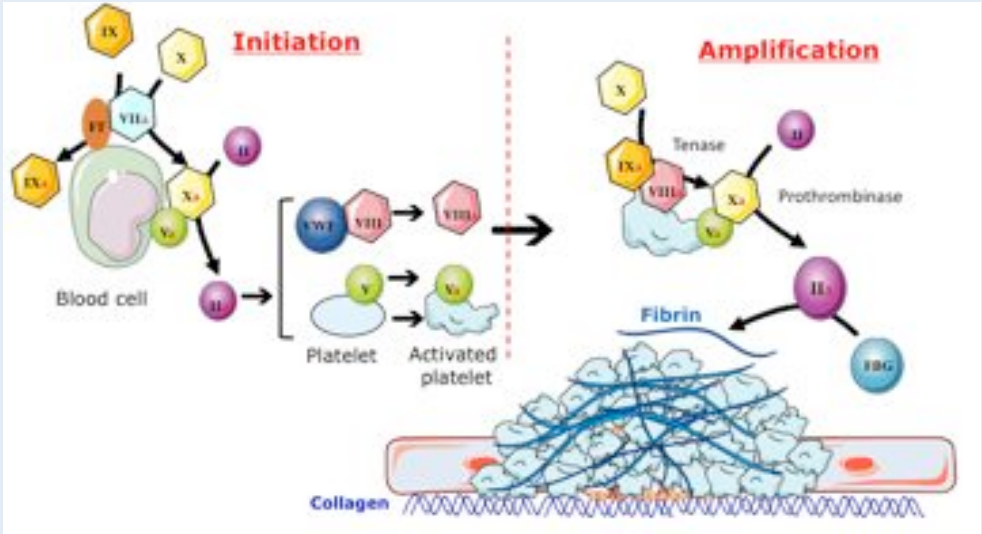
Four important steps can be distinguished:

3- Secondary hemostasis (or blood coagulation) achieving consolidation of the platelet plug by the formation of a fibrin network



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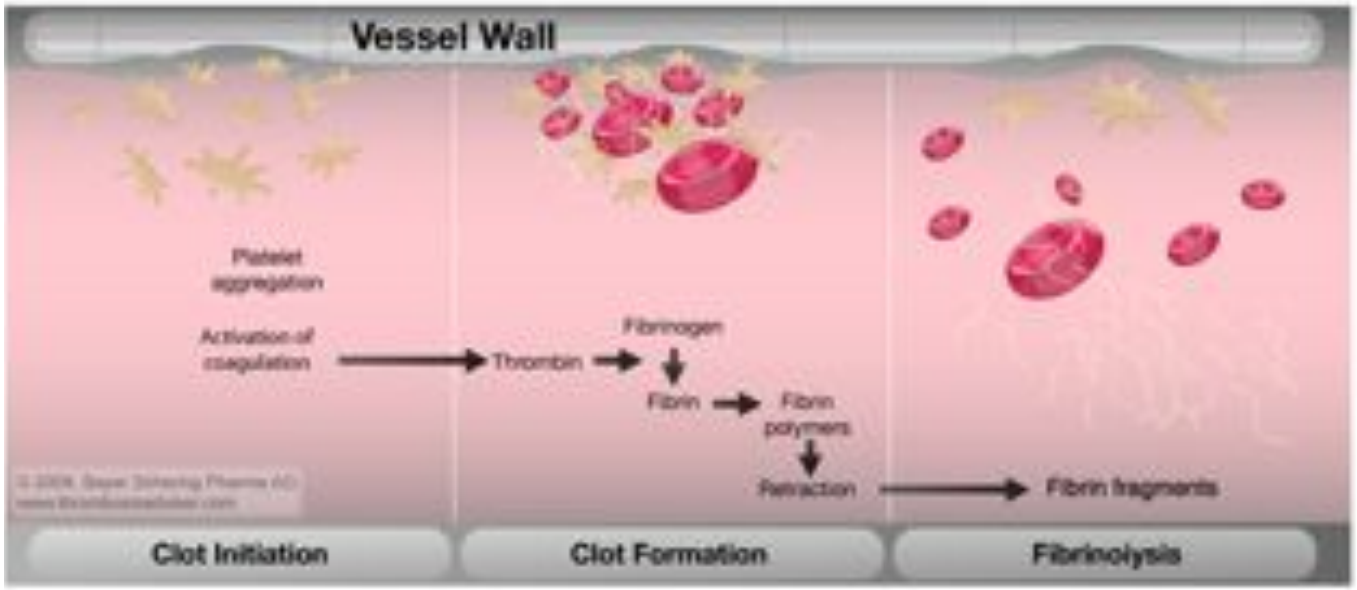
Four important steps can be distinguished:

4- Fibrinolysis inducing elimination of the clot during tissue reparation

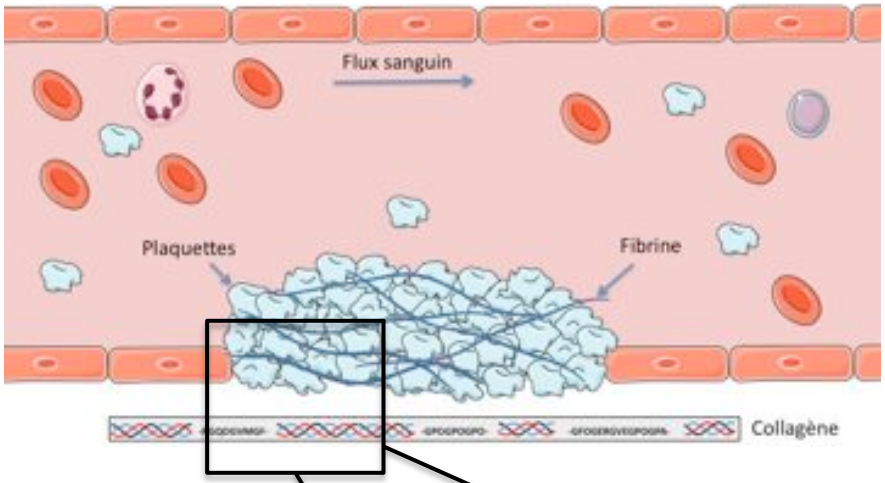


Hemostasis

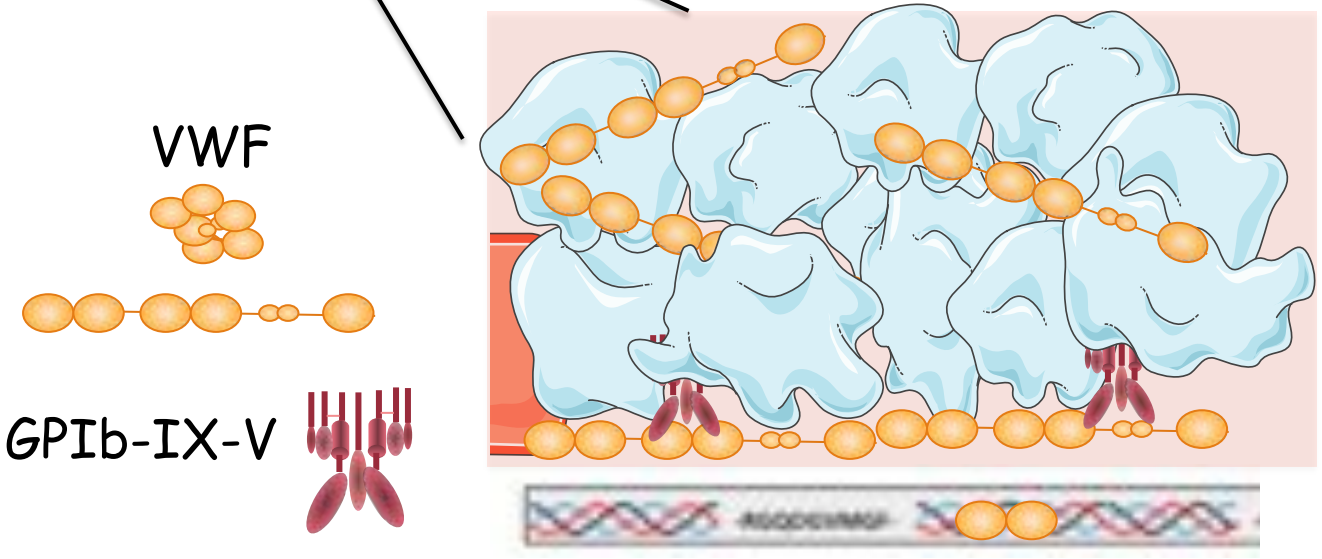
The major goal of the hemostatic system is to keep the blood in its fluid state in the vascular compartment, while preventing excessive blood loss following vessel injury

The hemostatic response must be quick, localized and carefully regulated. This process interconnects a series of physiological events involving vessels, blood cells, coagulation factors, coagulation inhibitors and fibrinolytic system.

Hemostasis and von Willebrand factor



Von Willebrand factor (VWF):
Key protein in primary hemostasis



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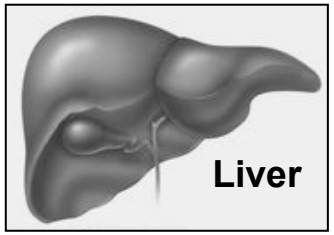
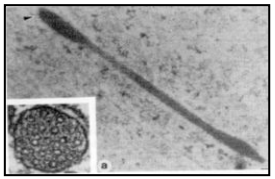
Life-cycle of von Willebrand factor (VWF)

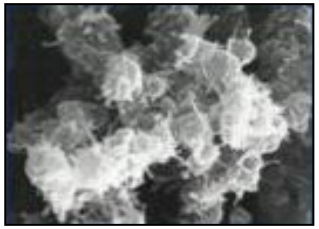


Clearance

Biosynthesis

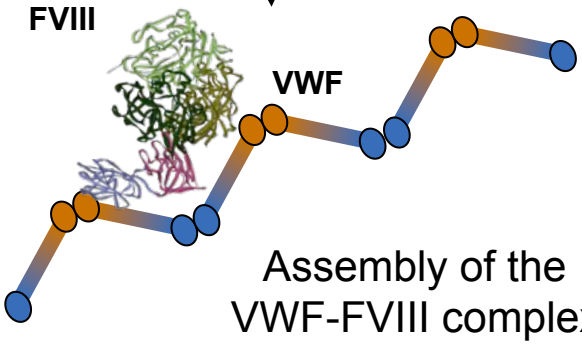
Storage and secretion in plasma





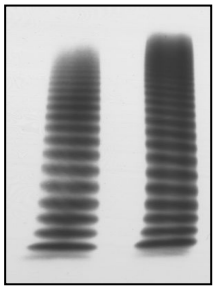
Formation of the platelet thrombus

Functions

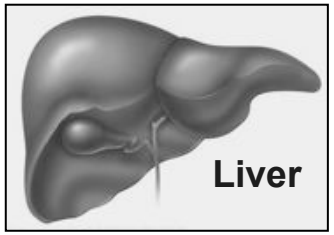


Assembly of the VWF-FVIII complex

Regulation of multimer size



Life-cycle of VWF



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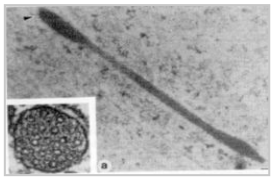

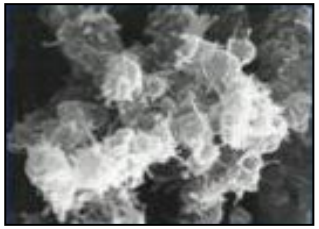

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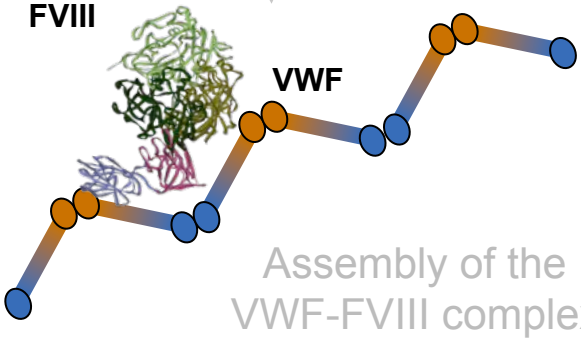

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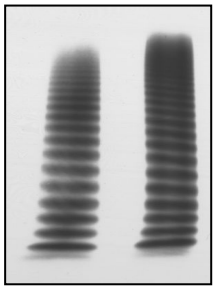
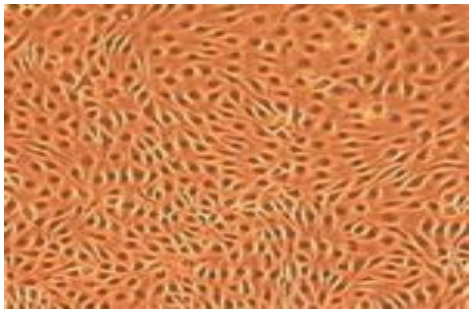
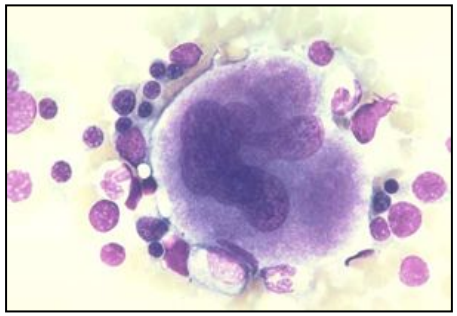
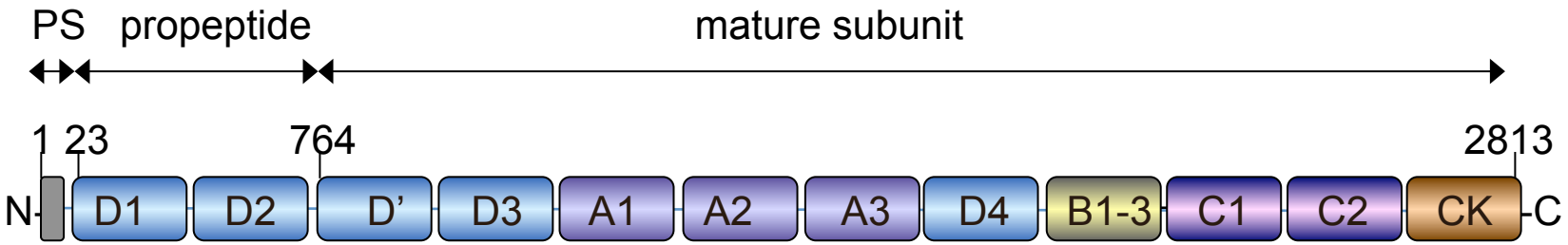


VWF biosynthesis

Endothelial cells and megakaryocytes







Endoplasmic reticulum → **Golgi** → **Trans Golgi network**

Peptide signal cleavage
N-Glycosylations
Dimerization

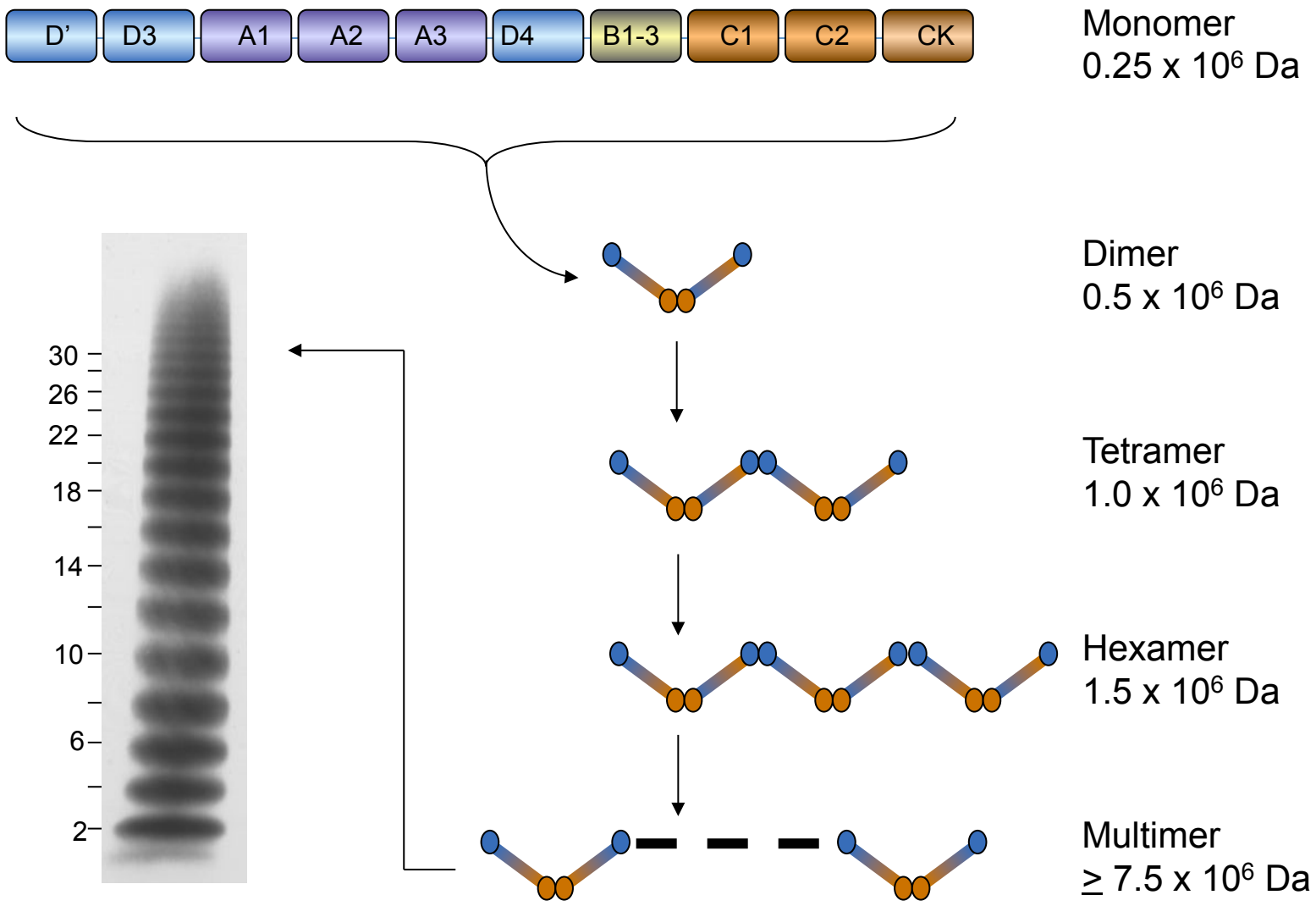
O-Glycosylations
Sulfatations
Multimerization

