Hemostasis:

methods in plasma and “in vivo”

Dr. Sara Calzavarini

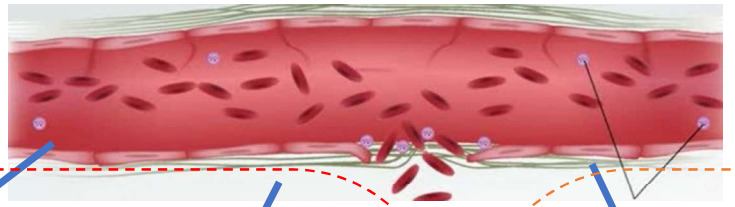
Dept. Hematology ,Dept. for Biomedical Research

Bern University -Switzerland-

Applied Biochemistry - Master Students

Ferrara, 26-28 November 2019

Hemostatic process: an overview



Vascular Injury

Collagen
vWF

Tissue factor

Vasoconstriction

Platelet activation

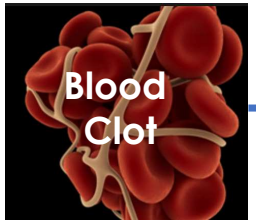
Coagulation cascade

vWF
Fibrinogen

Thrombin

Fibrinogen → Fibrin

Platelets plug



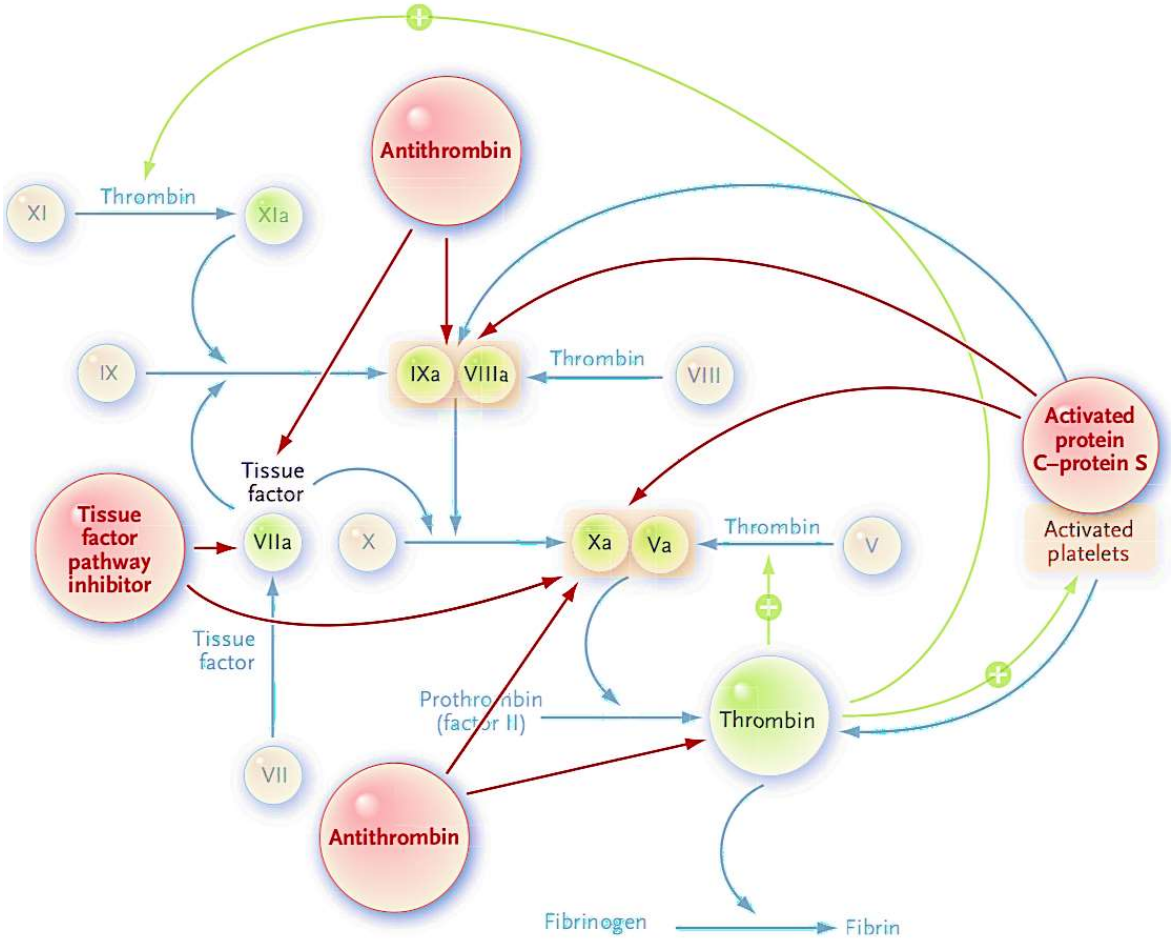
Plasmin

Fibrinolysis
&
Clot degradation

Primary Hemostasis

Secondary Hemostasis

Blood coagulation

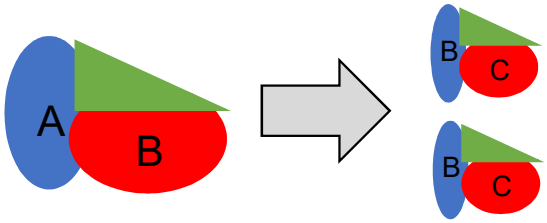


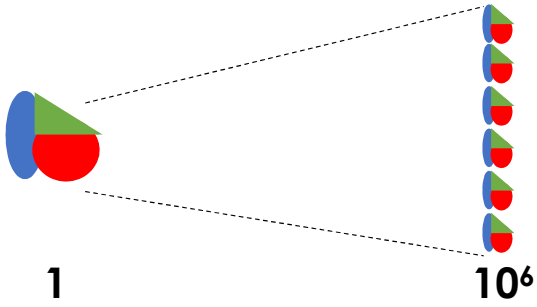
Thrombin is the key molecule!

Only if a certain level of thr is reached, fibrin can be formed only if the tg decrease below a certain threshold the fibrin formation is over too.

The cascade organization

Consequential enzymatic conversions of zymogens to activated enzymes

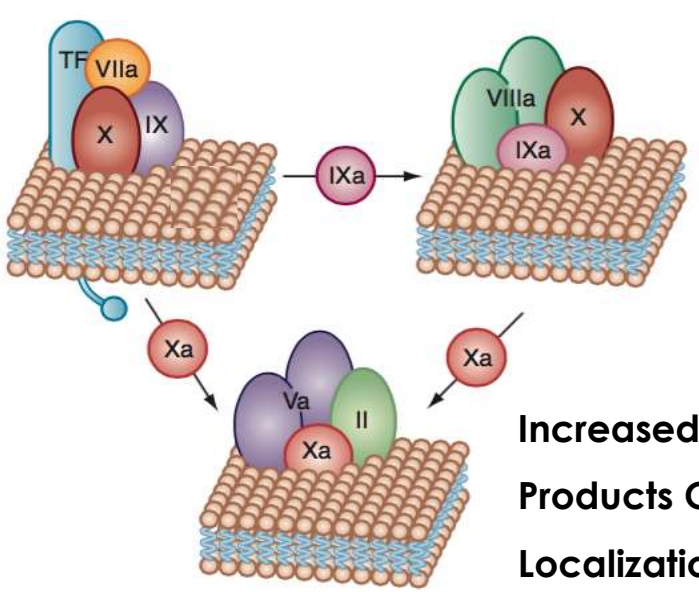




Huge Signal Amplification!

It takes place on ***macromolecular complex***:

Complex name	Enzyme (active)	Cofactor	Substrate (zymogen)	Catalytic Efficiency
Extrinsic Tenase	FVIIa	TF	FX	>15 x 10^6
Intrinsic Tenase	FIXa	FVIIIa	FX	>10^6
Prothrom binase	FXa	FVa	Prothrombin	>3 x 10^5