Sulfatations
Multimerization
Propeptide cleavage

2.7×10^5 Da

$0.5 - 20 \times 10^6$ Da

Multimeric structure of VWF



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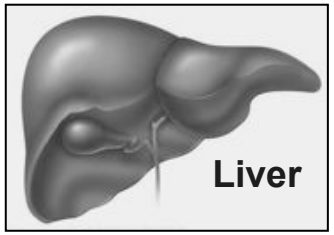

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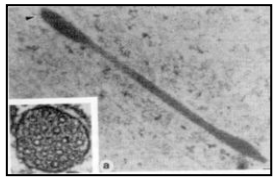

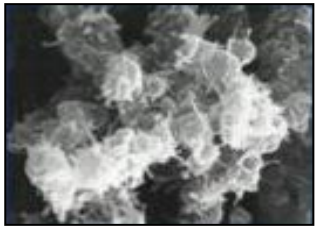

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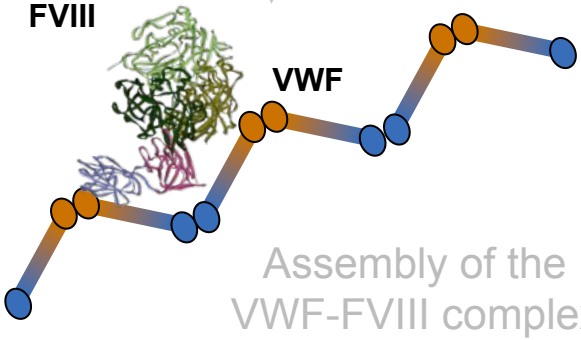

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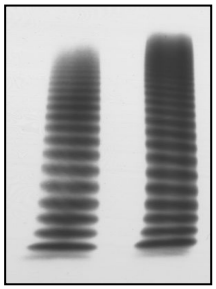
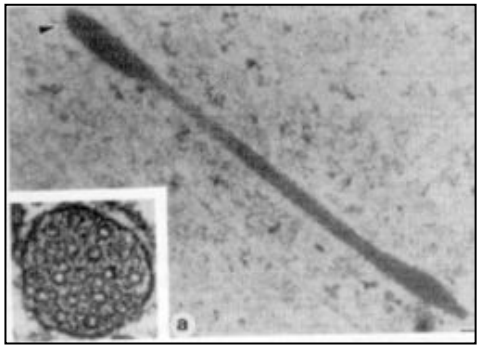
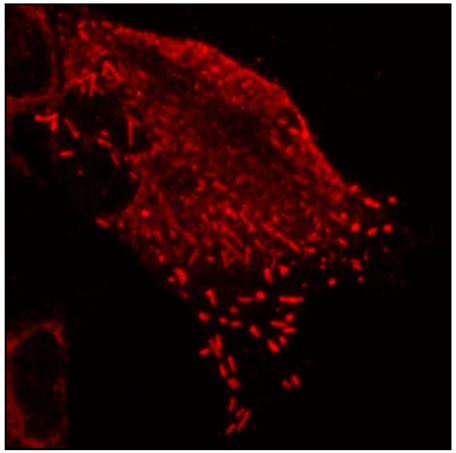


Storage and secretion of VWF

- ✓ Constitutive secretion: plasma (5 à 10 μg/mL) and subendothelium
- ✓ Regulated secretion: Platelet α granules and Weibel-Palade bodies of endothelial cells

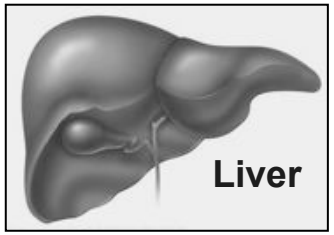




Anti-VWF-TRITC

- ✓ Rod shaped granules measuring 100 nm in width and 1-5 μm in length
- ✓ Presence of very high molecular weight multimers of VWF
- ✓ VWF is required for Weibel-Palade body formation
- ✓ Contain other proteins: P-selectin, angiopoietin-2, osteoprotegerin, IL-8
- ✓ Exocytosis: thrombin, shear stress, histamin, PMA, …

Life-cycle of VWF



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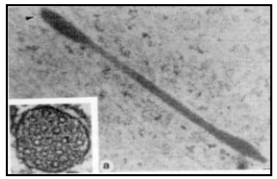

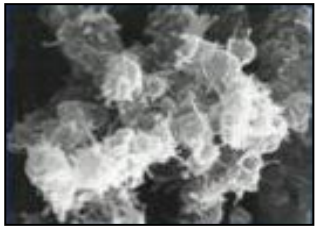

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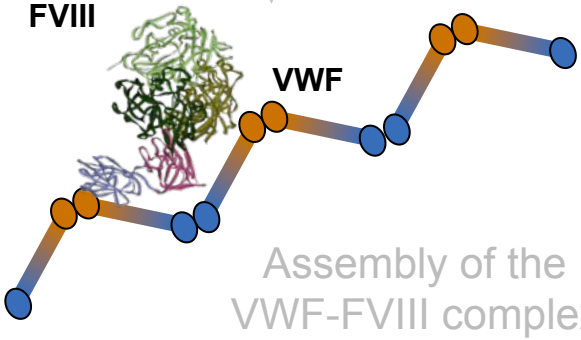

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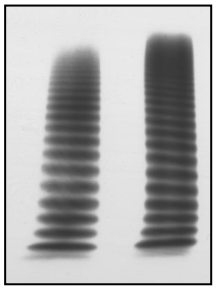
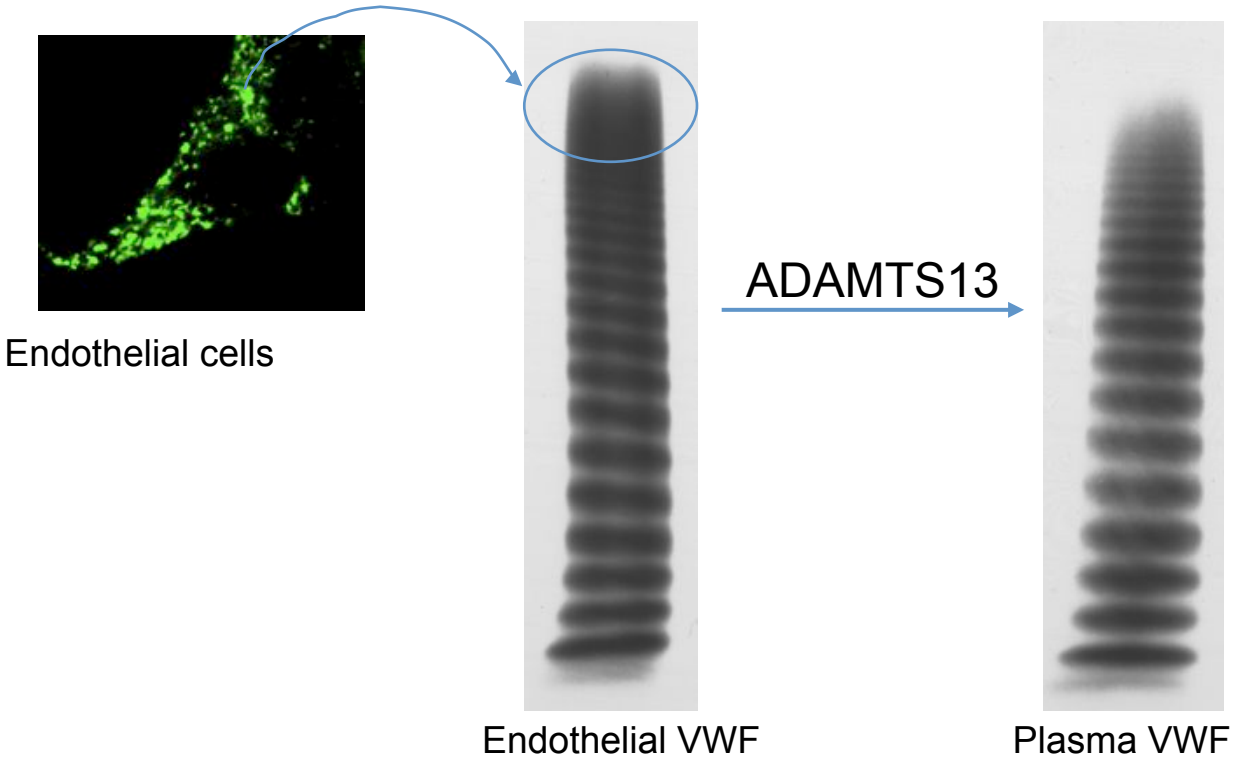
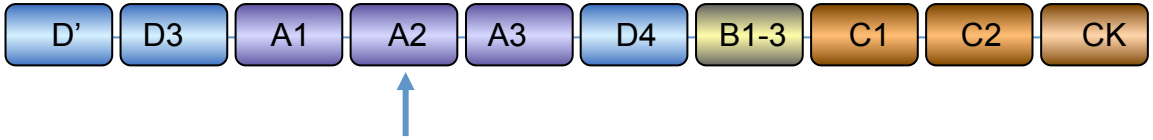


Regulation of VWF multimer size (1)

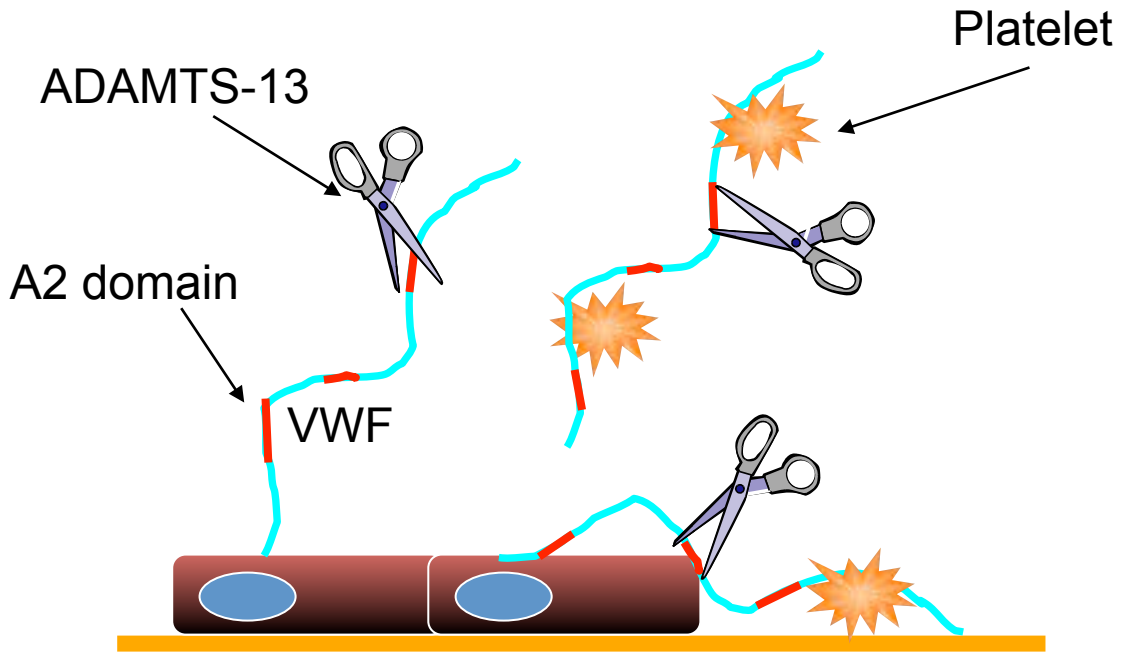


ADAMTS13: A Disintegrin And Metalloprotease with ThromboSpondin type 1 Repeats, member 13.

Regulation of VWF multimer size (2)

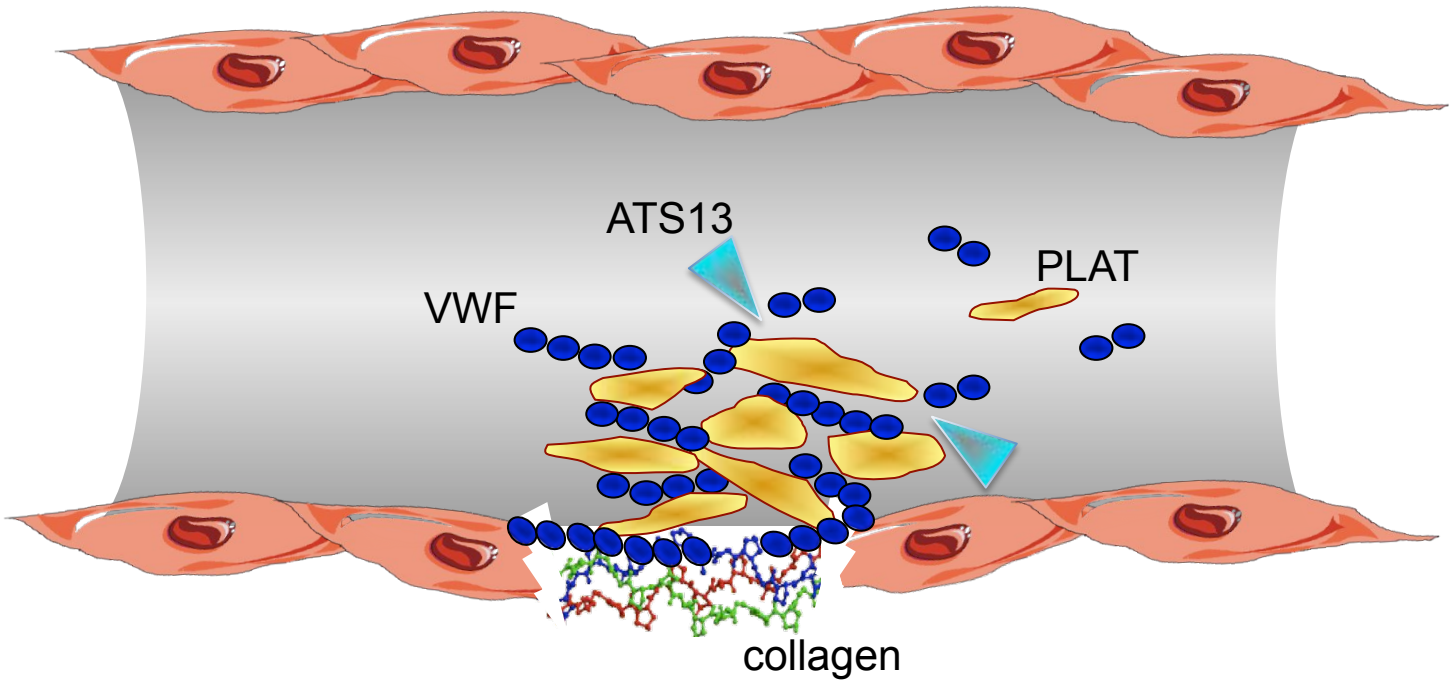


ADAMTS-13 cleaves VWF in its A2 domain between Tyr 1605 and Met 1606

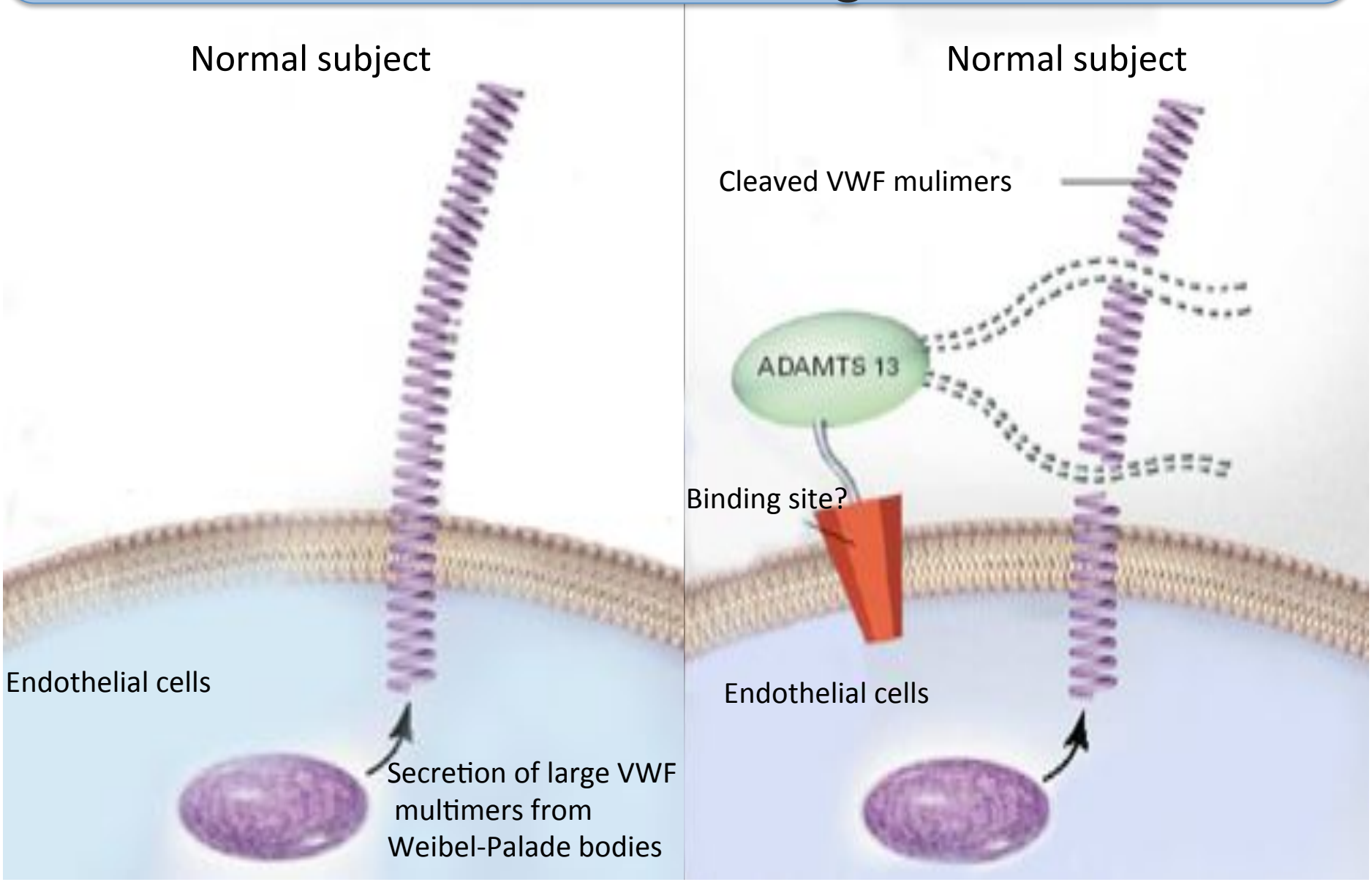


VWF as an ADAMTS13 substrate

- VWF circulating in plasma: NO
- VWF bound to endothelial cells: YES
- VWF bound to platelets in a thrombus: YES



Importance of ADAMTS13-mediated VWF cleavage



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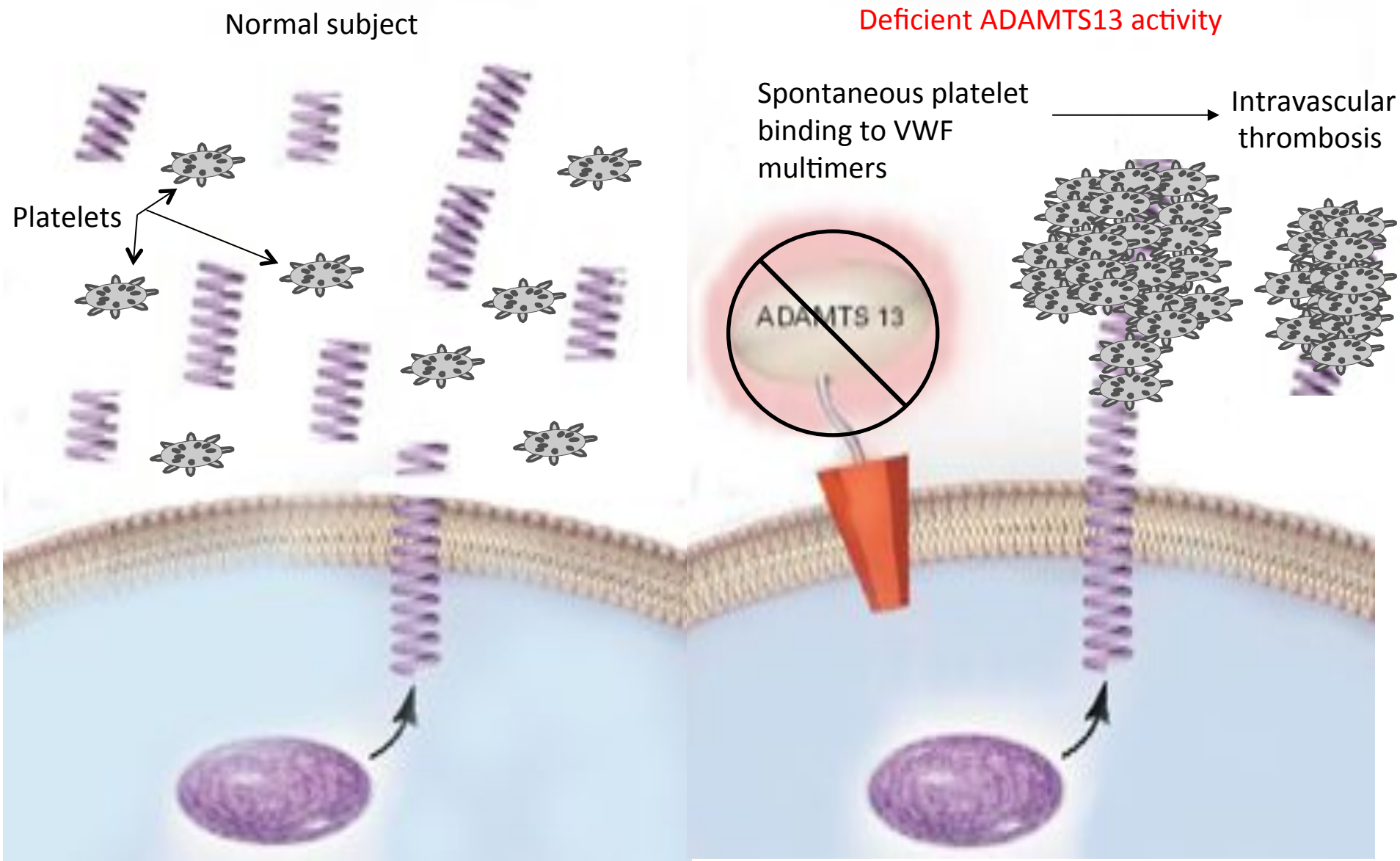
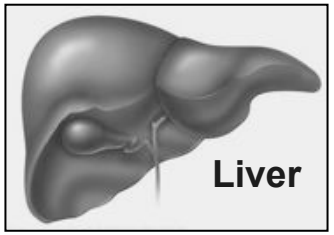


Life-cycle of VWF



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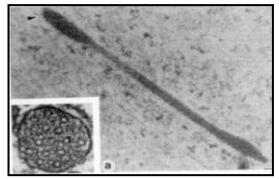

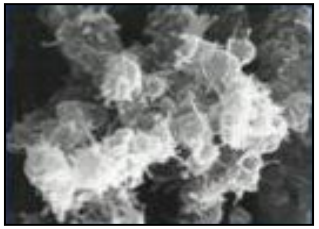

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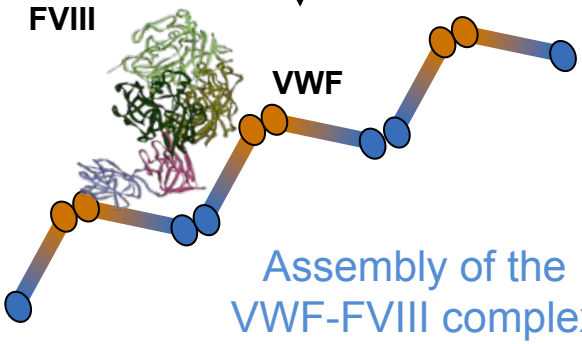

Assembly of the VWF-FVIII complex

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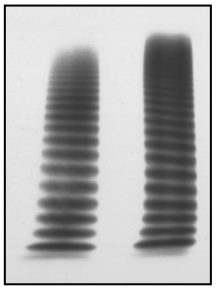
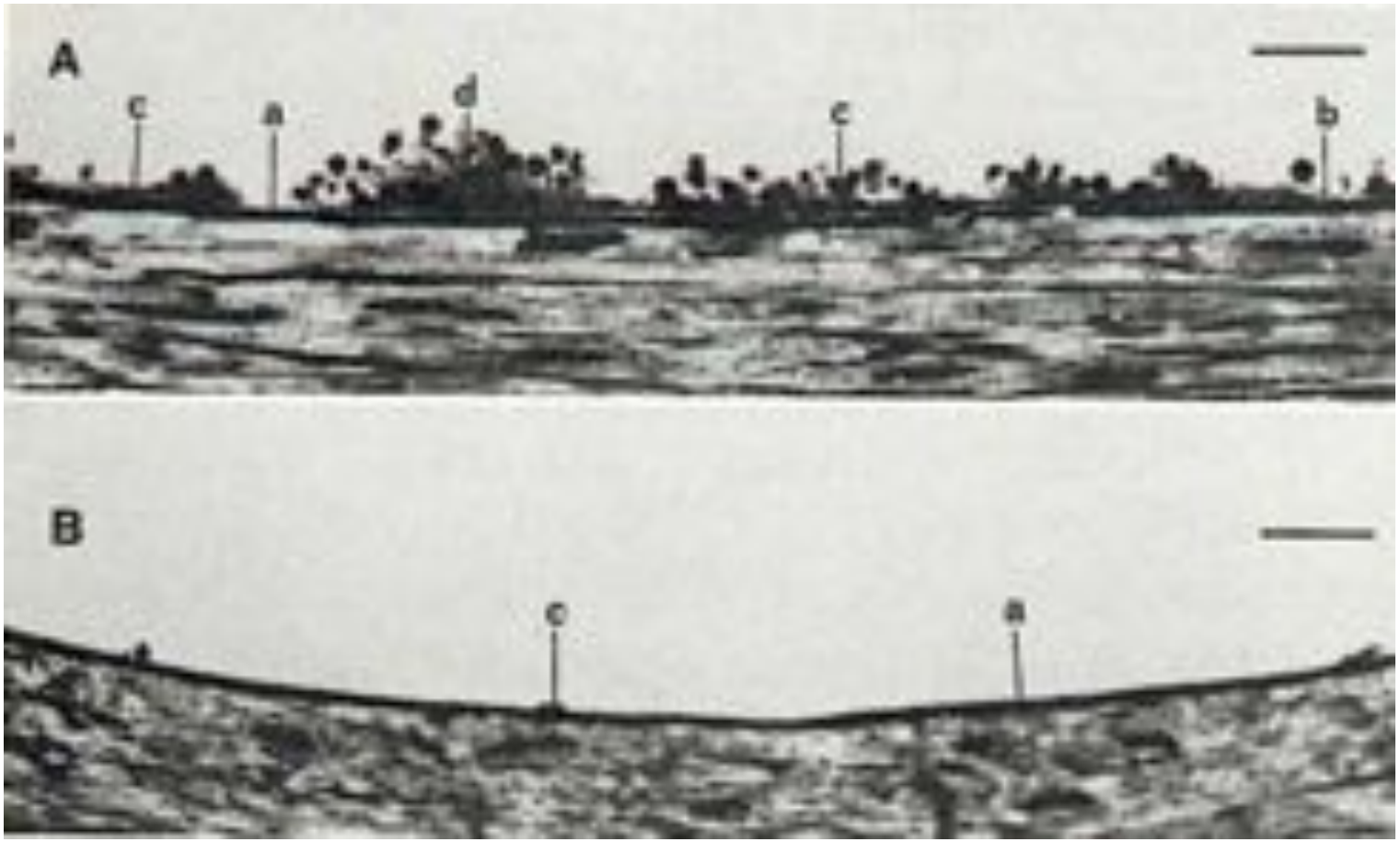


VWF role in platelet adhesion to the subendothelium at high shear rate

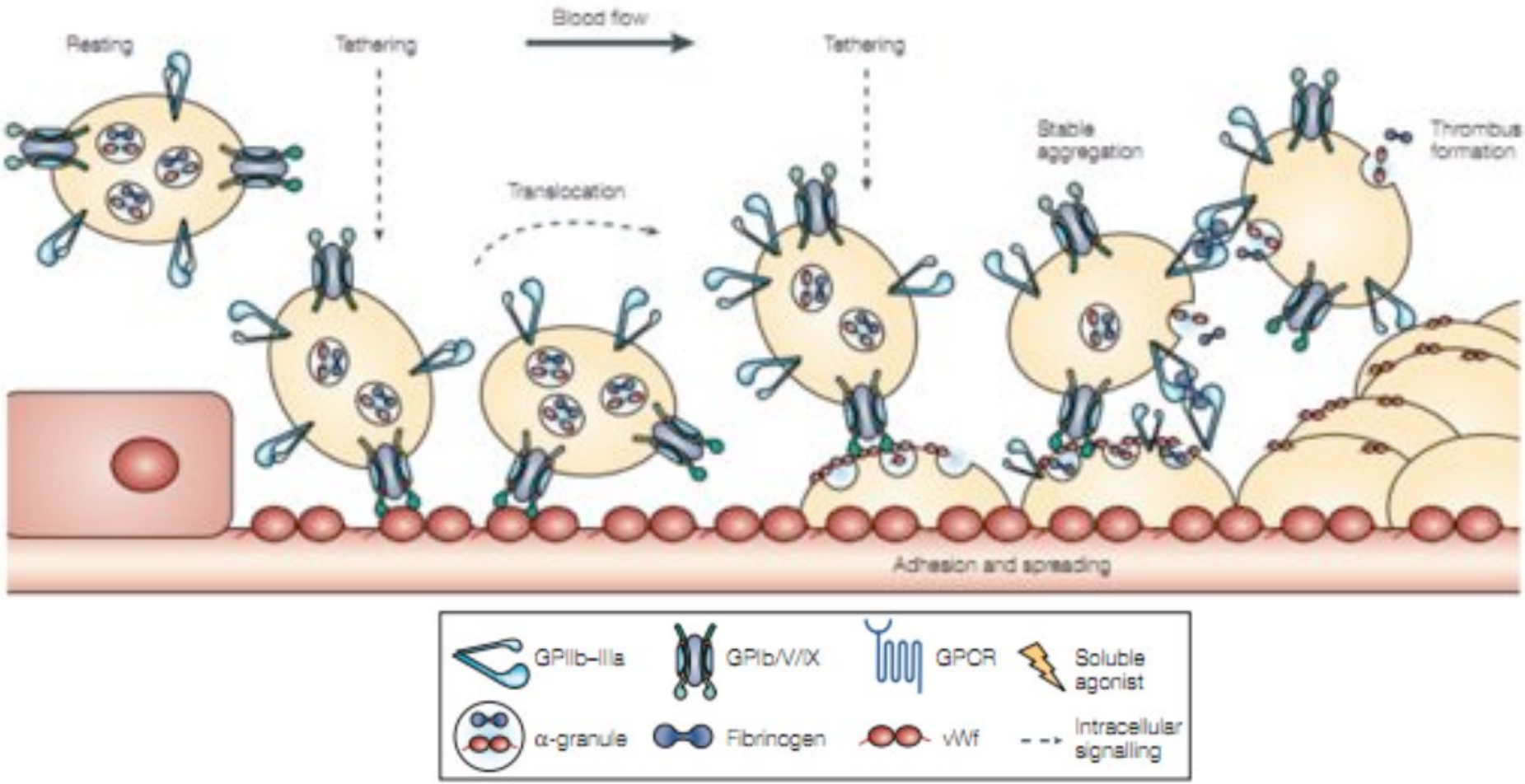


Control blood

VWF-deficient blood

Pictures taken by light microscopy of human platelets adhering to subendothelium from rabbit aorta
(Tschopp et al, J Lab Clin Med 1974, 83, 296-300)

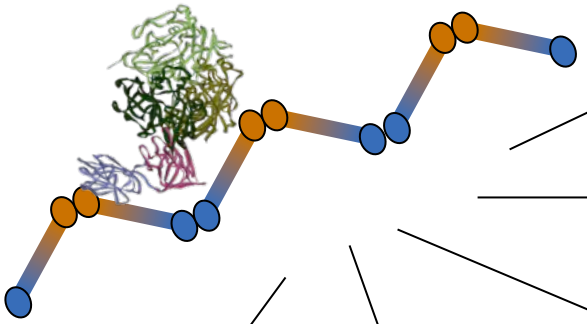
Platelet adhesion and aggregation at high shear rates

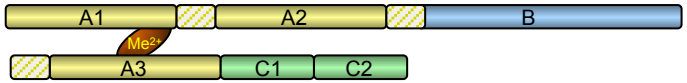


(Jackson SP and Schoenwaelder SM, 2003)

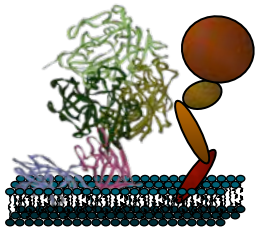
Interaction VWF-Factor VIII (FVIII)

FVIII/VWF complex

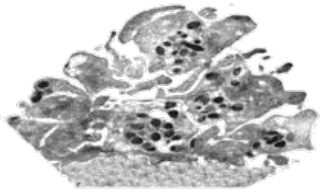




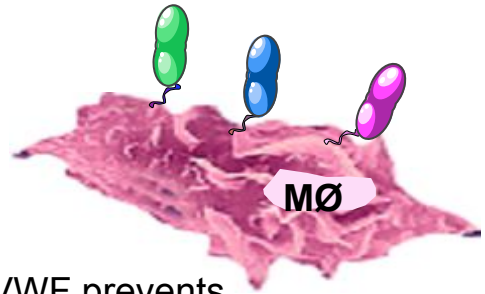
VWF stabilizes FVIII hetero-dimeric structure