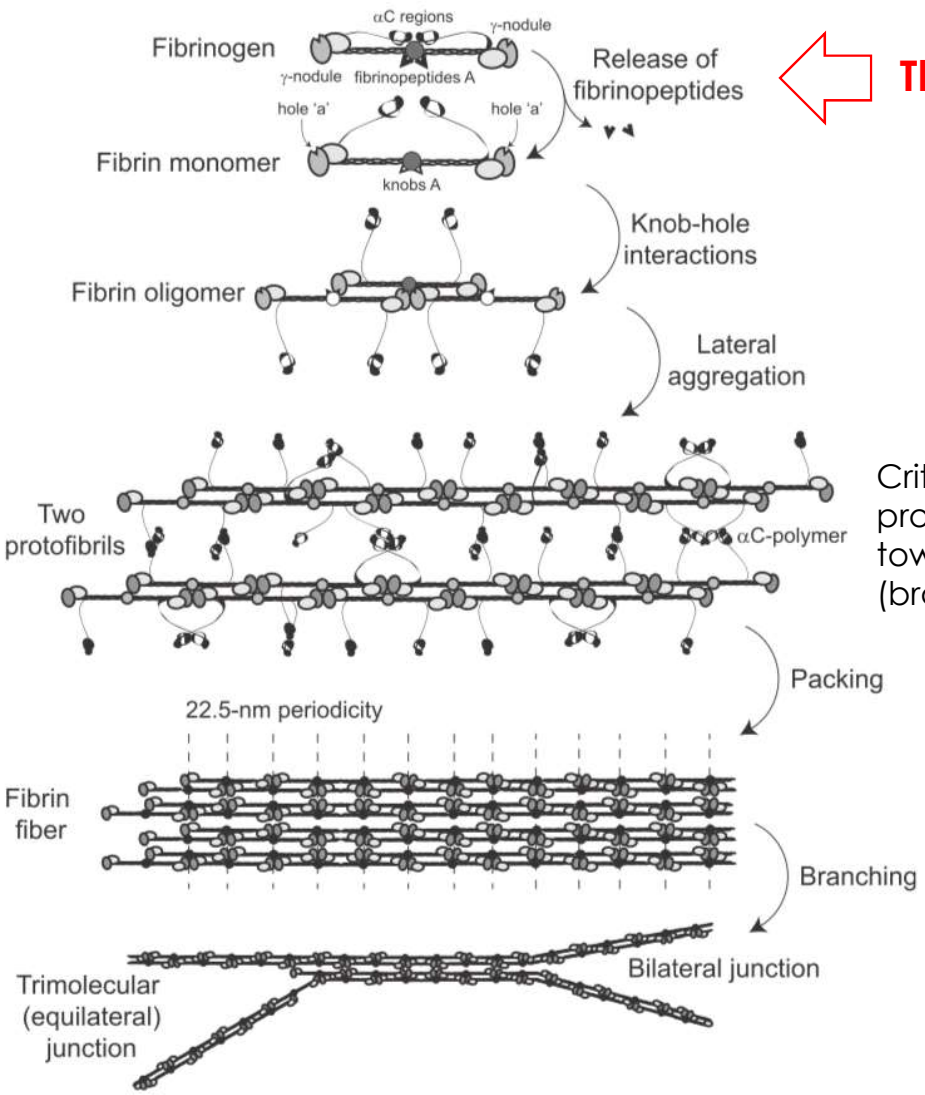


Increased catalytic efficiency

Products Channeling

Localization Control

Fibrinogen to fibrin



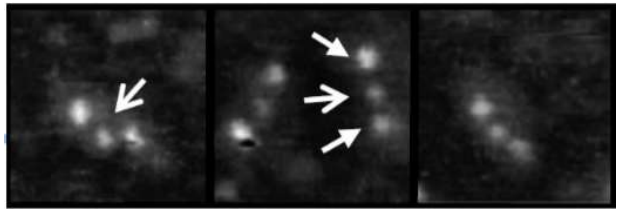
Thr

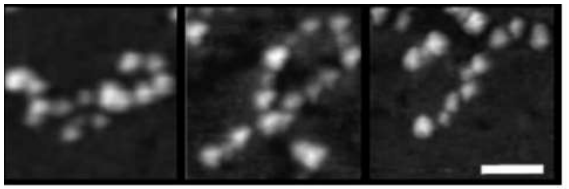
SOLUBLE

It occurs when only about 15–20 % of the fibrinogen is converted to fibrin!

INSOLUBLE

Critically important intermediate product, capable to undergo toward lateral aggregation (branching) -> 3D network



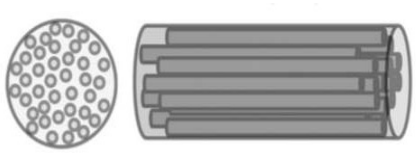


protofibril **50 nm**

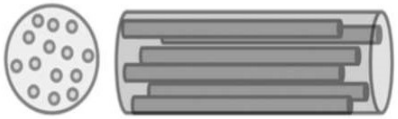
Fibrin fiber **1 μm**

Weisel JW and Litinov RI Blood 20013

Fibrin clot properties are clinically relevant



Clots with densely-packed fibers, increased stiffness and resistance to fibrinolysis



Low packing of fibers, reduced stiffness, more permeable to fibrinolytic molecules

Modifiers of fibrin clot:

Pro/anti-coagulants
Fibrinogen variants
Fibrinolytic agents
Metal ions
pH
Temperature
Blood cells
Vascular cells
Microparticles
Polyphosphates
DNA&histones
Heparin
Protamine
Blood flow
….

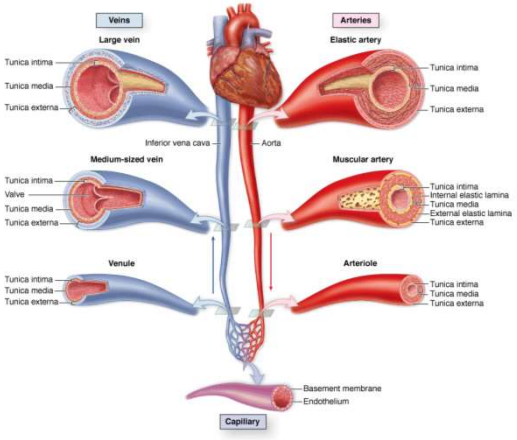
Diseases associated with abnormal fibrin clot:

Coronary Artery diseases
Myocardial Infarction
Ischemic Stroke
Venous thromboembolism
Aneurysm
Chronic kidney disease
Cirrhosis
Hemophilia
…
…

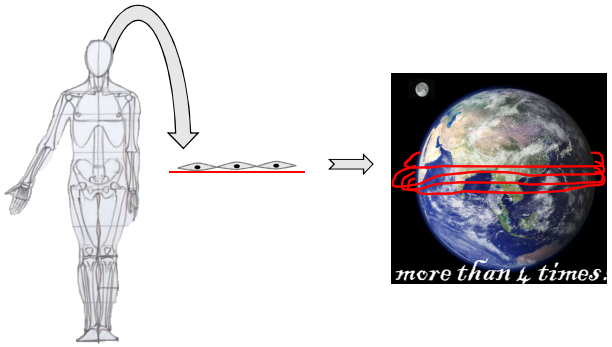
Domingues MM et al Blood 2016; Kattula S ATVB 2017

Which are the surface available for the blood clotting ?

The vascular endothelium

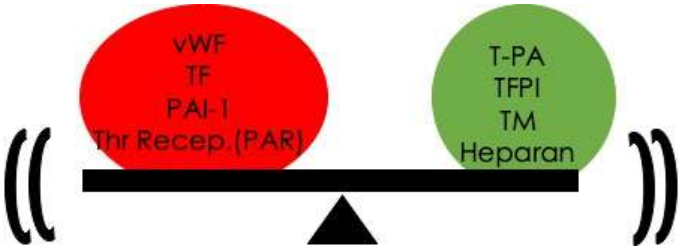


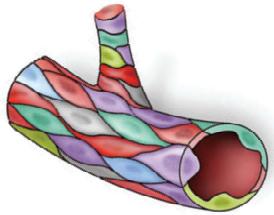
layer weighing approx 1 kg in an average-sized human

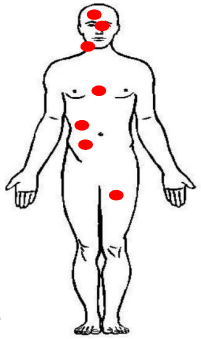


Highly metabolic active cell layer

, and very heterogeneous.





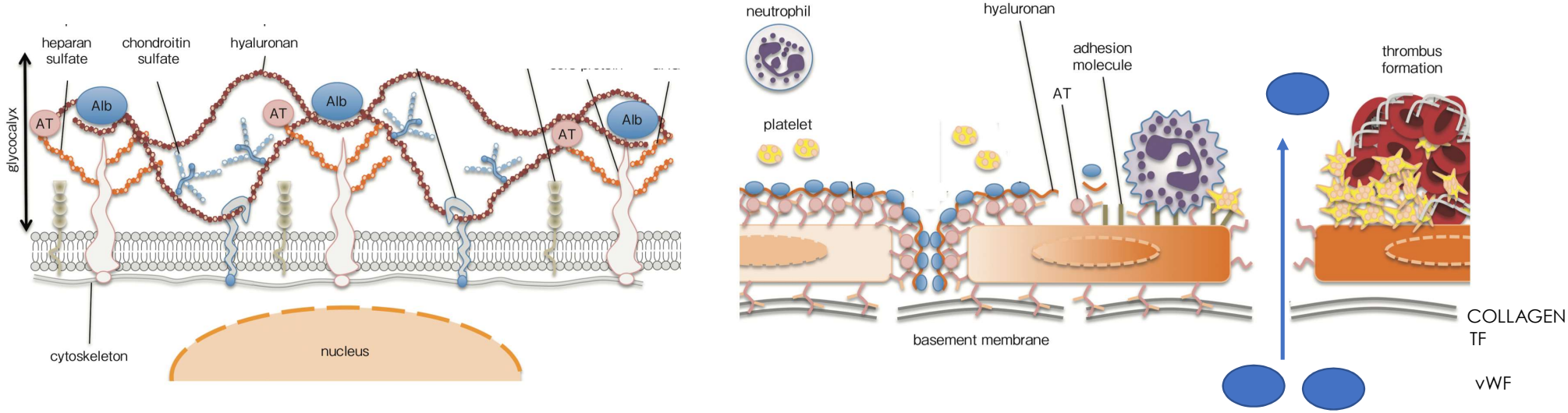


Hot spots for thrombosis

No 2 endothelial cells are identical…

The repertoire of EC-derived hemostatic factors varies between vascular beds :
→ changes in the systemic balance will have local different effects
→ site specific thrombotic phenotypes

The vascular endothelium



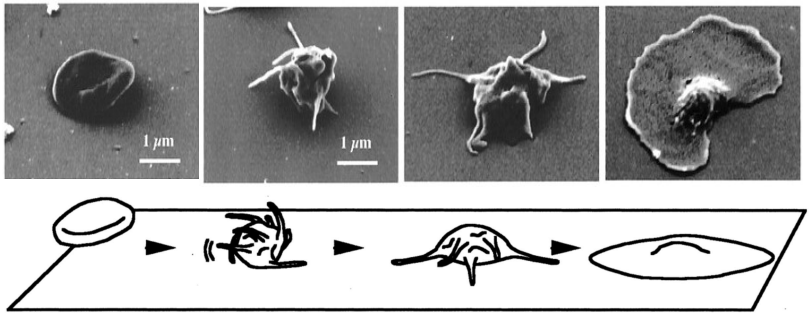
In response to injury, the phenotype of the endothelium transforms such as it promotes

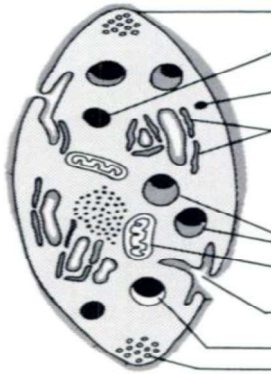
Recruitment, Adhesion and Activation of Platelets

In addition it exposes the **subendothelium** -> an highly thrombogenic surface

Iba T. Juntendo Medical Journal 2016

Platelets





Plasma membrane: ↑↑↑ several **receptors**
Lysosome λ granules
Peroxisome
Dense tubular system
α granules
Mitocondrion
δ granules
Microtubules

α granules (50-80/plt):
The most abundant secretory organelle (10% plt volume)
Contains Adhesive proteins (vWF, Fibrinogen,..), Pro and anticoagulant proteins (FV, PS TFPI), wound repair, inflammation and angiogenesis

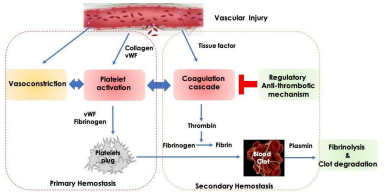
δ granules (3-6/plt):
The second most abundant organelle
Contains small molecules ADP,ATP, serotonin, calcium, pyrophosphate and polyphosphates

>1000 PLT/MEG; in adult 150-400 x10^9/L, diameter 2.6 um, half life is ~10 days

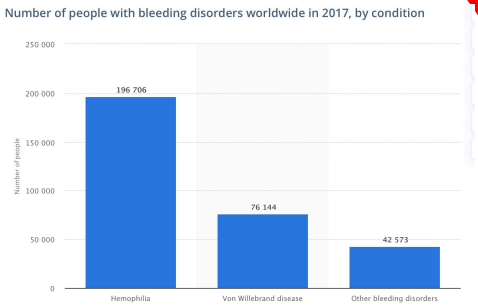
Versteeg et al. Physiol Rev. 2013, Mitsuhiro Kuwahara et al. Arterioscler Thromb Vasc Biol. 2002; Ruggeri Z. and Mendolicchio G.L. Hamostaseologie 2015

What's the interest in testing hemostasis?