VWF denies FVIII access to the factor X-activating complex



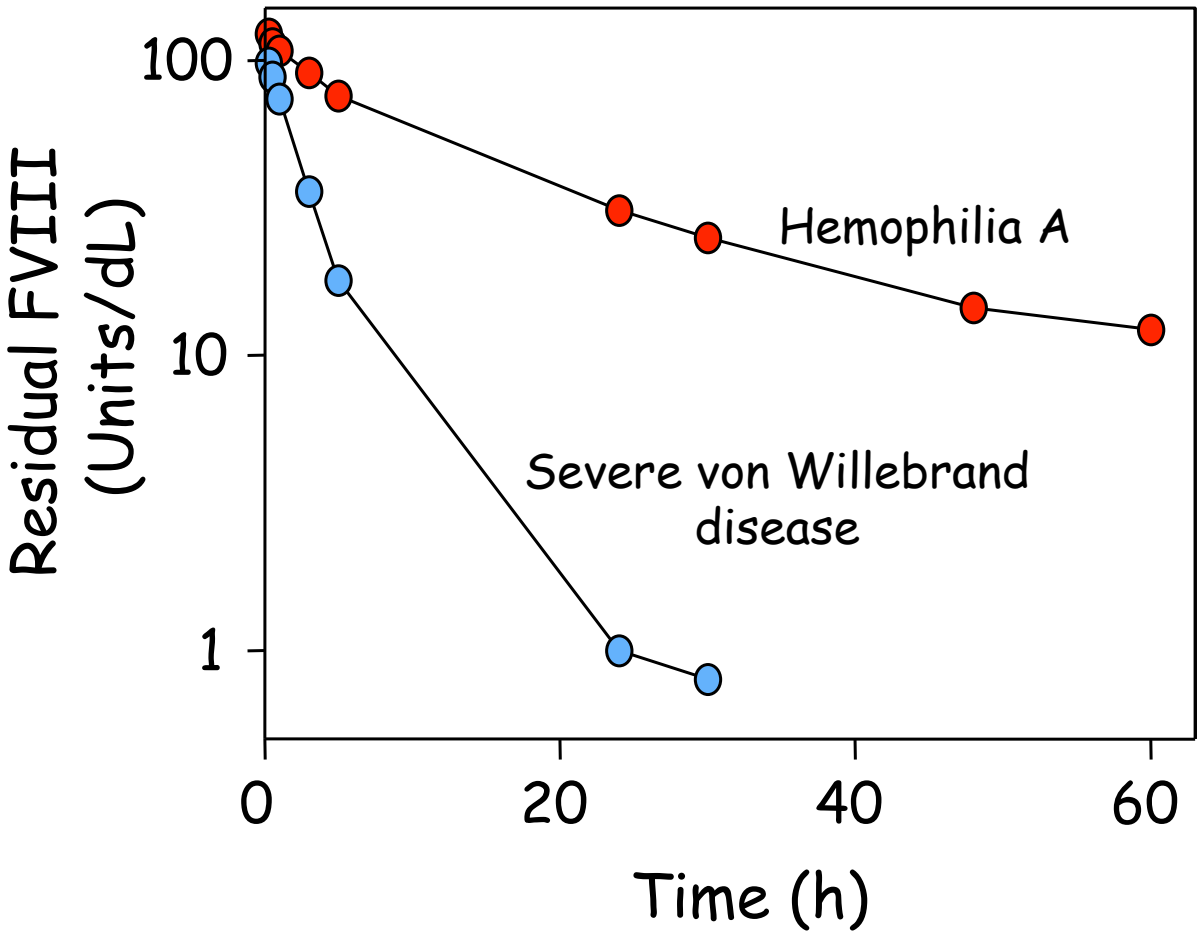
VWF inhibits binding of FVIII to phospholipid-surface of activated platelets



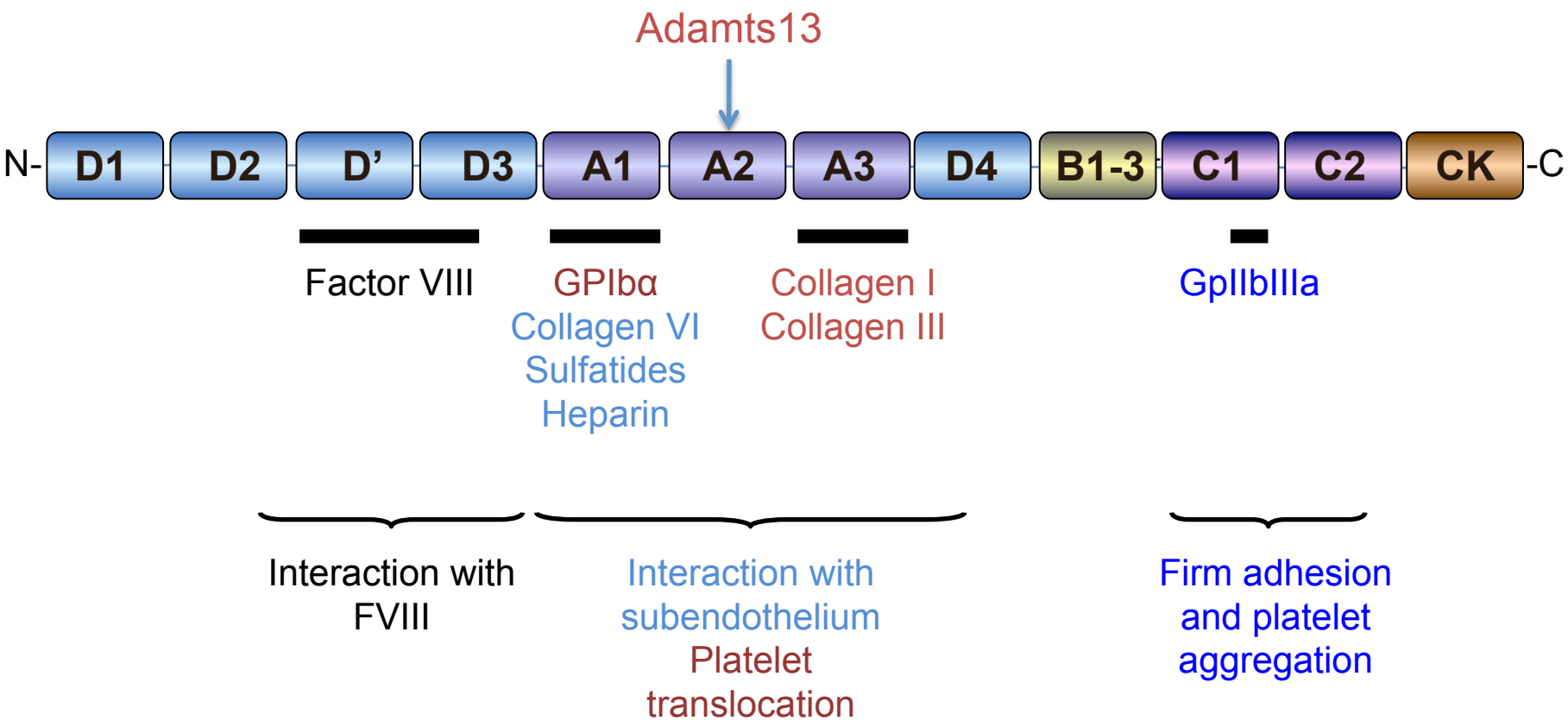
VWF prevents cellular uptake by clearance receptors

VWF protects FVIII from proteolysis by phospholipid-dependent proteases (APC, factor Xa)

Reduced survival of FVIII in the absence of VWF

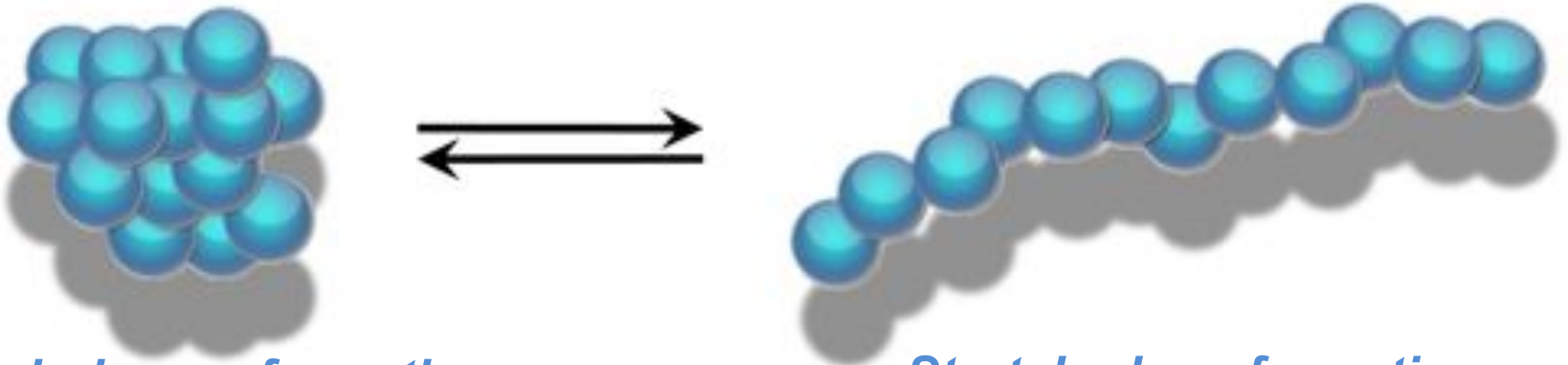


VWF functional domains



Regulation of VWF function

There are many regulators of VWF function. One of them is related to its conformation



Globular conformation
(inactive)

Circulating VWF

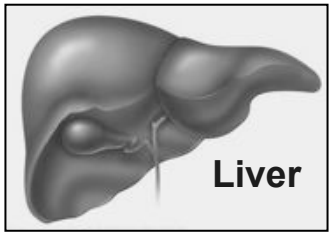
- *A1 domain is inaccessible for platelet binding*
- *ADAMTS13 cleavage site in the A2 domain is unavailable*

Stretched conformation
(active)

VWF immobilized on a surface or submitted to high shear stress

- *A1 domain is accessible for platelet binding*
- *ADAMTS13 cleavage site in the A2 domain is available*

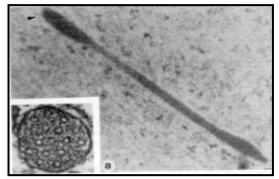
Life-cycle of VWF

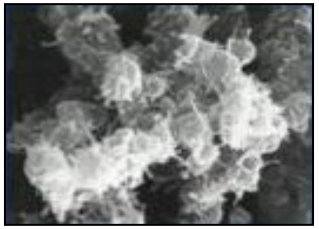


Clearance

Biosynthesis

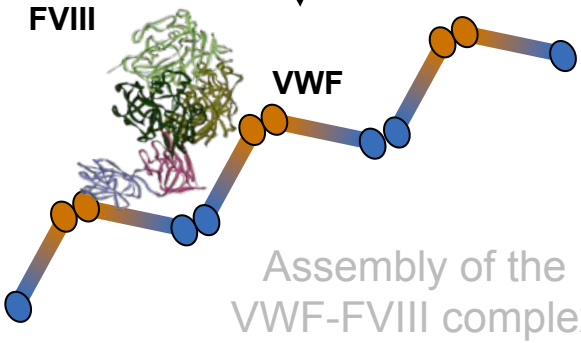
Storage and secretion in plasma





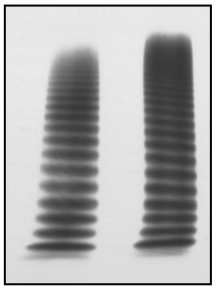
Formation of the platelet thrombus

Functions



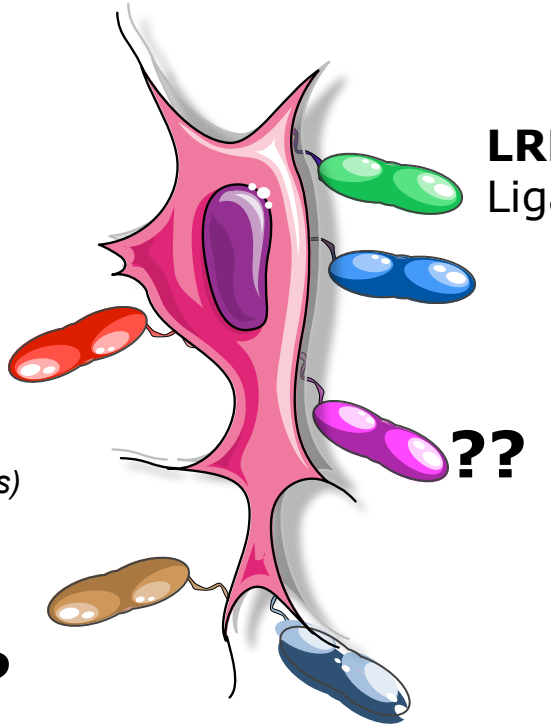
Assembly of the VWF-FVIII complex

Regulation of multimer size



Clearance of VWF

- ✓ VWF and the FVIII/VWF complex are targeted to liver and spleen macrophages
- ✓ VWF clearance is strongly influenced by its glycosylation profile
- ✓ Endocytosis receptor(s) are only partially identified



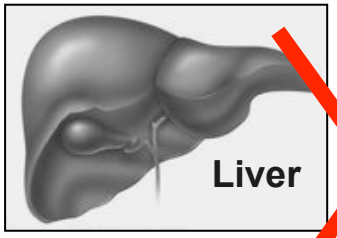
LRP1 *(LDL receptor related protein)*
Ligands: FVIII, FIXa, FXa, …

(Rastegarlari et al, Blood, 2012, 119: 216-2134)

Siglecs (Siglec-5)
(*sialic acid binding Ig-like lectins)*

(Pegon et al, Hematologica, 2012, In press)

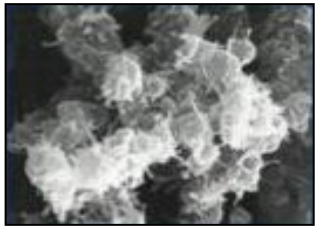
Life-cycle of VWF



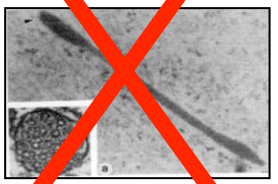
Clearance

Biosynthesis

VON WILLEBRAND DISEASE

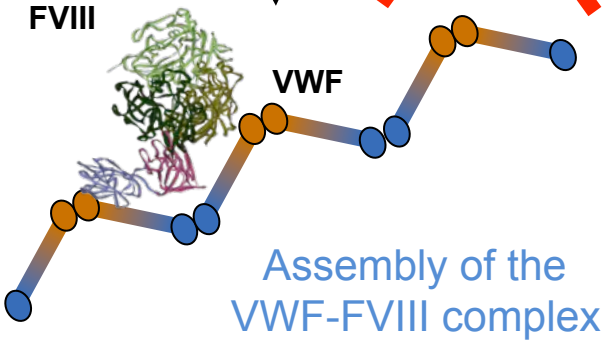


Formation of the platelet thrombus



Storage and secretion in plasma

Functions



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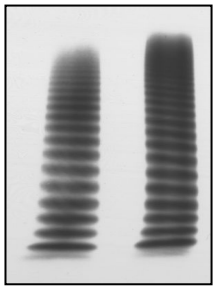


Presentation schedule

- ✓ Basic notions about hemostasis
- ✓ Von Willebrand factor: introduction
- ✓ Von Willebrand disease (VWD)
- ✓ Animal models of von Willebrand disease
- ✓ The use of hydrodynamic injection to generate new murine models

Classification of VWD

Genetic bleeding disorder

TYPE 1 (50-75% of cases)

- ✓ Partial quantitative defects, accounting for 70% of the cases
- ✓ Limited bleeding symptoms, normal activity of residual VWF
- ✓ Dominant transmission

TYPE 3 or severe (≤ 5% of cases)