Still not clear mechanism



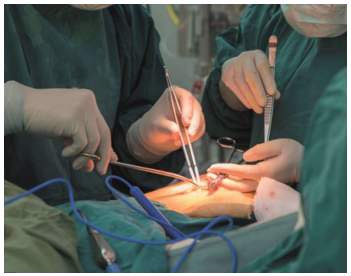
Bleeding disorders



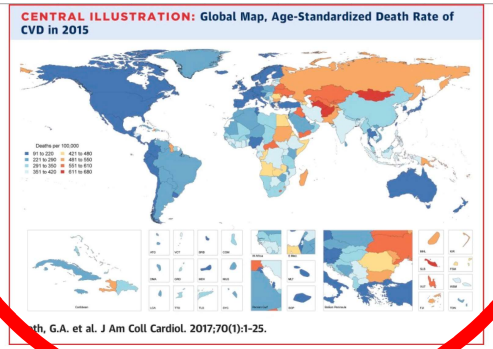
PREVENTION & THERAPY

Monitoring Drug therapy

Prior to surgery



Thrombotic disorders



How do we measure hemostasis?

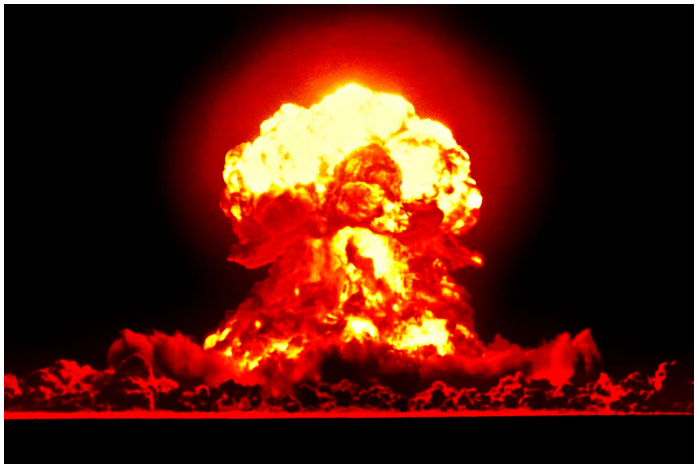
Primary hemostasis **Secondary hemostasis** **Fibrinolysis**

One component

Hemostasis lab : a clinical situation

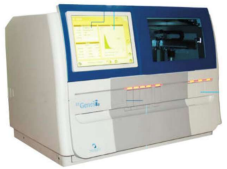
Arterial and Venous thrombophilia -individual clinical assays-

AT	Antiphospholipid Abs	FII	FIX
PC	Platelets	FV	FX
PS	Endothelium	FVII	FXI
TFPI	D-dimers	FVIII	TF
FVL	Fibrinolysis		PL



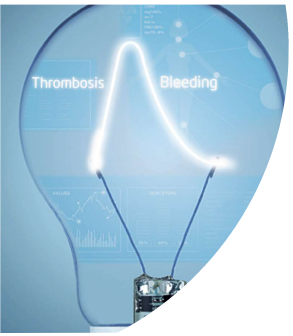
They will tell you ***how much gas in the tank is left***….. but not ***how fast the motor is running***!

What's new now?!

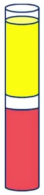


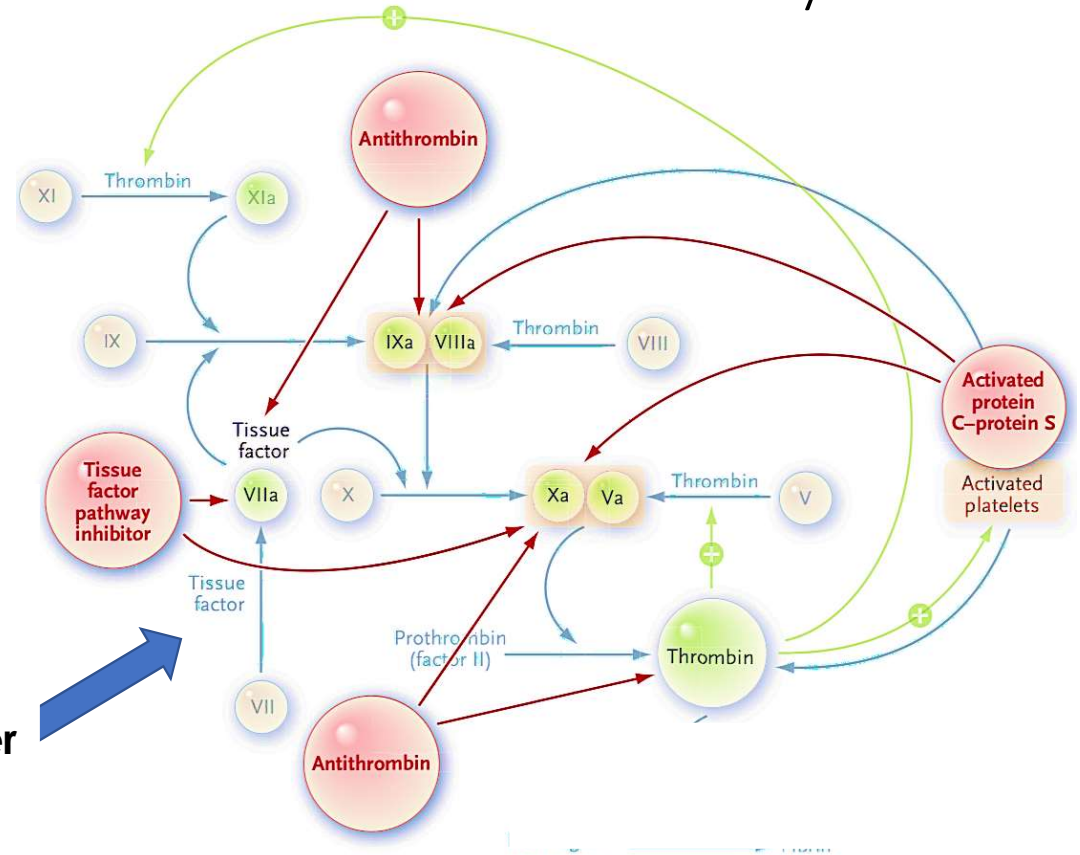
Primary hemostasis Secondary hemostasis Fibrinolysis

One component **All in one ?**



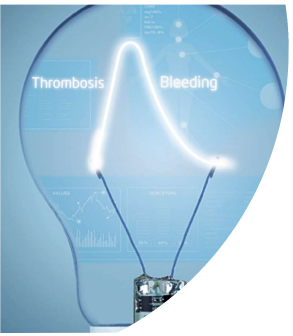
A global functional test for blood coagulation: Thrombin Generation Analysis



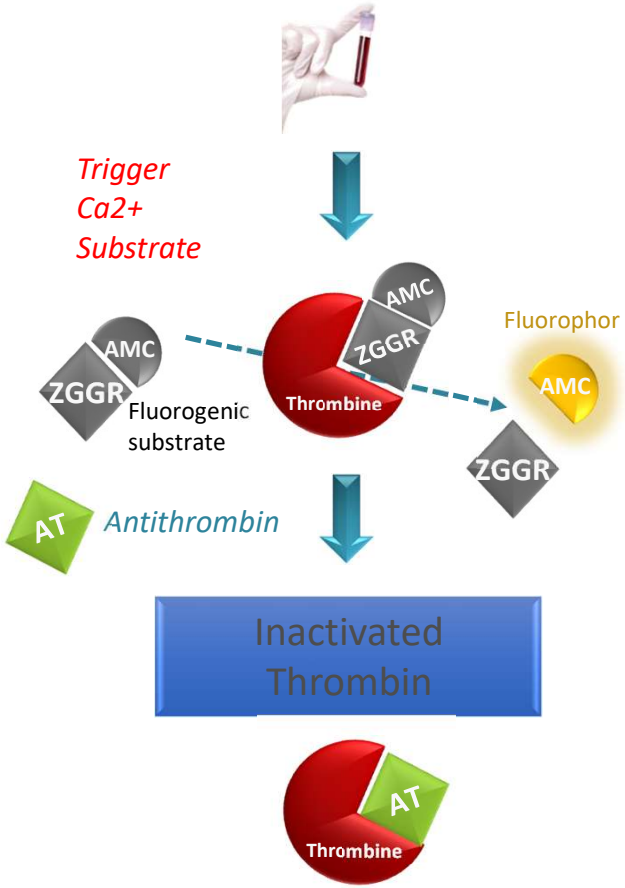


Modulating the concentration of the trigger , the overtime evaluation of the thrombin formed results more dependent by certain pro or anti coagulant forces.
This gives a better idea of the overall thrombin generation potential in a patient and can guide better the choice of the therapy.