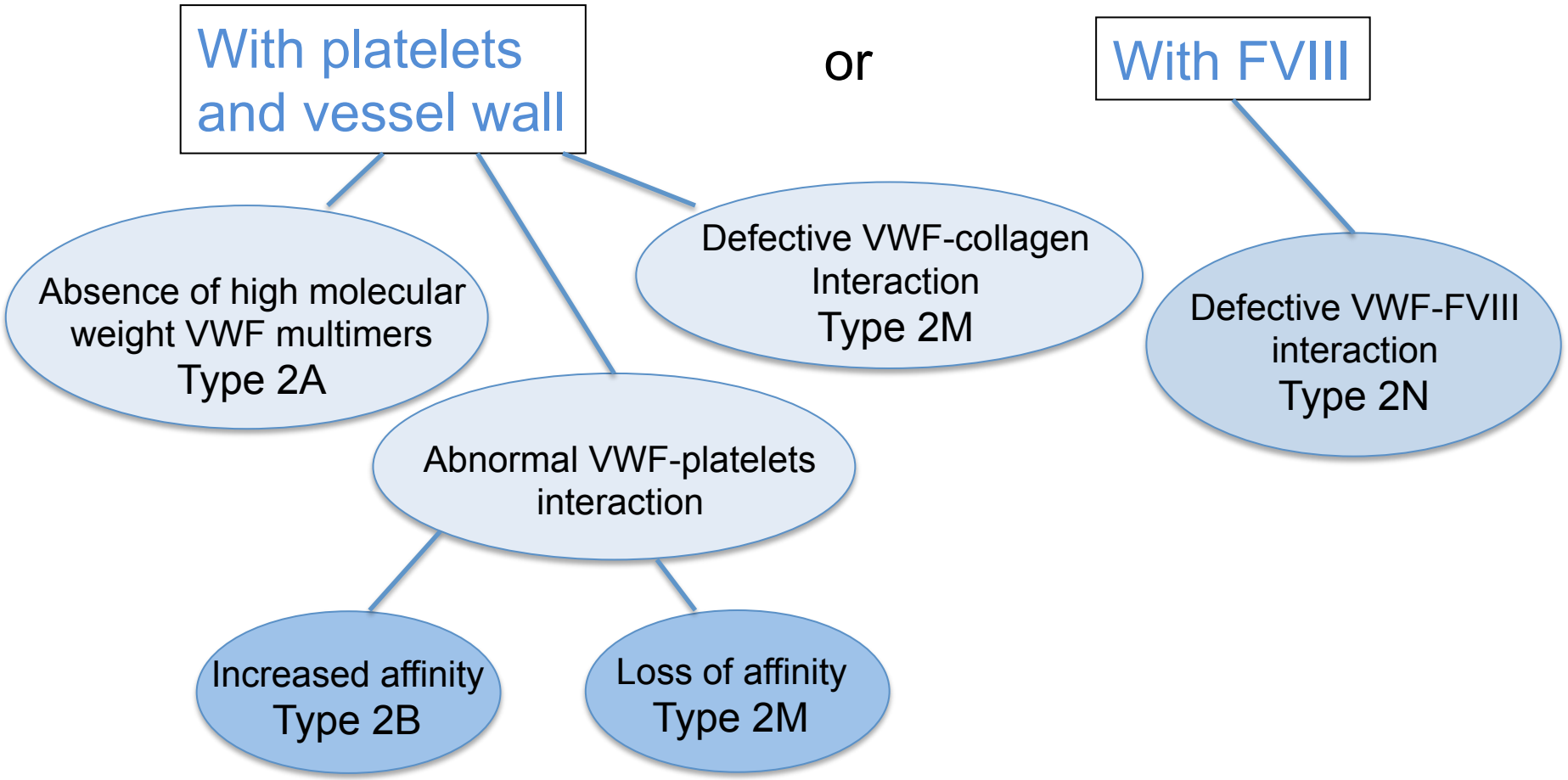
- ✓ Complete deficiency in VWF and strong reduction in FVIII
- ✓ Combined defects in primary hemostasis and coagulation leading to severe bleeding symptoms
- ✓ Recessive transmission

TYPE 2 (20-45% of cases)

- ✓ Qualitative defects
- ✓ Four main subtypes (2A, 2B, 2M et 2N) according to the multimeric profile and affected function
- ✓ Dominant or recessive transmission

VWD Type 2

Abnormal VWF interaction with its ligands



VWD: Epidemiology

- ✓ Prevalence in the general population
  ≈ 1 p. 100
  (Rodeguiero et al, Blood 1987)

- ✓ Prevalence of symptomatic subjects
  ≈ 1 p. 10 000
  (Joint WHO/ISTH Meeting, London 1998)

- ✓ Prevalence of severe form
  ≈ 0.5 to 5 p. 1 000 000

In France (65 millions inhabitants): 7000 to 8000 symptomatic patients
50 to 100 patients with VWD type 3

Globally, there is a overrepresentation of women and of individuals with blood group O

Hemorrhagic symptoms in VWD

Symptoms	VWD (n=264)	NORMAL (n=500)
Epistaxis	62.5%	4.6%
Menorrhagia	60.0%	25.3%
Bleeding following tooth extraction	51.5%	4.8%
Gum bleeding	34.8%	7.4%
Post-surgical bleeding	28.0%	1.4%

Sylwer et al, Acta Paediatr Scand, 1973

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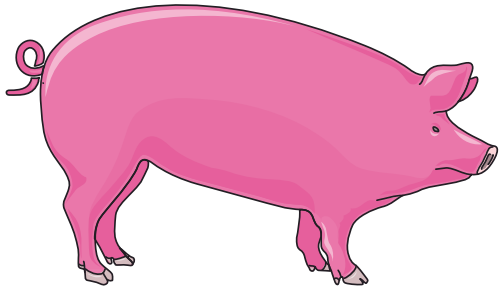
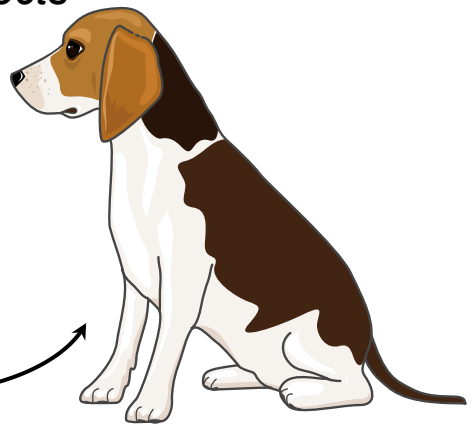
Animal models of VWD



Canine type 1, 2, 3 VWD
(1970)
Various genetic defects



Porcine type 3 VWD
(1941)

Reduced transcription

Post-translational defects

VWD models

Murine type 1, 2 and 3 VWD
(1990; 1998; 2008)

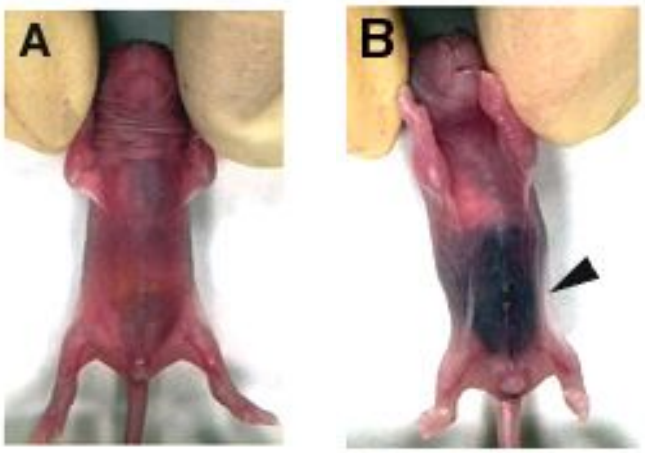
Presence of gene modifiers (type 1 VWD)

Genetically-induced (type 3 VWD)

Hydrodynamic gene transfer (type 2 VWD)

Murine model of VWD

VWF +/+ **VWF -/-**



- Spontaneous hemorrhage at the abdominal level in 10% of the mutant neonates
- Prolonged bleeding time

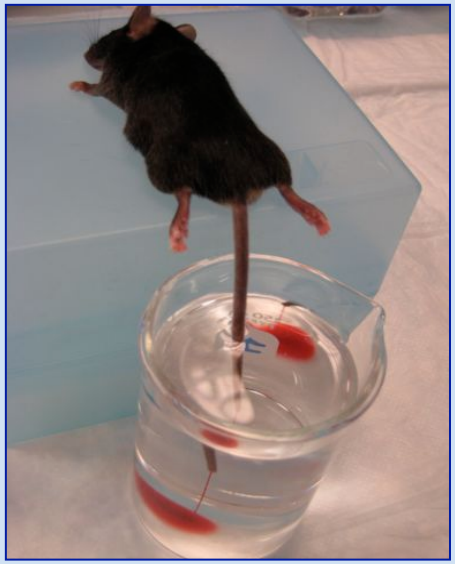
VWF -/- mice represent a good model of human severe VWD

(Denis et al, PNAS, 1998, 95: 9524-9529)

In vivo tests of VWF function

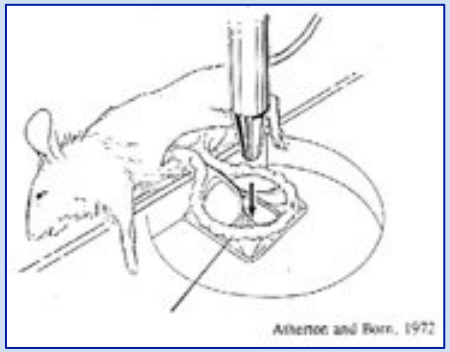
Bleeding time: Tail clip assay

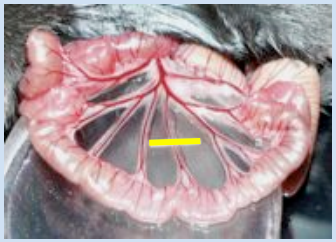
- Mouse anesthetized
- Cut 3 mm of the tail extremity with a scalpel
- Immerse tail in 37°C saline
- Measure time to 1st arrest of bleeding
- Stop after 10 min if no spontaneous stop

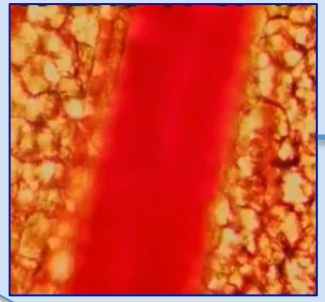


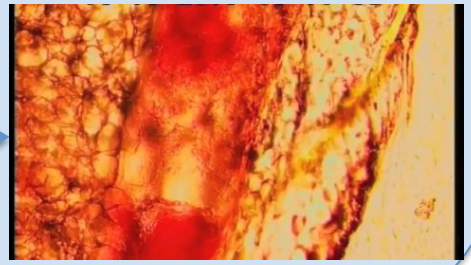
Thrombosis model: $FeCl_3$-induced injury in mesenteric vessels

- Mouse anesthetized
- Inject rhodamine 6G IV to label platelets
- Exteriorize mesenteric vessels
- Put mouse under microscope
- Place filter paper saturated with with 7.5% $FeCl_3$ on vessels for 5 min to induce vessel injury
- Measure time to vessel occlusion









Presentation schedule

- ✓ Basic notions about hemostasis
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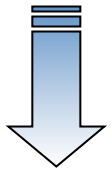
Aim of the study

To develop murine models to study

VWF structure-function relationships

in vivo

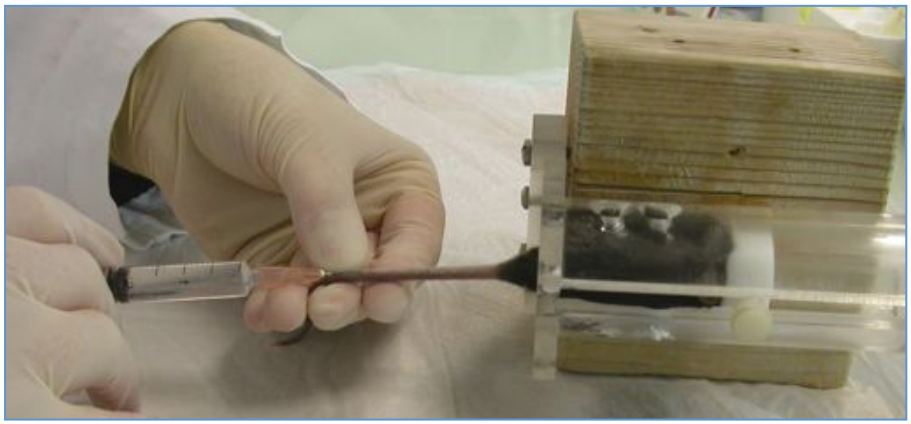


VWF-deficient mice

Hydrodynamic injection

Hydrodynamic injection (1)

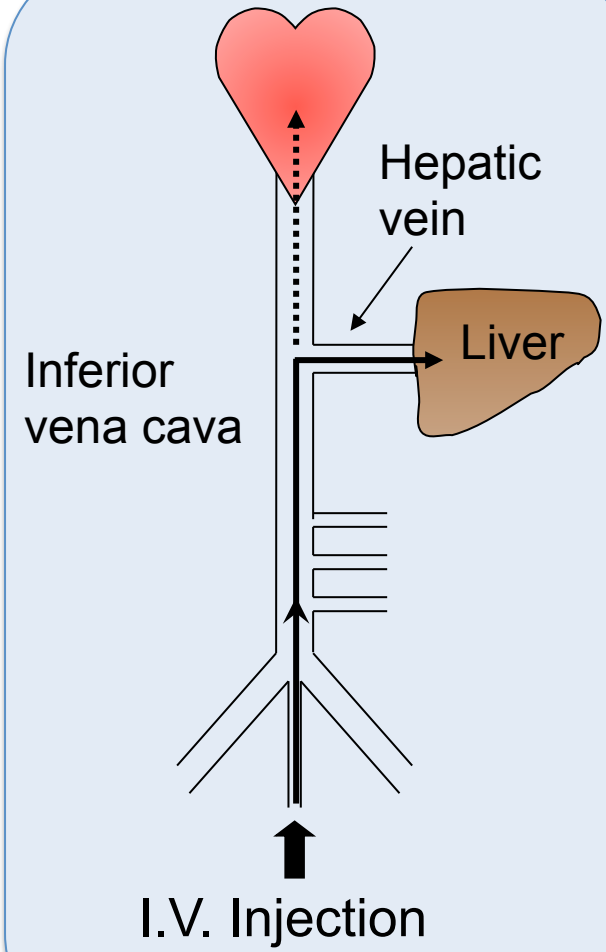
In vivo transfection allowing transient expression of a transgene by the liver



A rapid injection of a large volume of plasmid DNA via the tail vein of mice

- *Injection volume: 10% of the bodyweight*
  (i.e 2ml for a 20g mouse)
- *Injection time: ~ 5 sec*

Hydrodynamic injection (2)



Mechanism

- Exceeding the cardiac output
- Development of high pressure
- Back flow of the solution towards organs connected to the vena cava
- The liver absorbs most of the injected solution
- 10-40% of hepatocytes are transfected

Zhang et al, Human Gene Therapy, 1999, 10:1735; Liu et al, Gene Therapy, 1999, 6:1258

Hydrodynamic injection toxicity?

- ✓ Very little, if any, deaths following injection
- ✓ Animal growth is not affected in the days following hydrodynamic injection
- ✓ Concentration of ions (Na+,K+, Cl-), total protein concentration, albumin and bilirubin concentrations are not affected
- ✓ Concentration of liver specific enzymes such as alkaline phosphatase and aspartate aminotransferase (AST) remains unchanged

- ✓ Concentration of alanine aminotransferase (ALT) is increased 4-20 fold at 1 day post-injection but goes down to baseline within 3 days
- ✓ Liver necrosis is apparent in 50% of the mice at 1 day post-injection and affects between 5-10% of hepatocytes
- ✓ Platelet counts drop by 30% at 1 day post-injection

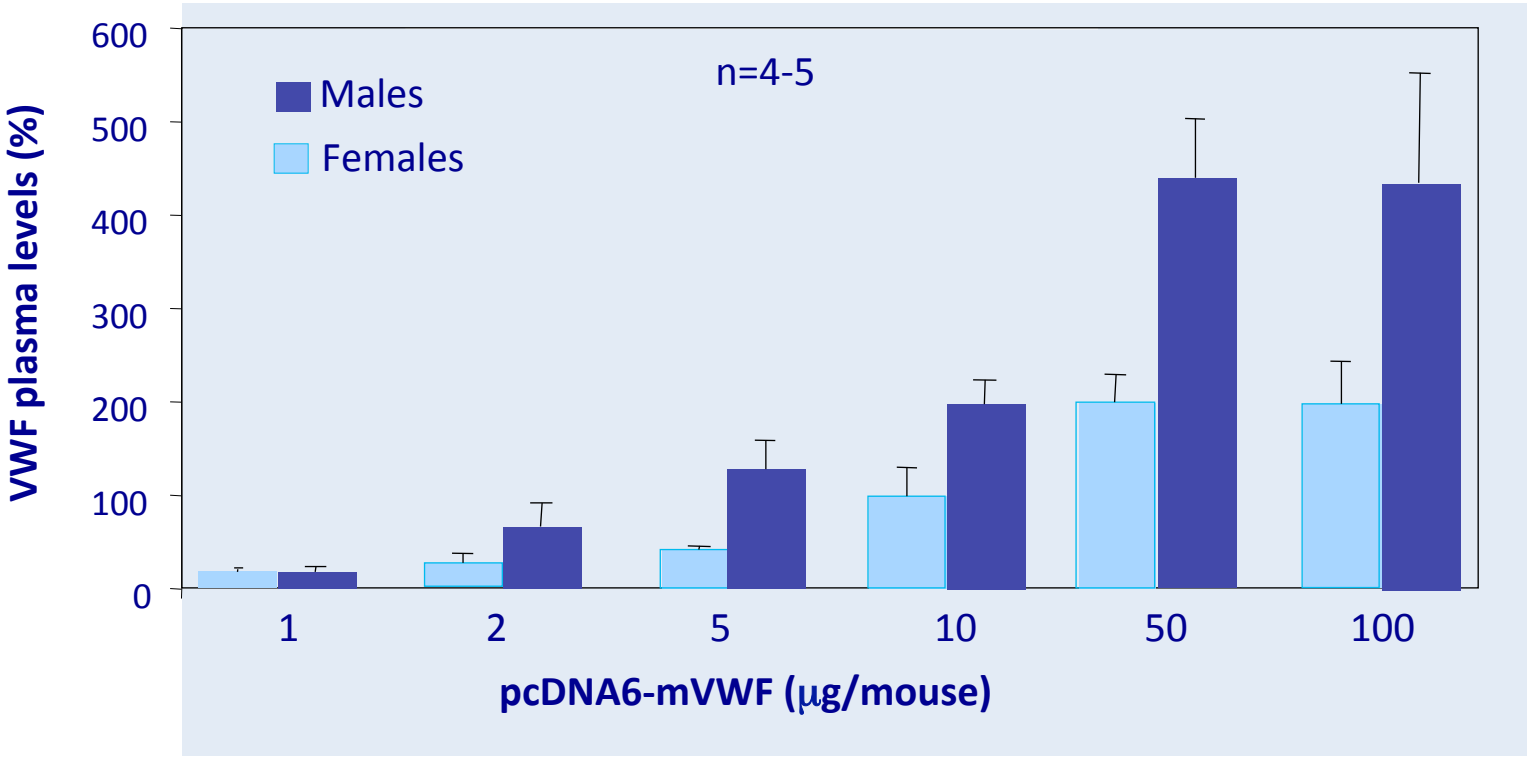
Expression in liver hepatocytes

- ✓ Injection of 50 μg murine von Willebrand factor (VWF) cDNA in a VWF-/- mouse
- ✓ Liver isolation 24H after injection

VWF expression is switched from endothelial cells in wild-type mice to hepatocytes in VWF knockout mice injected with mVWF cDNA

VWF expression levels following hydrodynamic injection

- ➔ Injection of 1-100μg of murine VWF cDNA in VWF-/- mice
- ➔ Blood collection 24h after injection
- ➔ ELISA assay to measure VWF plasma levels

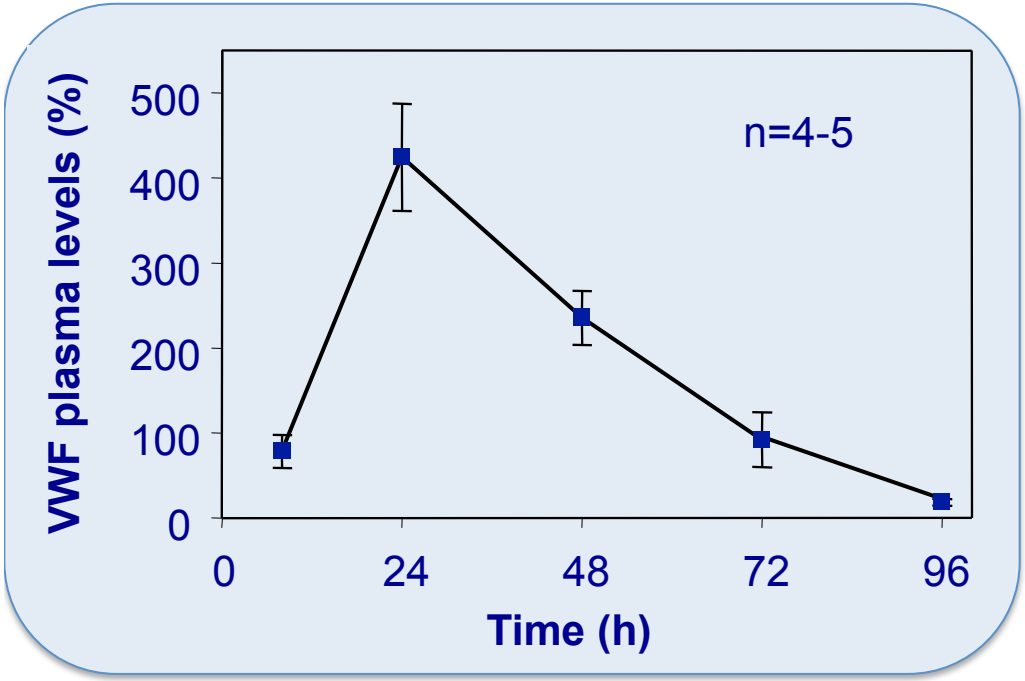


Kinetic of expression of VWF after hydrodynamic injection

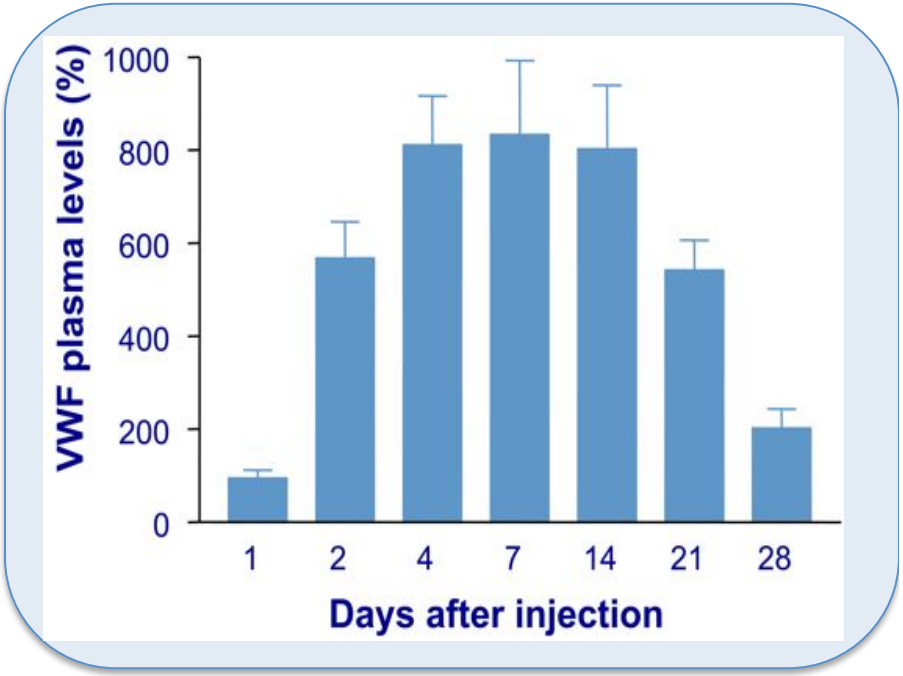
➔ Injection of 50μg murine VWF cDNA in VWF-/- mice

➔ Blood collection at different time points after injection

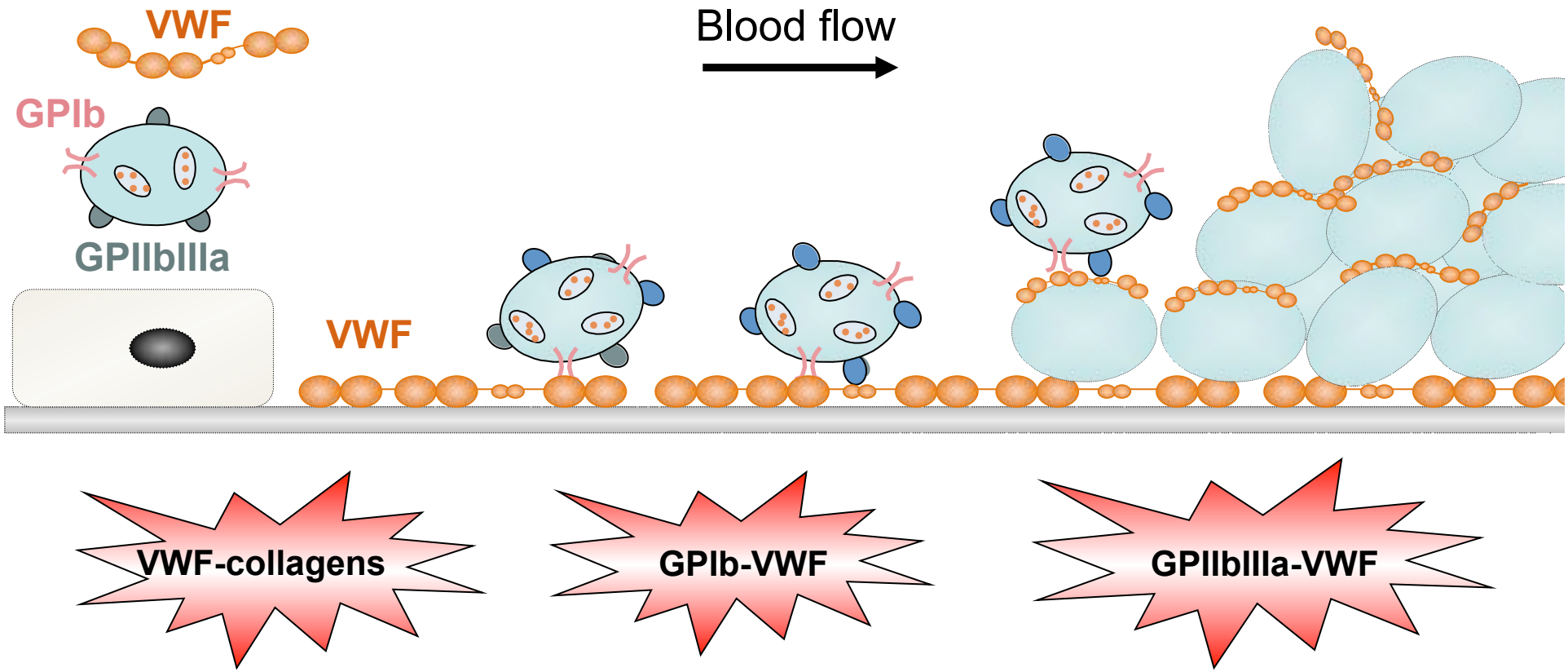
pcDNA6 vector (CMV promoter)



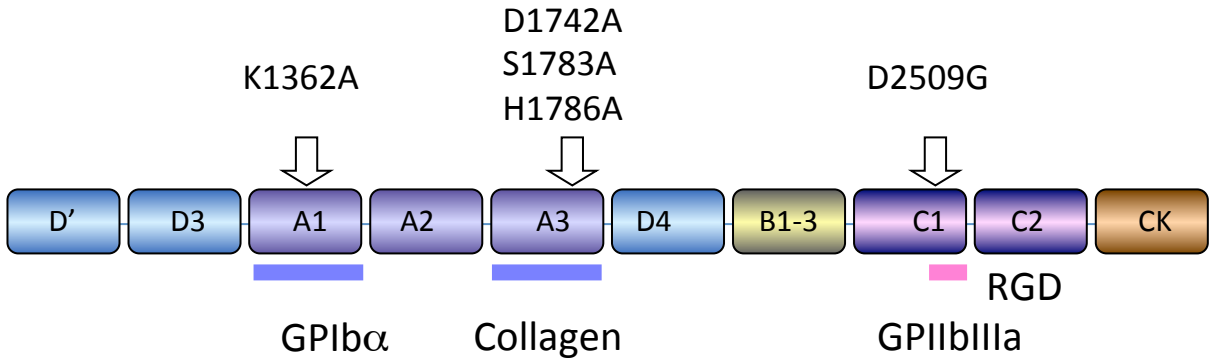
pLIVE vector (albumin promoter)



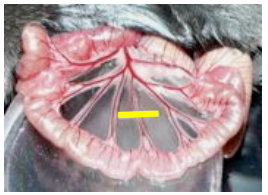
VWF mutants expressed in vivo



Analysis of VWF carrying mutations in its main binding sites



VWF-/- mice



Murine cDNA injected	Tail bleeding	$FeCl_3$-induced thrombosis
None	Infinite	Minimal
Wild type	Normal	Normal
GPIbα-binding mutant	Infinite	Minimal
Collagen–binding mutant	**Normal**	**Reduced**
GPIIbIIIa-binding mutant	**Normal**	**Reduced**

Marx et al, ATVB, 2008, 28, 419

Marx et al, Blood, 2008, 112, 1704

Summary analysis of mutants

Collagen and GPIIbIIIa binding mutants

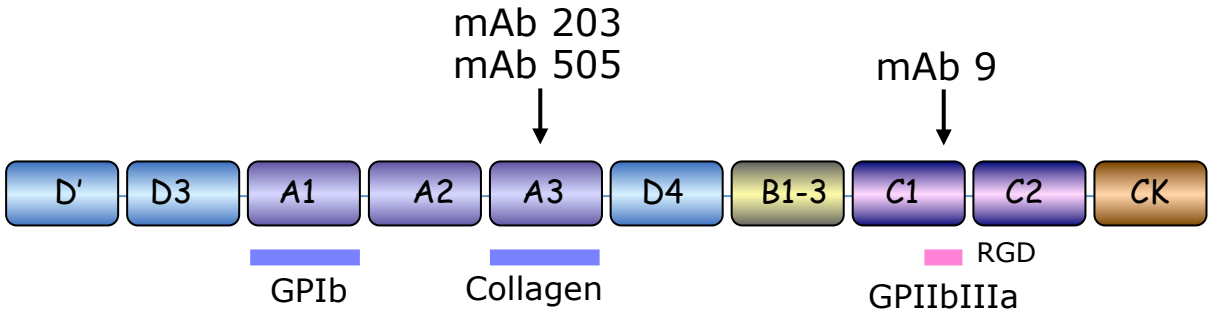
- ✓ Delayed vessel occlusion
- ✓ Complete correction of bleeding time

VWF interactions with collagens type I and III and with GPIIbIIIa are more important in the pathological thrombotic process than in the physiological process of hemostasis

Interesting targets for anti-thrombotic therapy

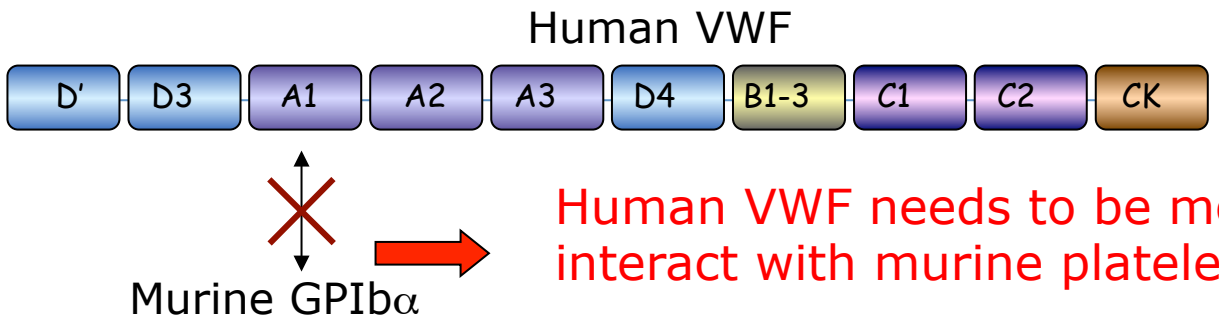
Anti-thrombotic potential of mAbs to VWF

To test anti-thrombotic properties in mice of tools targeting VWF binding to collagens or to GPIIbIIIa



These mAbs are directed against human VWF and do not cross-react with murine VWF

→ Hydrodynamic injection of human VWF cDNA in VWF$^{-/-}$ mice :

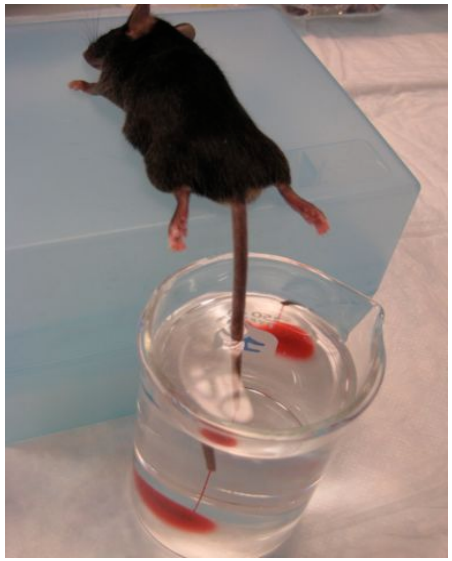


Human VWF needs to be modified to interact with murine platelets

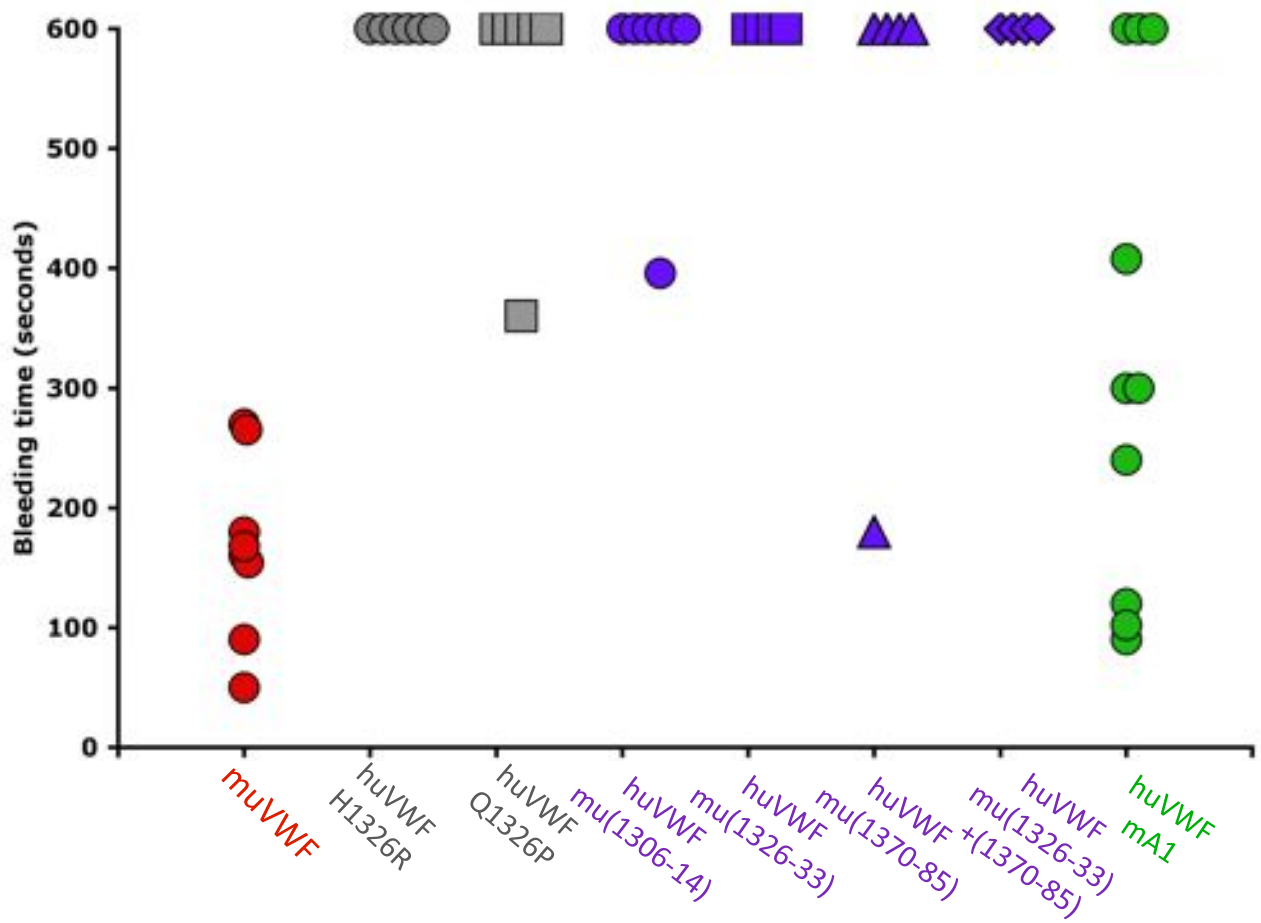
Can human VWF be modified to bind murine GPIb?