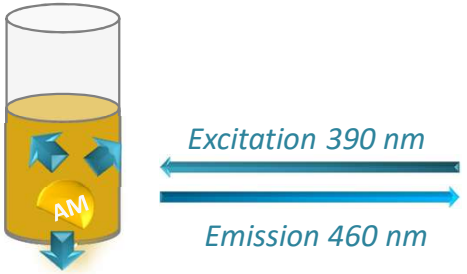
Modified from Tripodi A NEJM 2011

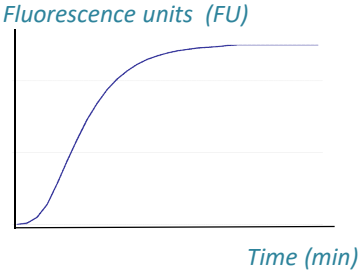


Thrombin generation assay: method

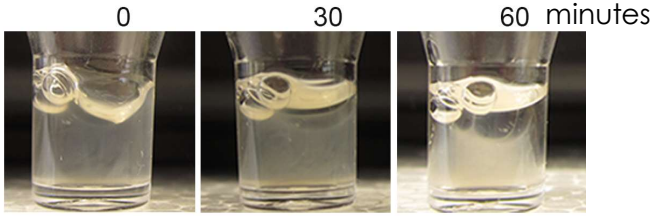


AMC = Amino-Methyl-Coumarin



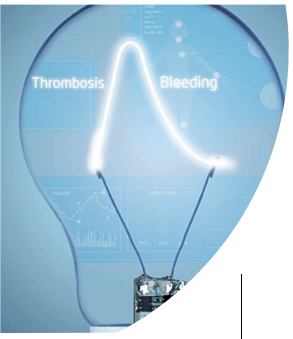


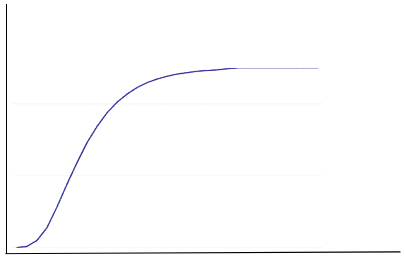
What is the main advantage of using fluorochrome instead of chromogenic substrate?



the fluorogenic substrate is not perturbed by the turbidity of a forming clot

Thrombin generation assay: method summary

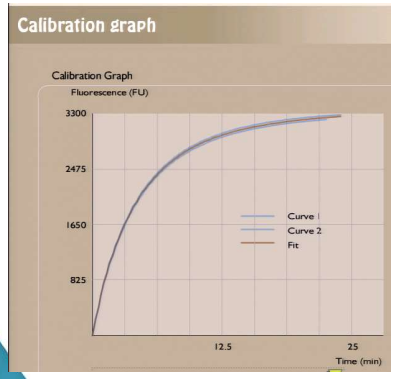


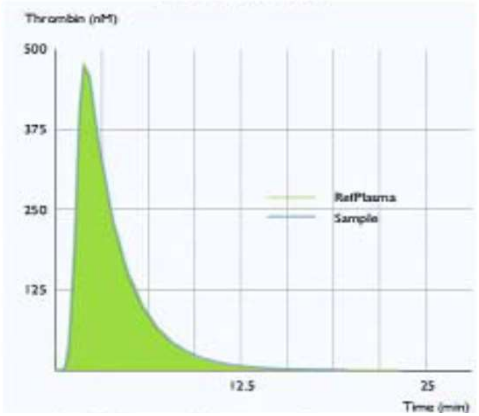


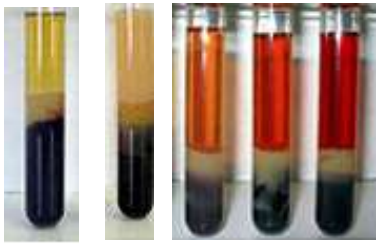
Time)

The fluorescence intensity is not linear with the concentration of the fluorophore

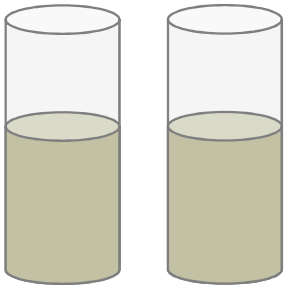
First derivative







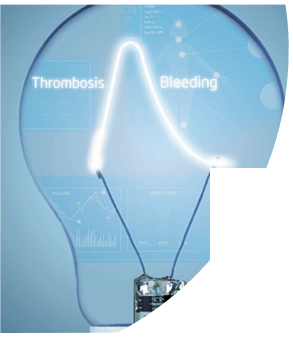
Proceed or not proceed?!



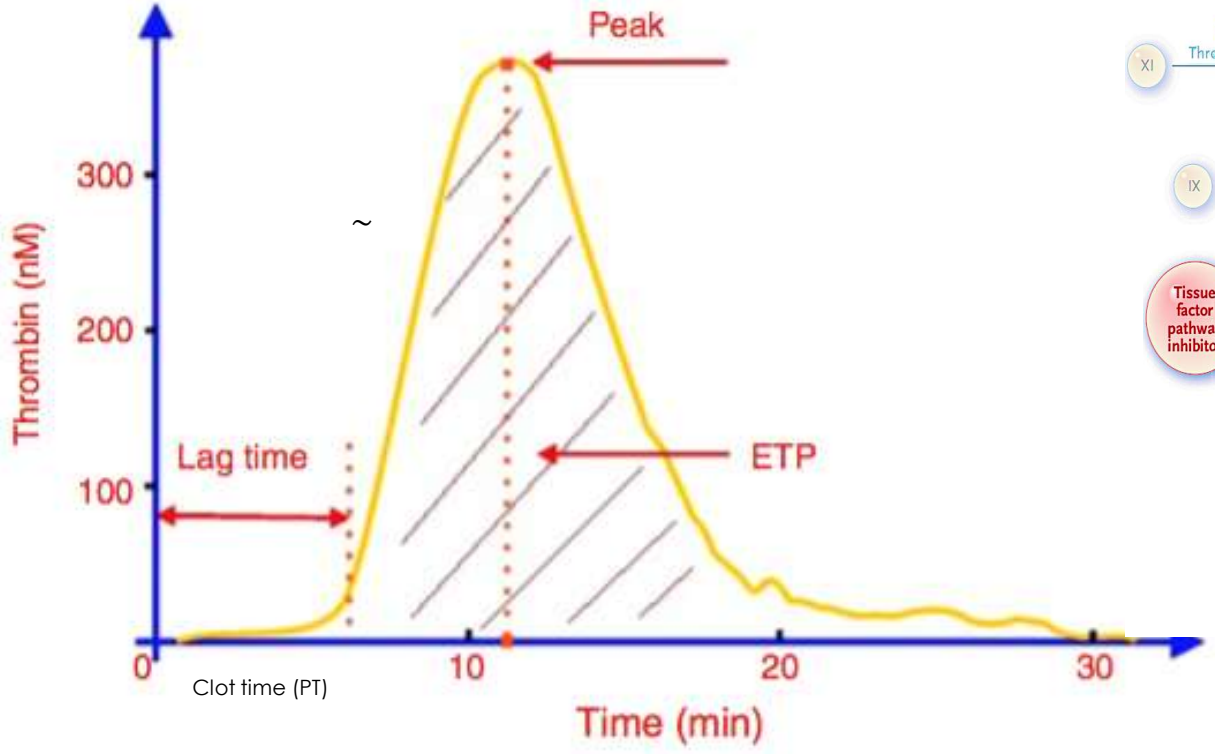
Reference

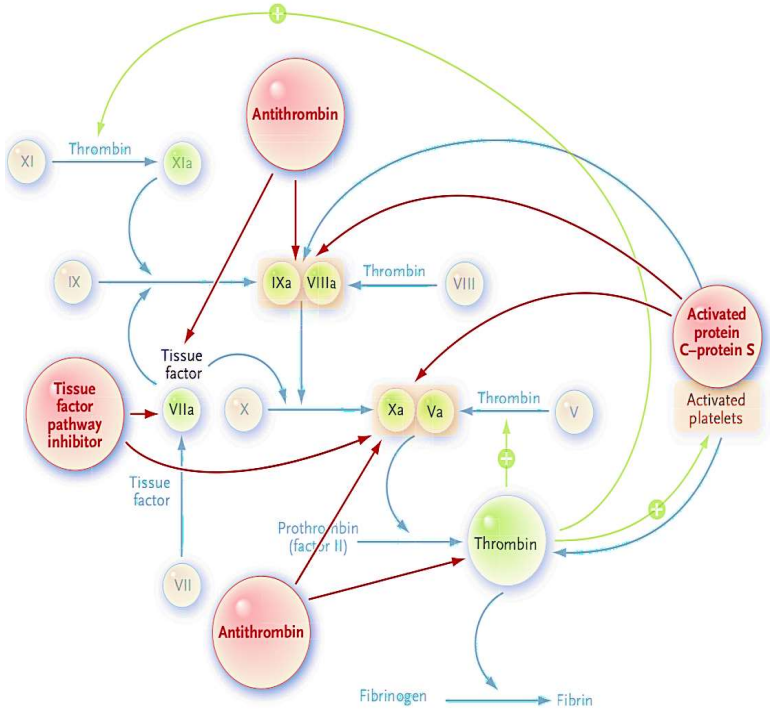
80 µL STG-ThrombiCal
Known [Thrombin]

+ 40 µL STG-FluoSet
Known [AMC]



Thrombin generation curve and their determinants



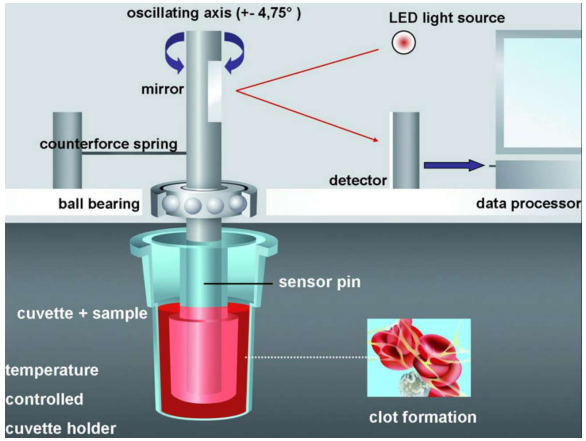


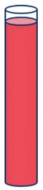
95% Thrombin
is produced
during
Propagation phase

Modified from Tripodi A NEJM 2011

Thromboelastometry: ROTEM

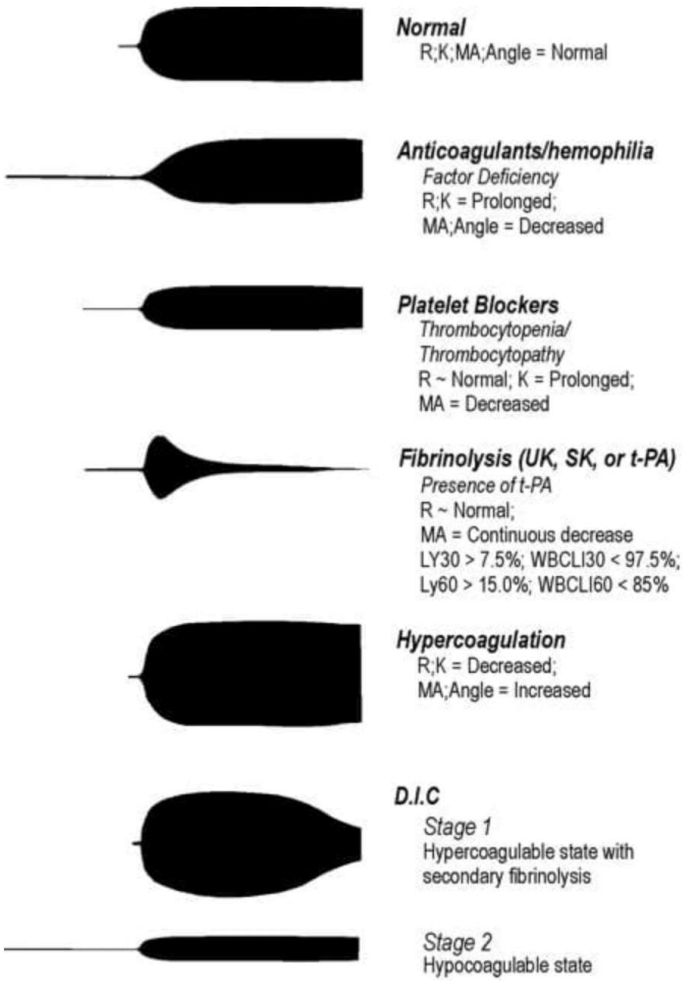
Viscoelastic tests enables the analysis of clot formation, clot elasticity development and its dissolution in real time