- ✓ Mutagenesis of single residues potentially involved in the interaction between human VWF and murine GPIb or substitution of larger murine sequences into the A1 domain of human VWF
- ✓ Hydrodynamic injection of the Vwf cDNA variants to VWF -/- mice
- ✓ One day later bleeding time test

pCDNA6 mu VWF
pCDNA6 hu VWF H1326R
pCDNA6 hu VWF Q1391P
pCDNA6 hu VWF mu 1306-14
pCDNA6 hu VWF mu 1326-33
pCDNA6 hu VWF mu 1370-85
pCDNA6 hu VWF mu 1326-33+1370-85
pcDNA6 VWF hu VWF/mu A1



In vivo function of VWF chimeras (bleeding time)

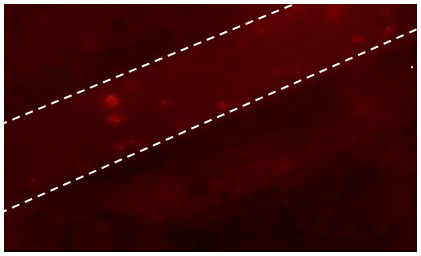


Only a chimera containing the entire murine A1 domain was able to correct the bleeding phenotype of VWF $^{-/-}$ mice

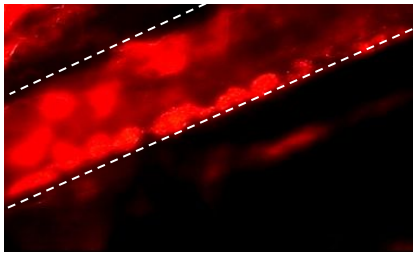
Functionality of huVWF/mu-A1 chimera in $FeCl_3$-induced thrombosis model

Hu-VWF/mu-A1

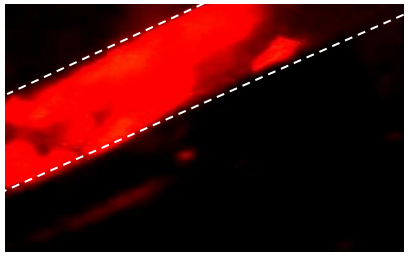
5 min



10 min

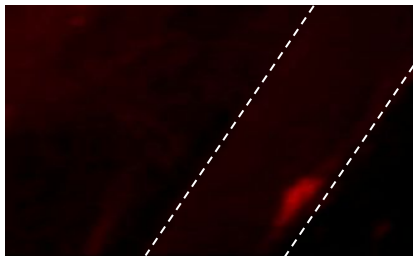


20 min

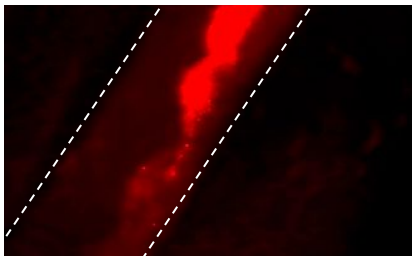


mu VWF

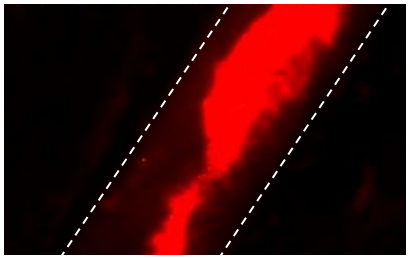
5 min



10 min

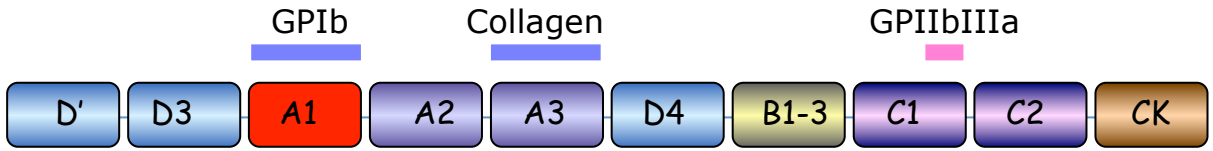


20 min



Hu-VWF/mu-A1 chimera was able to restore thrombus formation in VWF KO mice

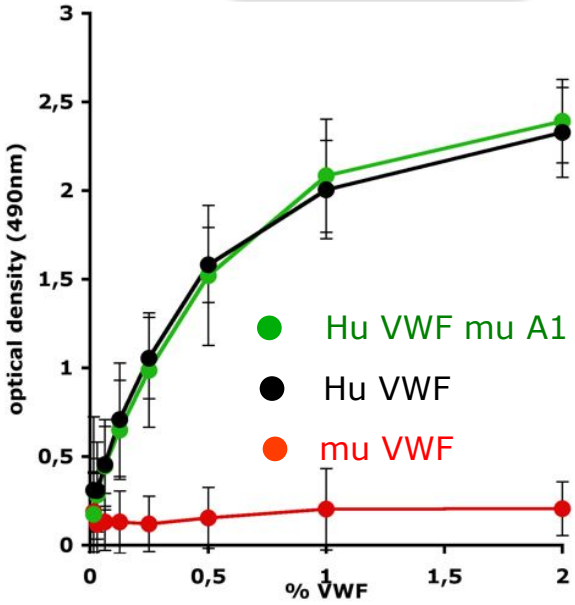
Recognition of huVWF/mu-A1 chimera by mAbs to human VWF

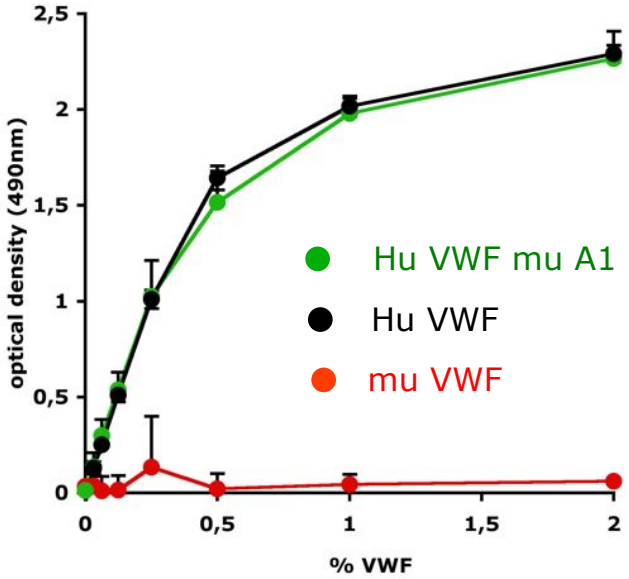


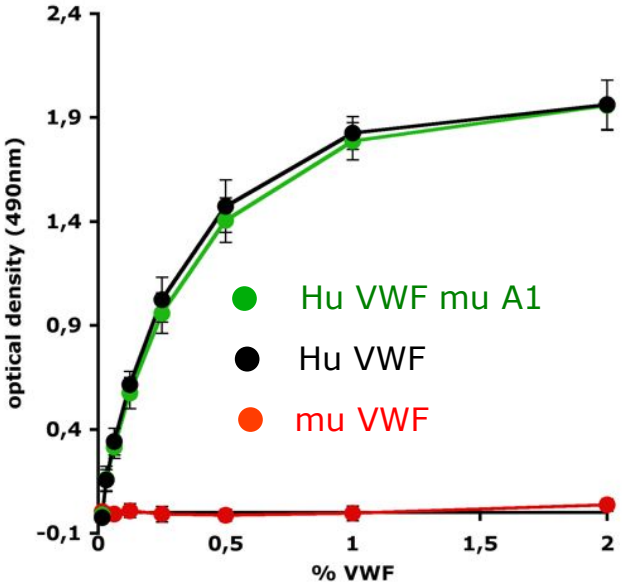
mAb 203

mAb 505

mAb 9

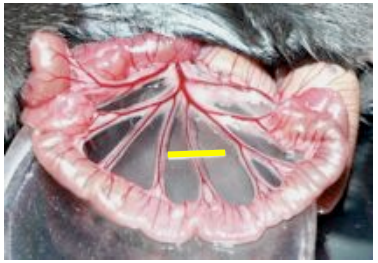






Antibodies efficacy in a thrombosis model

- ✓ Hydrodynamic injection of hu-VWF/mu-A1 cDNA to VWF KO mice
- ✓ 4 days later: IV injection of 100 µg of mAbs 9, 203, 505 or control isotype
- ✓ Platelets labeling by retro-orbital injection of rhodamine 6G
- ✓ Induction of vascular injury in mesenteric vessels by application of $FeCl_3$



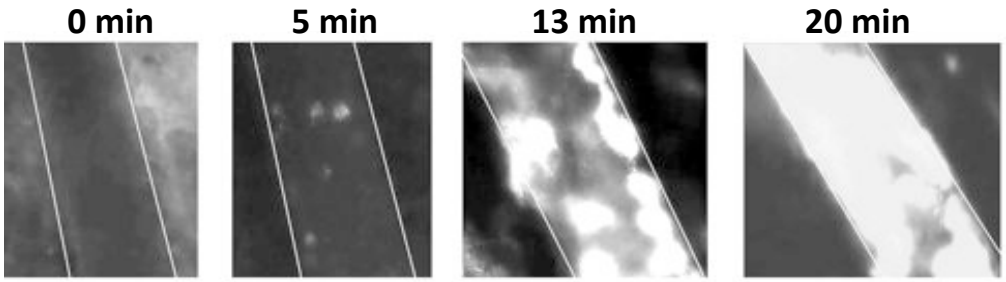
Results Mabs efficacy in a thrombosis model

Antibody	Number of mice reaching venous occlusion	Number of mice reaching arterial occlusion
Control isotype	8 out of 8 tested	8 out of 8 tested
mAb 203 (anti VWF-collagen)	5 out of 8 tested	1 out of 8 tested
mAb 505 (anti VWF-collagen)	4 out of 8 tested	3 out of 8 tested
mAb 9 (anti VWF-GPIIbIIIa)	6 out of 8 tested	3 out of 8 tested

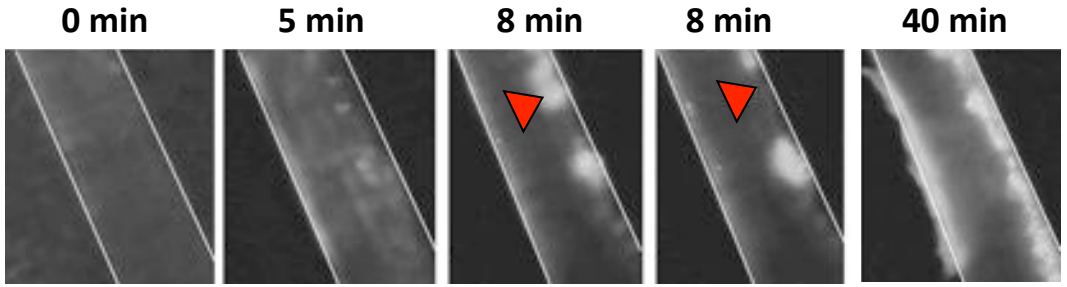
Effect of the mAbs anti VWF was stronger on thrombus formation in arteries than in veins when used preventively

Thrombus growth in arteries of mice pre-treated with anti-VWF mAbs

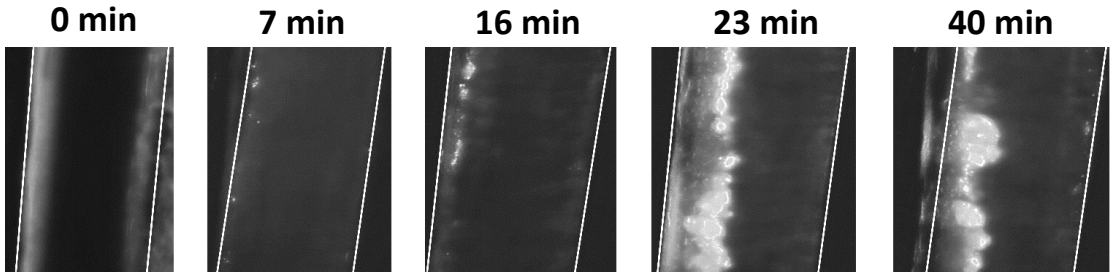
mAb Control isotype
Arteries occlusion:
8 out of 8 treated mice



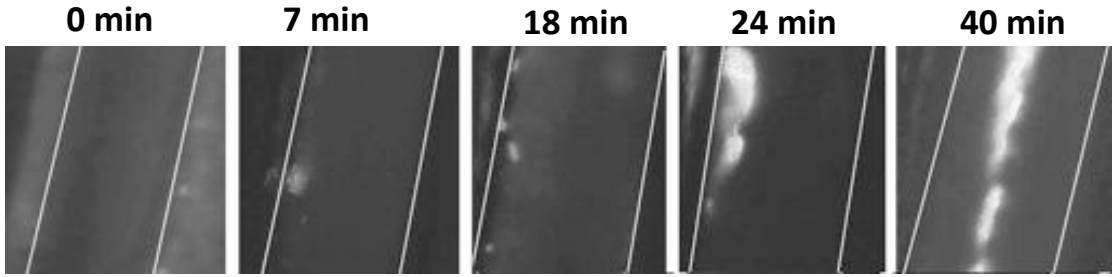
mAb 9 (anti VWF-GPIIbIIIa)
Arteries occlusion:
3 out of 8 treated mice



mAb 203 (anti VWF-collagen)
Arteries occlusion:
1 out of 8 treated mice



mAb 505 (anti VWF-collagen)
Arteries occlusion:
3 out of 8 treated mice



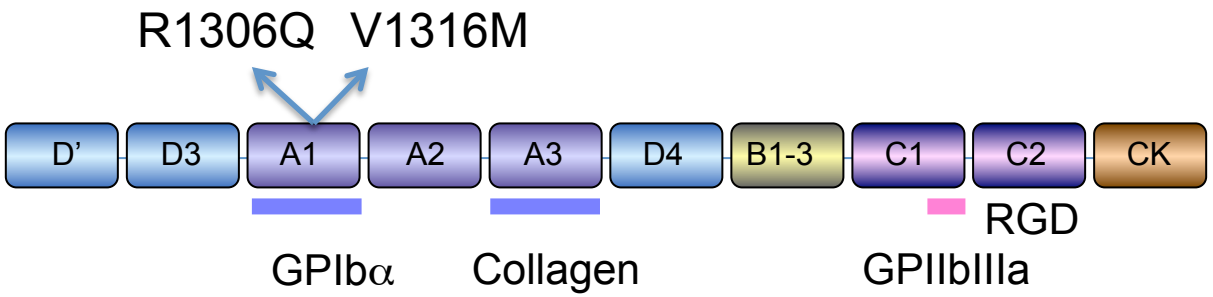
Summary antibodies study

- ✓ A model has been developed allowing to test therapeutic agents targeting human VWF in mouse
- ✓ All the antibodies tested inhibiting the interactions of VWF with fibrillar collagen and platelet GPIIbIIIa were able to prevent thrombus formation in mesenteric arteries without affecting the bleeding phenotype of the treated animal.
- ✓ Effect of the anti VWF mAbs was stronger on thrombus formation in arteries than in veins.
- ✓ Inhibition of the VWF-collagen and VWF-GPIIbIIIa axes is a promising strategy that deserves further investigations (other thrombosis models, other inhibiting agents…).