Assay	Activator/Inhibitor	Information Provided	Liquid Reagents
INTEM	Contact activation	Fast assessment of clot formation, fibrin polymerization, and fibrinolysis via the intrinsic pathway	In-tem
HEPTEM	Contact activation + heparinase	ROTEM analysis without heparin influence: specific detection of heparin (compared to INTEM), assessment of clot formation in heparinized patients	Hep-tem
EXTEM	Tissue factor activation	Fast assessment of clot formation, fibrin polymerization, and fibrinolysis via the extrinsic pathway	Ex-tem
FIBTEM	Tissue factor activation + platelet inhibition	ROTEM analysis without platelets: qualitative assessment of fibrinogen status	Fib-tem
APTEM	Tissue factor activation + aprotinin	In vitro fibrinolysis inhibition: fast detection of lysis when compared with EXTEM	Ap-tem
NATEM	Recalcification only = classical TEM (thromboelastometry)	Very sensitive assessment of the equilibrium of coagulation activation or inhibition	Star-tem

Thromboelastometry: data



Normal
R;K;MA;Angle = Normal

Anticoagulants/hemophilia
Factor Deficiency
R;K = Prolonged;
MA;Angle = Decreased

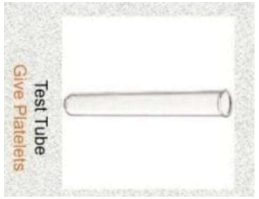
Platelet Blockers
Thrombocytopenia/
Thrombocytopathy
R ~ Normal; K = Prolonged;
MA = Decreased

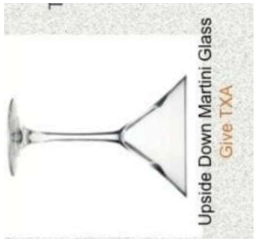
Fibrinolysis (UK, SK, or t-PA)
Presence of t-PA
R ~ Normal;
MA = Continuous decrease
LY30 > 7.5%; WBCLI30 < 97.5%;
Ly60 > 15.0%; WBCLI60 < 85%

Hypercoagulation
R;K = Decreased;
MA;Angle = Increased

D.I.C
Stage 1
Hypercoagulable state with
secondary fibrinolysis

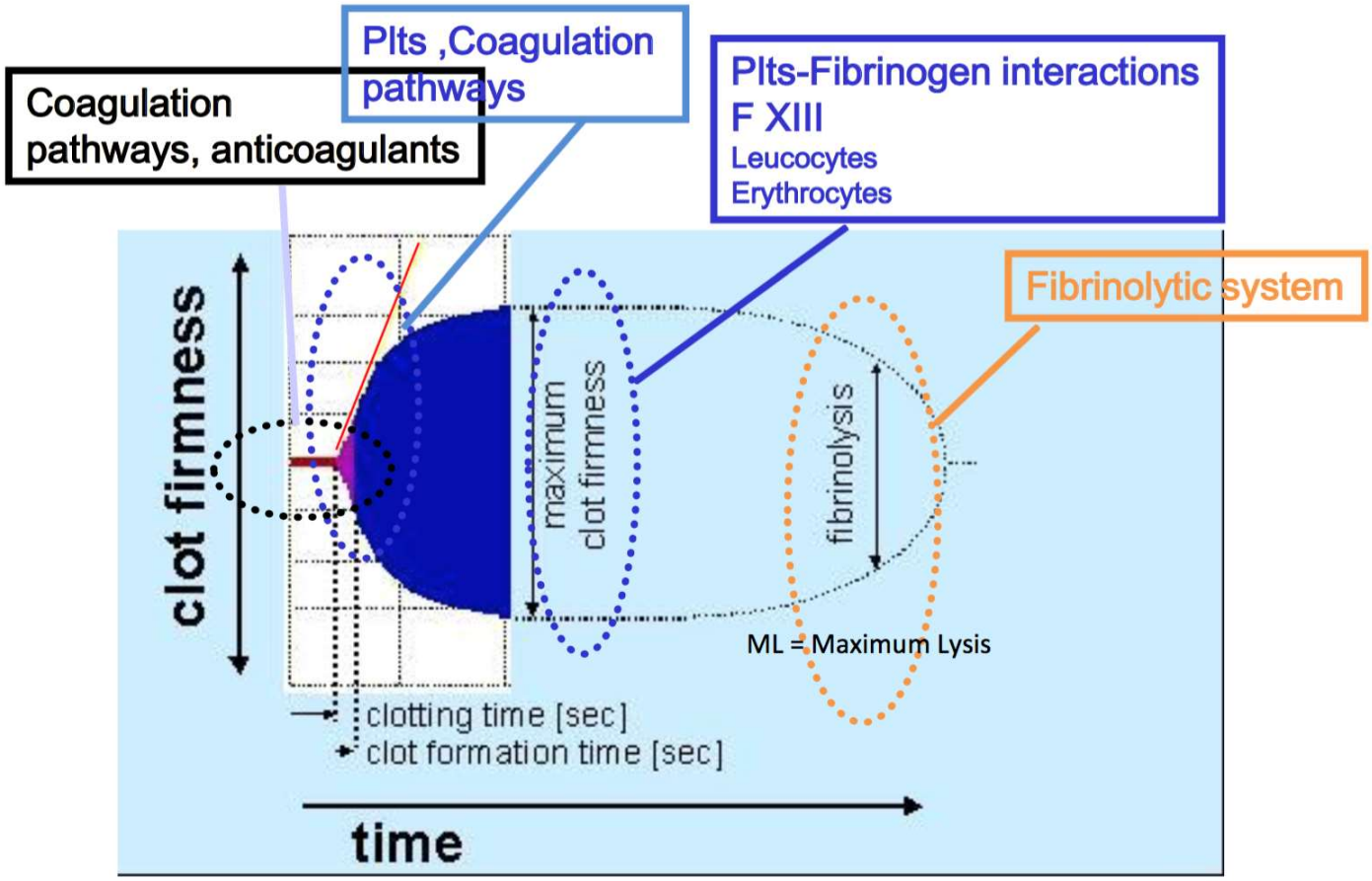
Stage 2
Hypocoagulable state



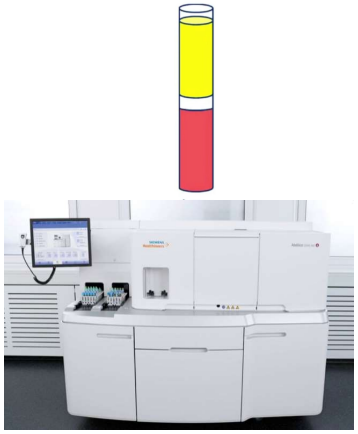


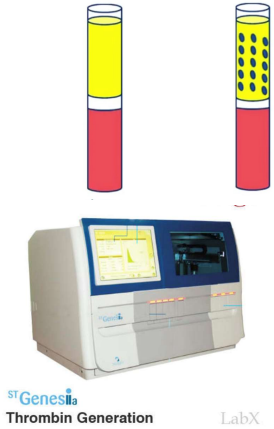
Viscoelastic tests enables the analysis of clot formation, clot elasticity development and its dissolution in real time

Viscoelastic tests of hemostasis



There is still space for improvement….

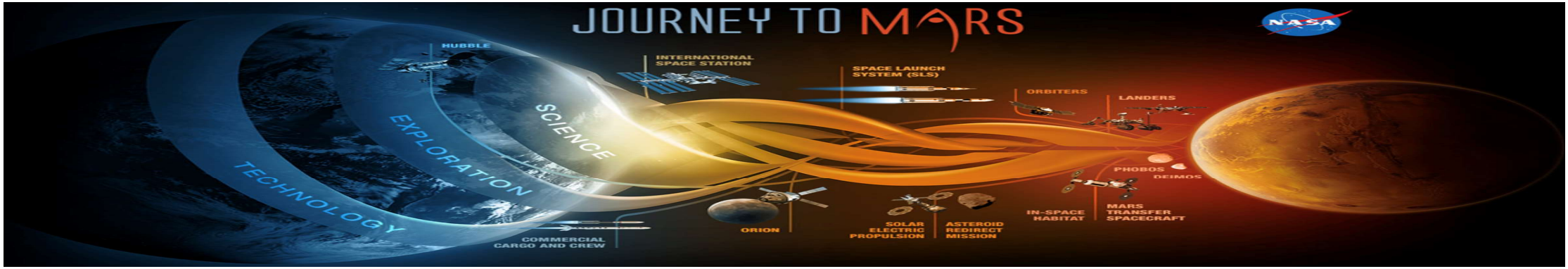




Primary hemostasis **Secondary hemostasis** **Fibrinolysis**

One component **All in one ?**

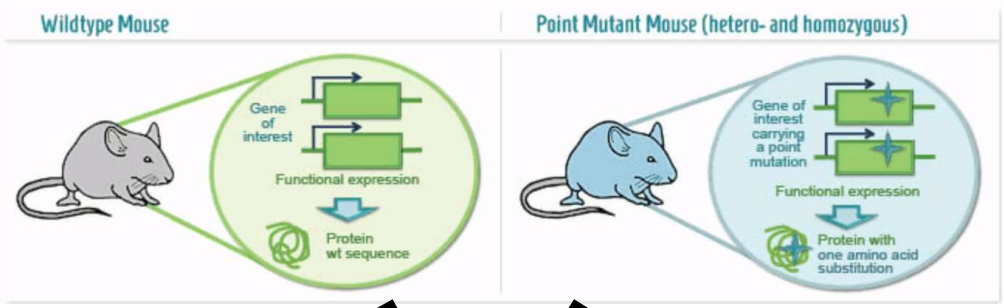
Endothelium
Flow
Plts
Severe-> mild deficiency of
Pro/anti coag
Fibrinolytic



How do we investigate *in vivo* the hemostatic process?