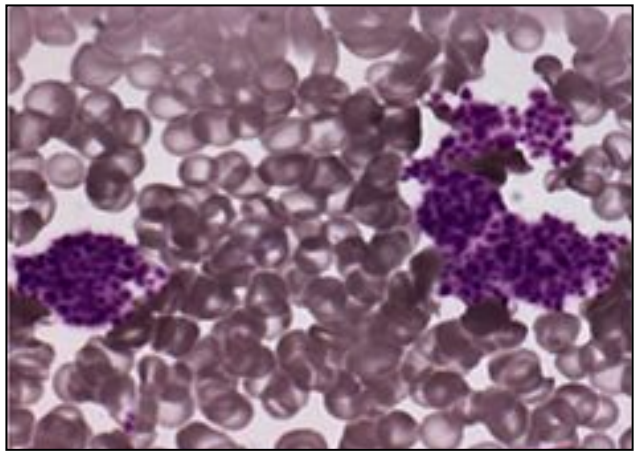
(Navarrete et al, Blood, 2012, 120, 2723-2732)

Type 2B VWD

- ✓ Characterized by increased VWF-GPIbα binding due to “gain of function” mutations
- ✓ Leads to fluctuating thrombocytopenia and loss of high molecular weight VWF multimers
- ✓ Different mutations lead to variable phenotype



Human type 2B patient



Federici et al, Blood, 2009, 113, 526

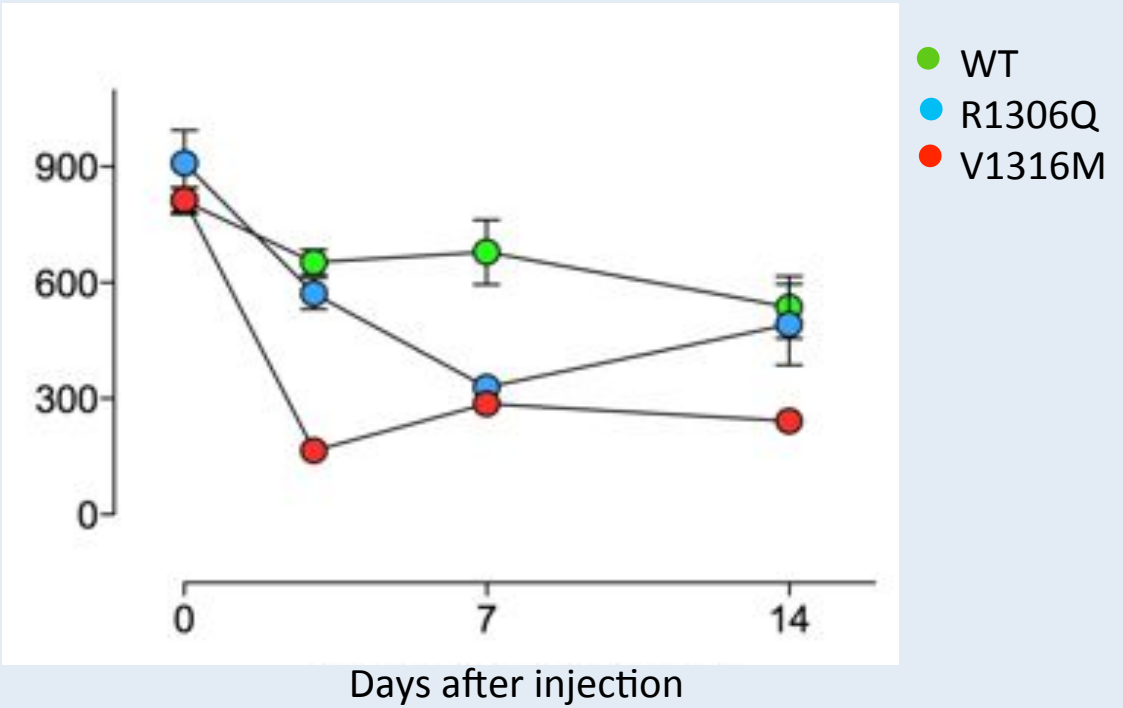
Generation of new models of VWD
Comparison of two type 2B mutations: R1306Q and V1316M on thrombocytopenia

Blood. 2010;115(23):4870-4877

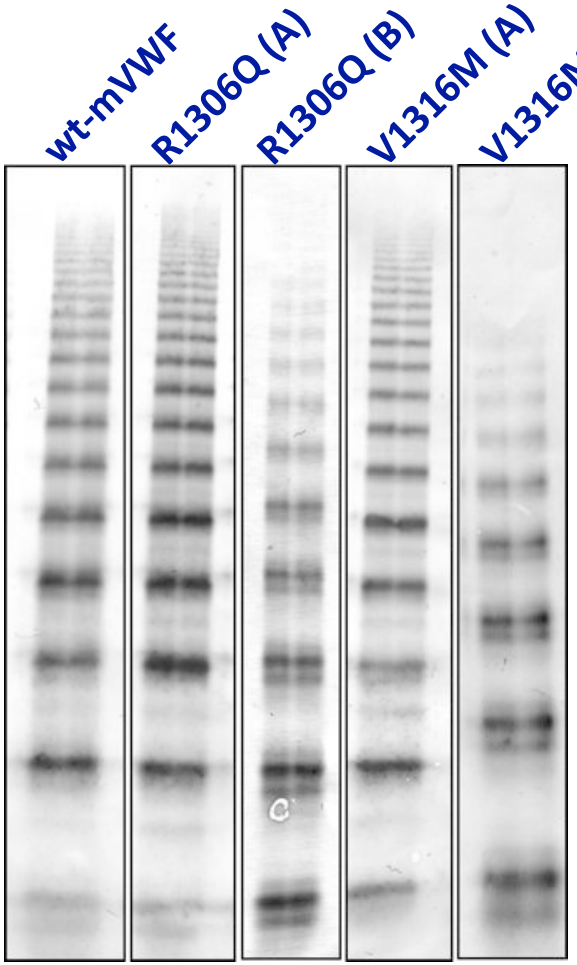
Mutation and ADAMTS13-dependent modulation of disease severity in a mouse model for von Willebrand disease type 2B

*Julie Rayes,[1] *Martine J. Hollestelle,[2] Paulette Legendre,[1] Isabelle Marx,[1] Philip G. de Groot,[2] Olivier D. Christophe,[1] Peter J. Lenting,[1] and Cécile V. Denis[1]

Injection mVWF cDNA carrying type 2B mutations To VWF-/- mice



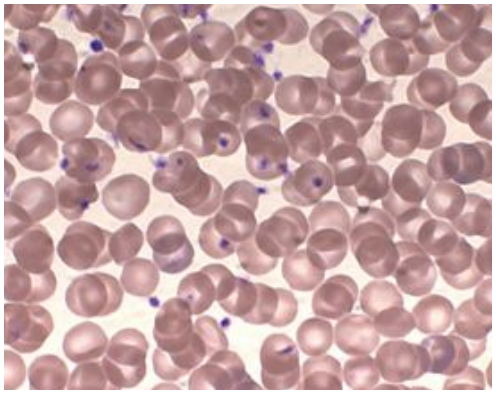
Comparison of two type 2B mutations: R1306Q and V1316M: Effect on VWF multimer profile



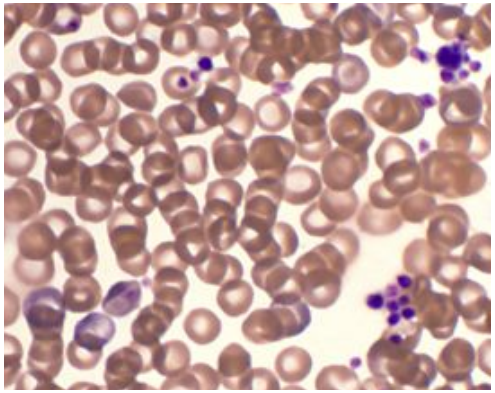
- ✓ Loss of high molecular weight multimers is associated with type 2B mutations in about 50% of the mice
- ✓ The loss of high molecular weight multimers is more pronounced with V1316M mutation

Comparison of two type 2B mutations: R1306Q and V1316M: Effect on platelet aggregates

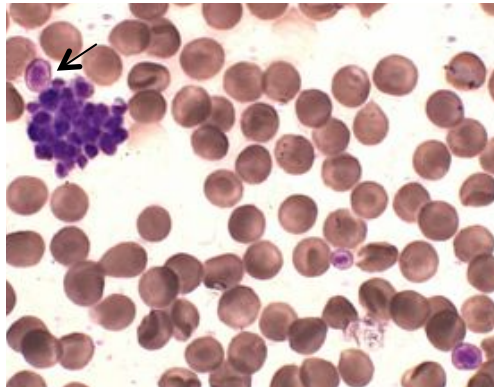
WT-mVWF



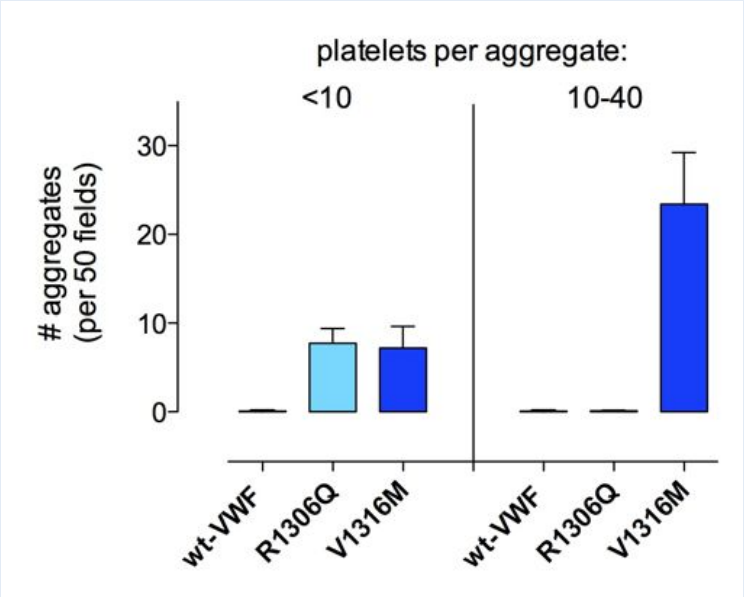
R1306Q



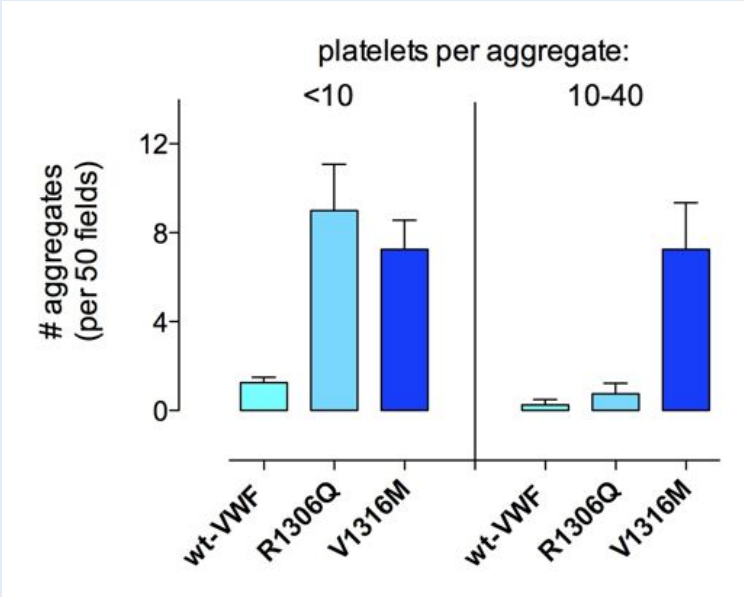
V1316M



3 days post-injection

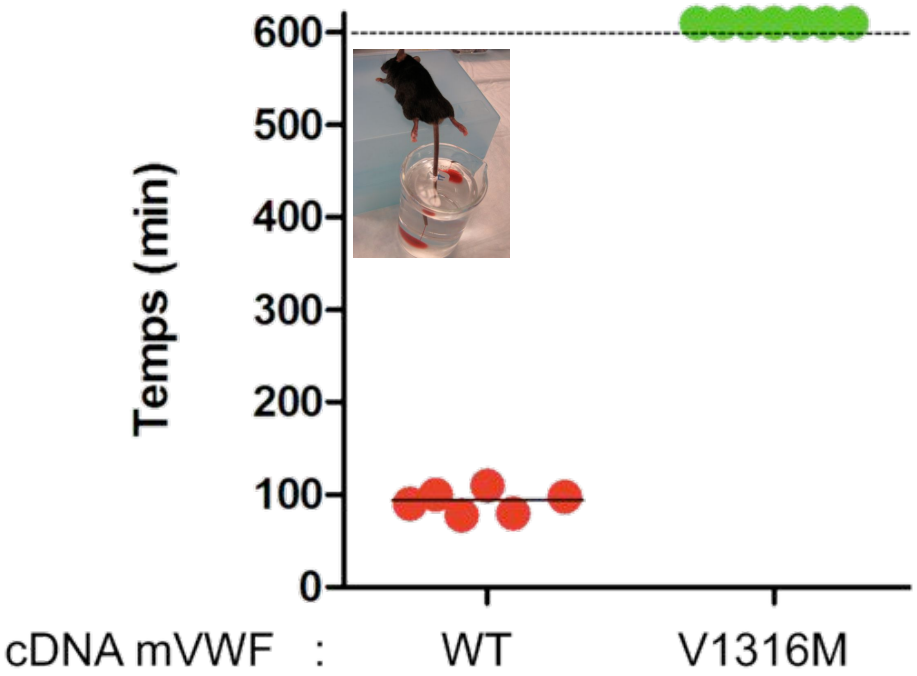


7 days post-injection



Type 2B mice resemble the human phenotype: Impaired hemostasis

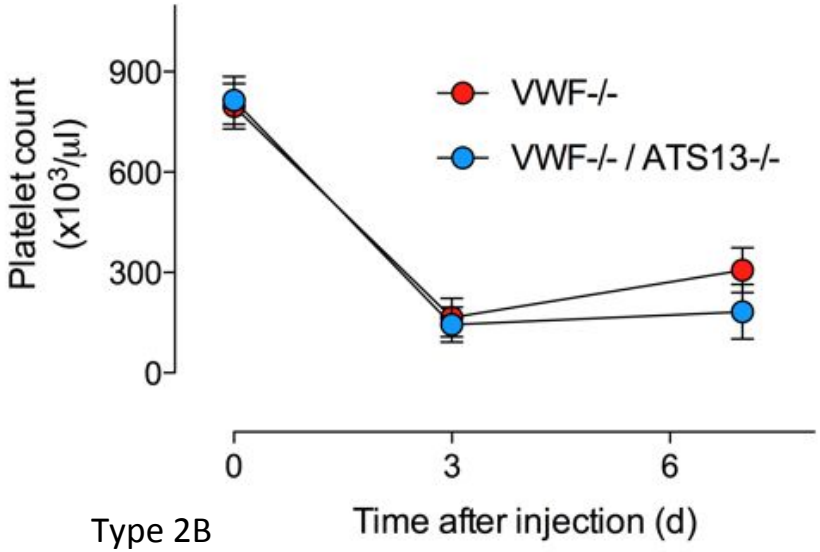
Tail-clip bleeding

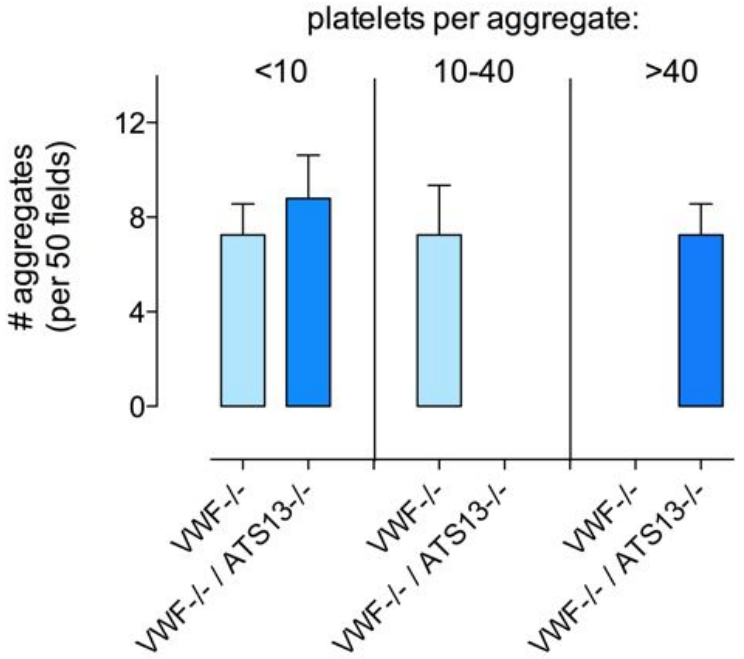


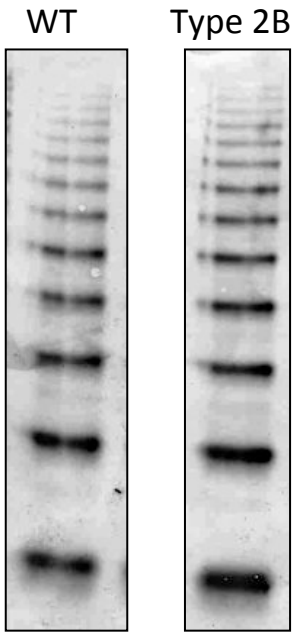
FeCl3-thrombosis model

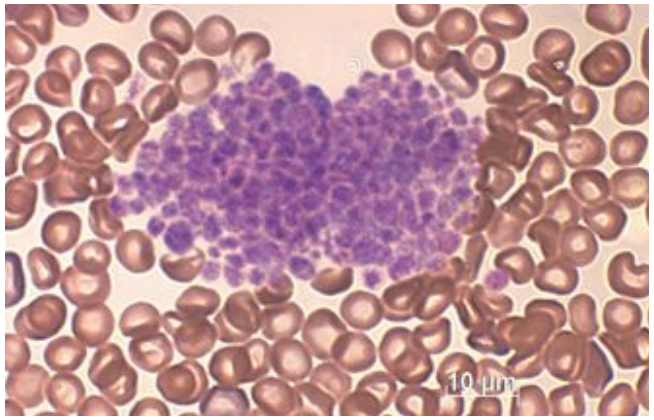
	wt	V1316M
Mesenteric venules		
- thombi >50 μm	7/7	1/5
- occlusion	6/7	0/5
Mesenteric arterioles		
- thombi >50 μm	7/7	0/5
- occlusion	6/7	0/5

ADAMTS13 deficiency worsens type 2B phenotype









Summary murine models of VWD

- ✓ Hydrodynamic injection of mutant VWF allows reproduction of human VWD phenotypes
- ✓ The technique is sensitive enough to reproduce phenotype subtleties associated with the expression of different mutations
- ✓ Quick method to assess causative effect of mutations in the expression of the disease