An experience in a mice based lab

Mice models in hemostasis

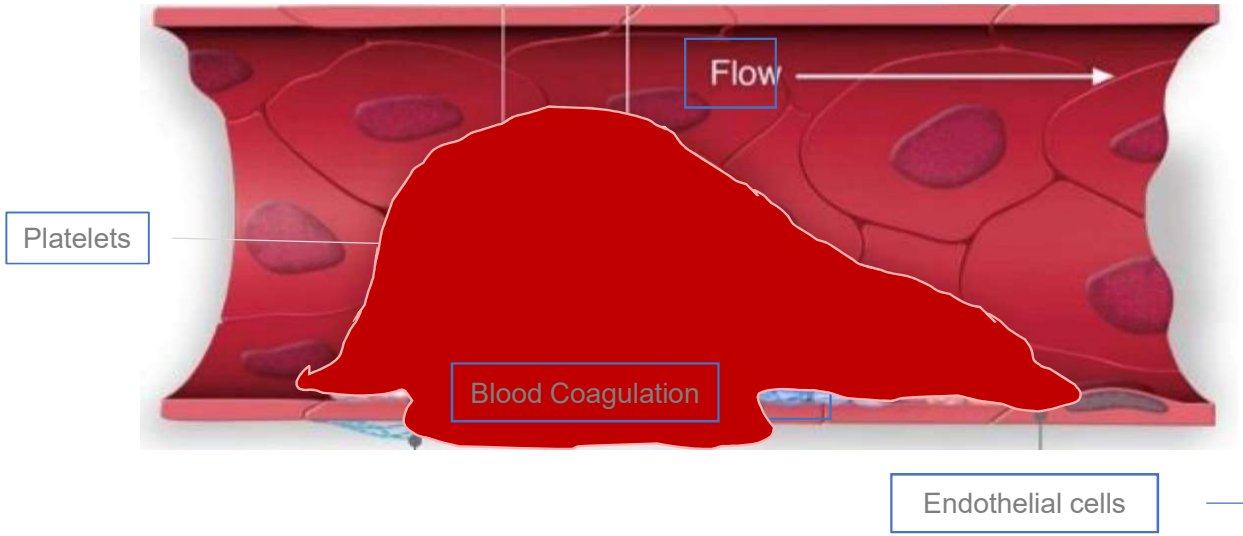


Hemorrhagic

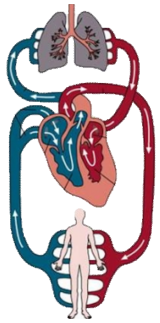
Thrombosis

Clot formation is a fast and microscopic process

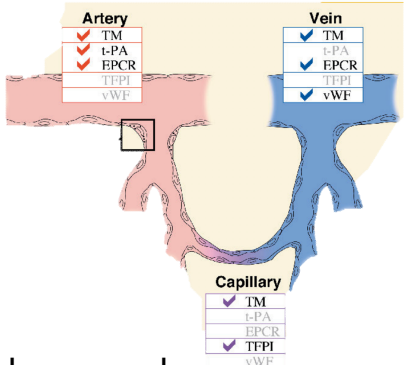
The study of thrombus formation is complicated by the fact that thrombi form rapidly, are highly variable, and do not respond in a predictable manner to injury. Quantitation of thrombus formation in live animals is therefore challenging.



CLOSED
HIGH-PRESSURE
SYSTEM



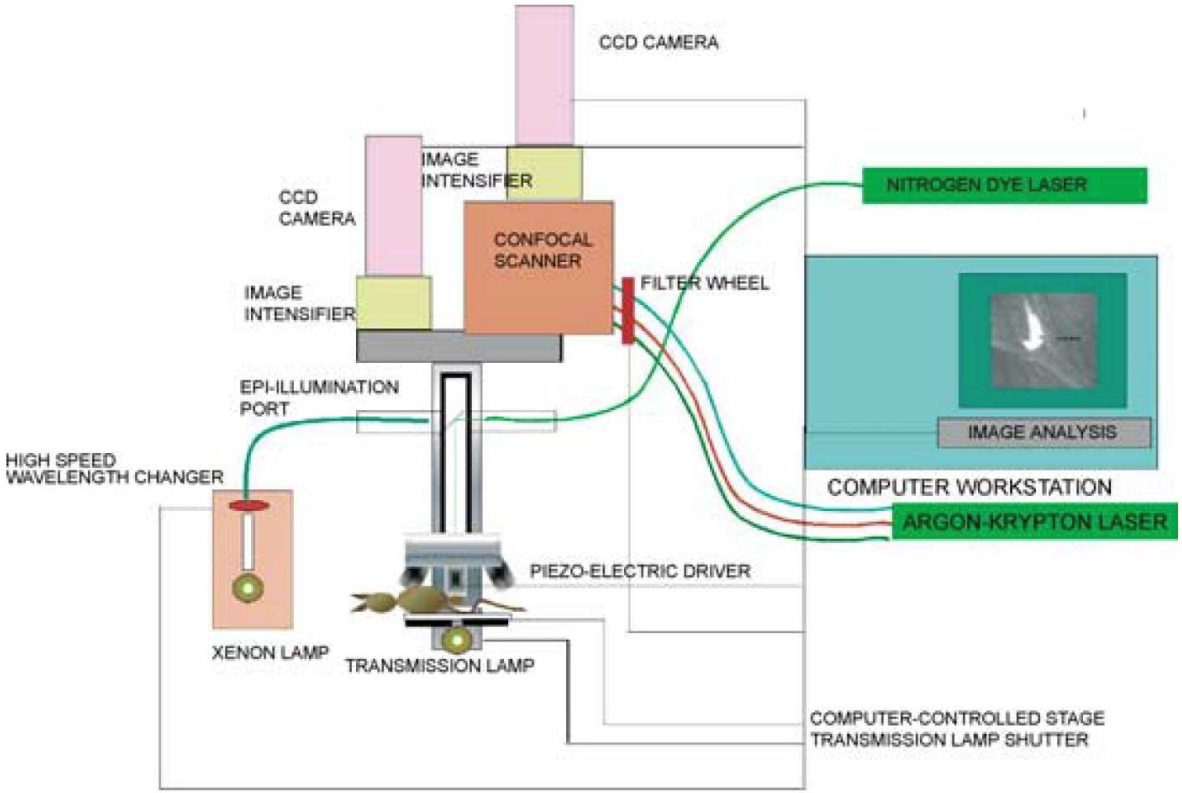
↑ HETEREOGENEITY

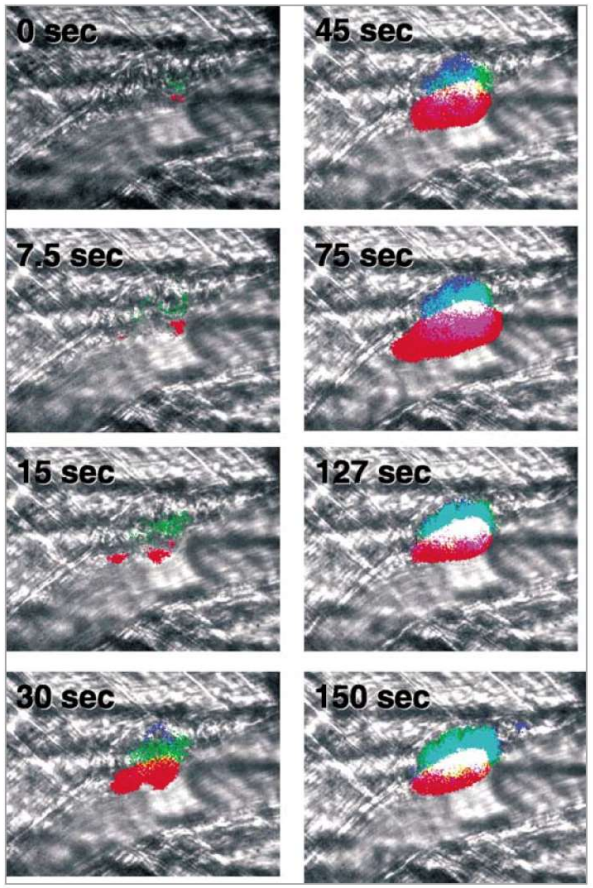


Although it has been studied since at least 1881[1], only recently have developments in intravital microscopy began to unravel mechanisms behind

Flaumenhaft R. Blood 2014; [1] Bizzozero G. Su di un nuovo elemento morfologico del sangue deimammiferi e sulla sua importanza nella trombosi e nella coagulazione.Osserv Gazz Clin 1881;17:785–
Google credits;

In vivo imaging of thrombosis and hemostasis





Platelets
tissue factor
fibrin

tissue factor + fibrin

Platelets+ fibrin

platelets + fibrin+tissue factor (white).

Real time imaging

3d thrombus reconstruction

Celi A. JTH 2002

Types of blood vessels used

Ear vasculature

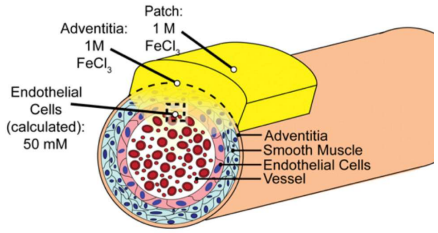
Carotid Artery

Mesenteric vessels

Cremaster muscle vasculature

Methods of induction of injury

1. **Ferric chloride**



Filter paper 1x1 mm
saturated with $FeCl_3$(5-20%) x 3-5 min

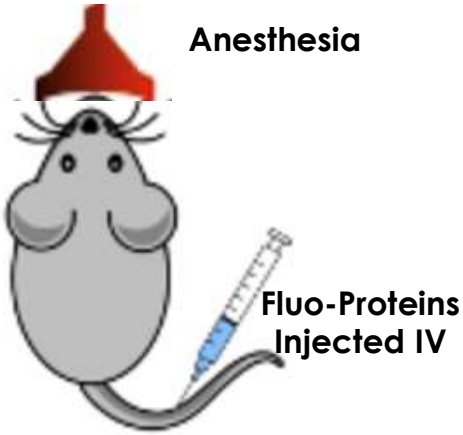
1. **Laser induced injury**

2. Mechanical injury

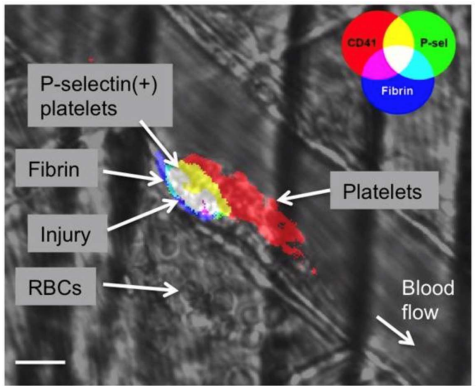
3. Electrolytic injury

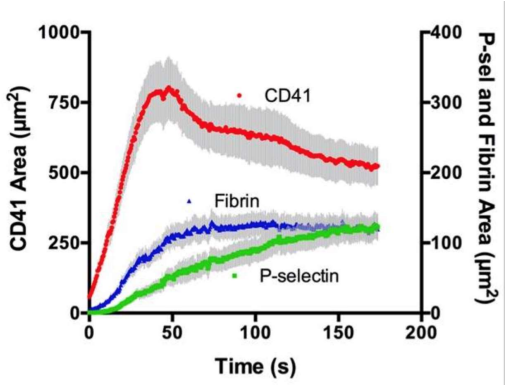
Methods of visualizing and quantifying the thrombus growth

For multichannel microscopy, platelet and plasma proteins can be labeled simultaneously with different fluorochromes.



-> take into account the tissue autofluorescence
and crossover of signals between 2 or more fluorochromes





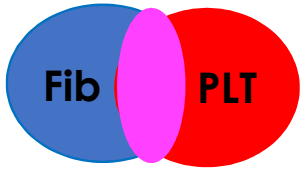
To quantitively asses thrombus growth in real time:

- interaction plt-vessel wall (fluorescence at the vessel wall segment during 1 min)
- Time required for thrombus formation larger than 30-50 um
- Thrombus growth rate
- Thrombus stability- > evaluation of embolization
- Time to occlusion

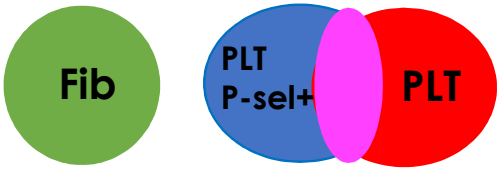
Experimental setting in mouse: *In vivo thrombus formation*

Laser-induced injury on mesenteric arterioles

Part 1



Part 2

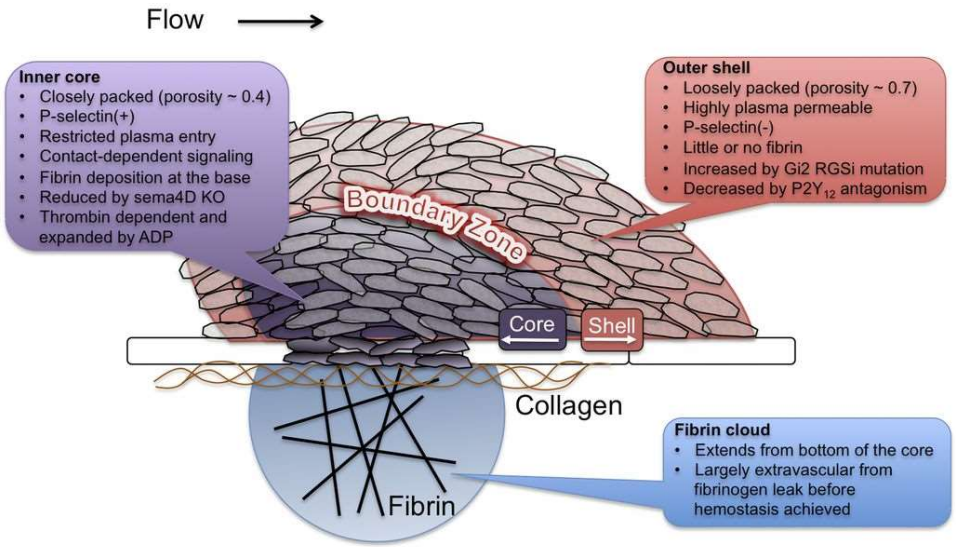


More activated

Less activated

Stalker T et al. Blood 2013

What we have recently learned

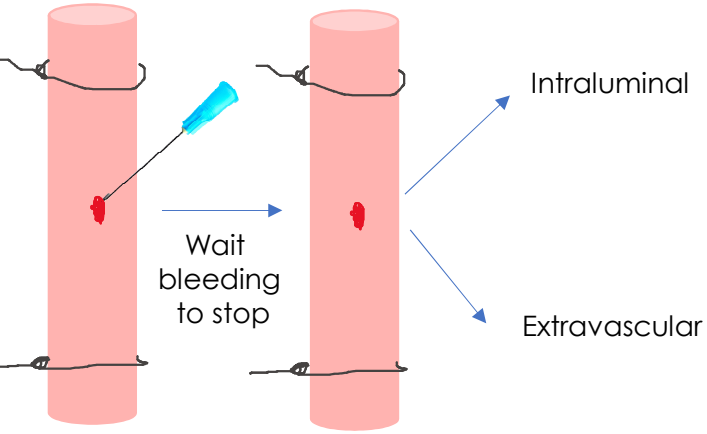


A REGIONAL ARCHITECTURE as a REGULATORY MECHANISM

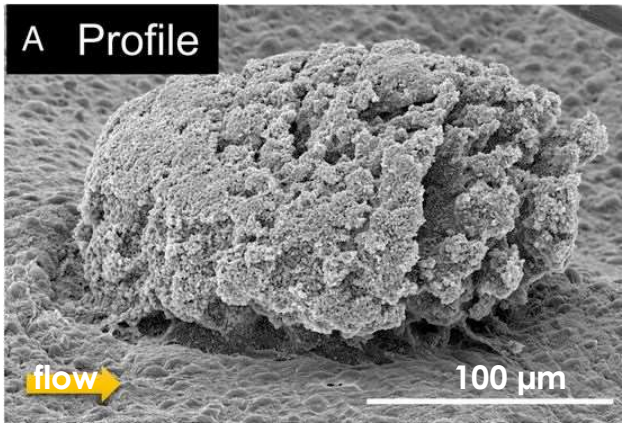
Rather than a mass of uniformly activated platelets contained in a fibrin meshwork, hemostatic plug formed in vivo develop a regional architecture where not all platelets are activated in the same way and fibrin is distinctly localized.

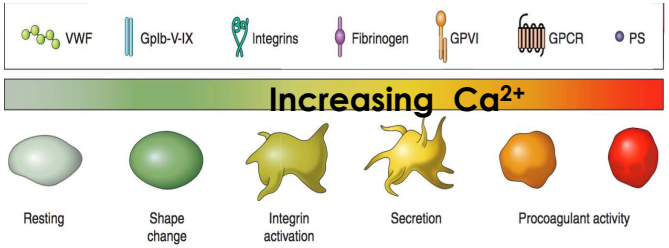
Tomaiuolo M et al. Intervent Cardiol Clin 2017

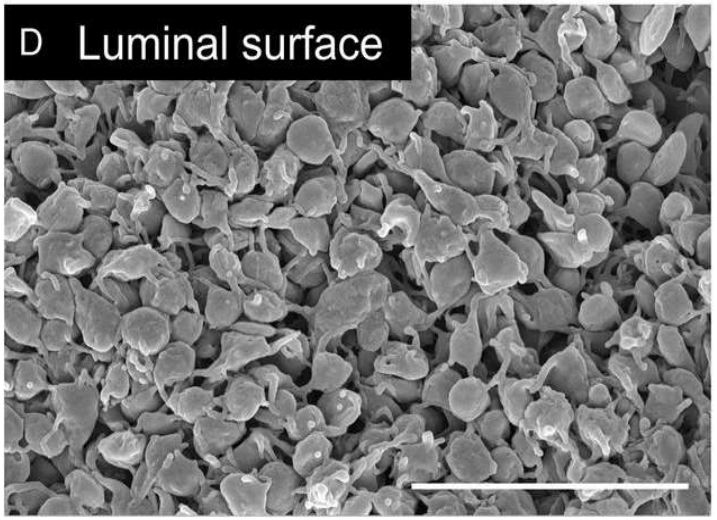
Architecture of the hemostatic clot

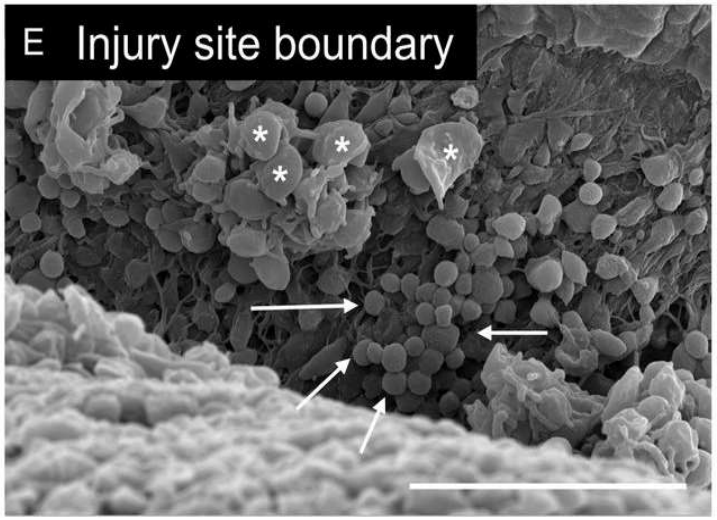


INTRALUMINAL MORPHOLOGY –JUGULAR VEIN-



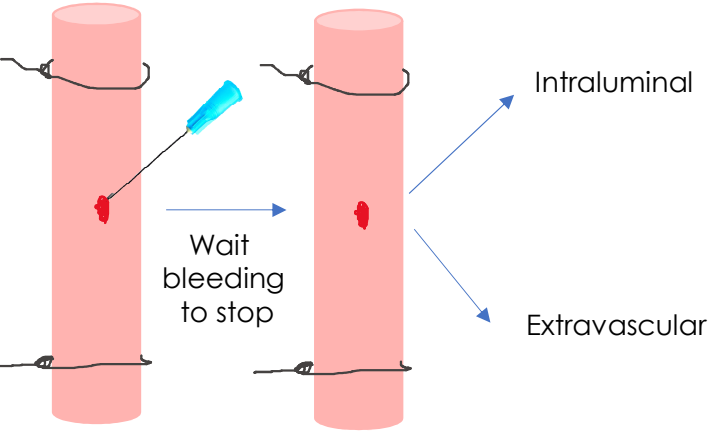






Tomaiuolo M. et al. PNAS 2018

Architecture of the hemostatic clot

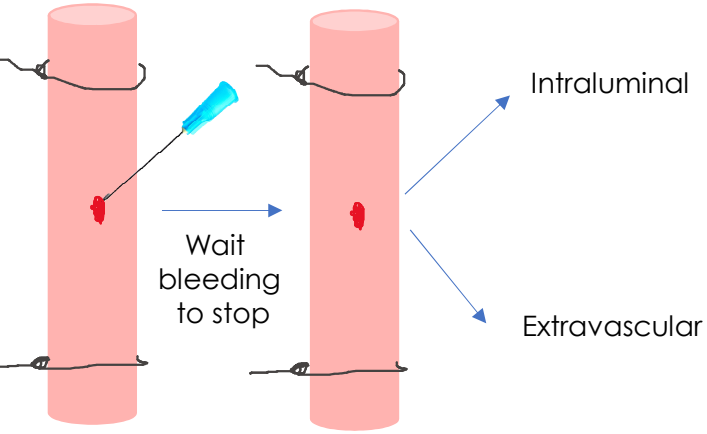


INTRALUMINAL MORPHOLOGY –JUGULAR VEIN-

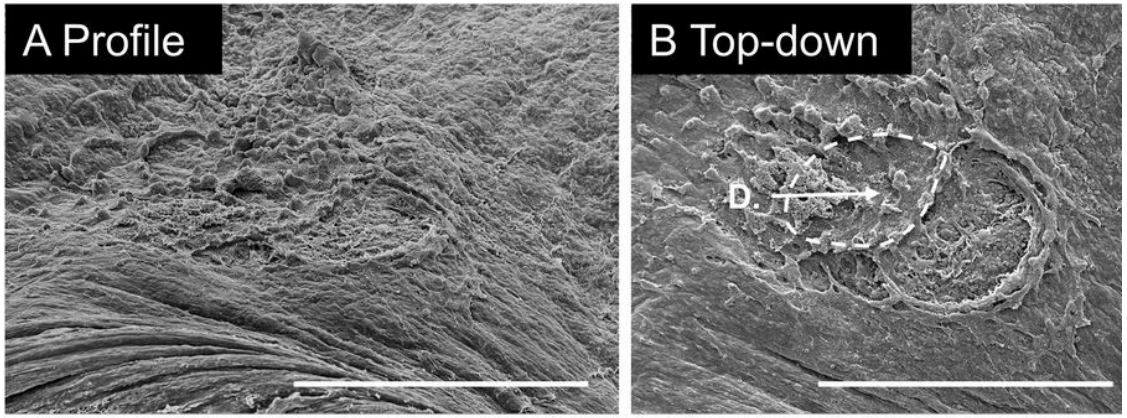
3D reconstruction of the intravascular portion of a jugular vein hemostatic plug. Anti-CD41 (red) and anti-P-selectin (green) antibodies were infused prior to injury.

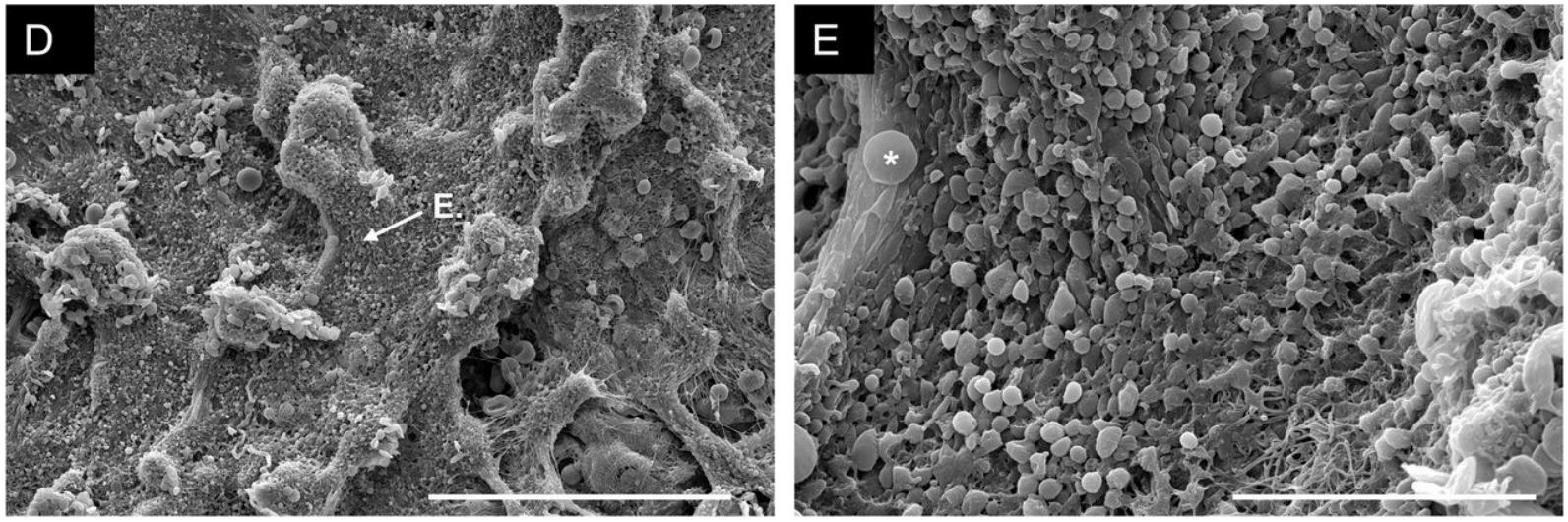
Tomaiuolo M. et al. PNAS 2018

Architecture of the hemostatic clot



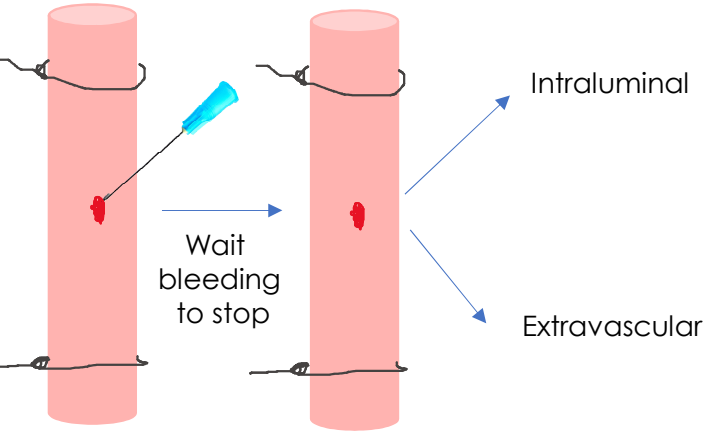
EXTRAVASCULAR MORPHOLOGY –JUGULAR VEIN-





Tomaiuolo M. et al. PNAS 2018

Architecture of the hemostatic clot

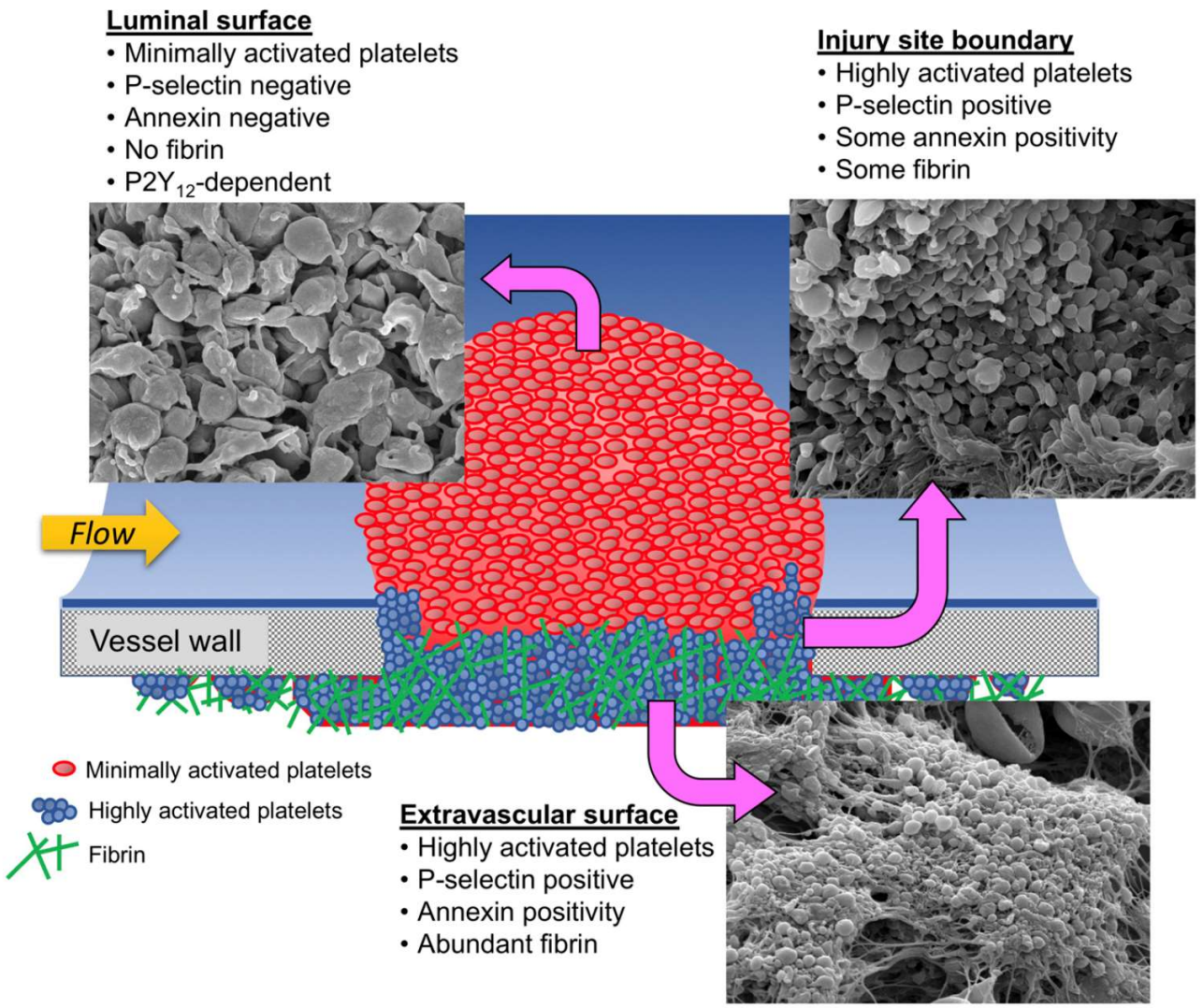


EXTRAVASCULAR MORPHOLOGY –JUGULAR VEIN-

3D reconstruction of the extravascular portion of a jugular vein hemostatic plug. Anti-CD41 (red) and anti Fibrin(ogen) (green) antibodies were infused prior to injury.

Tomaiuolo M. et al. PNAS 2018

Architecture of the hemostatic clot



Tomaiuolo M. et al. PNAS 2018