Laboratory investigations of hemostatic disorders

Hemostasis

The object of the complex hemostatic process is:

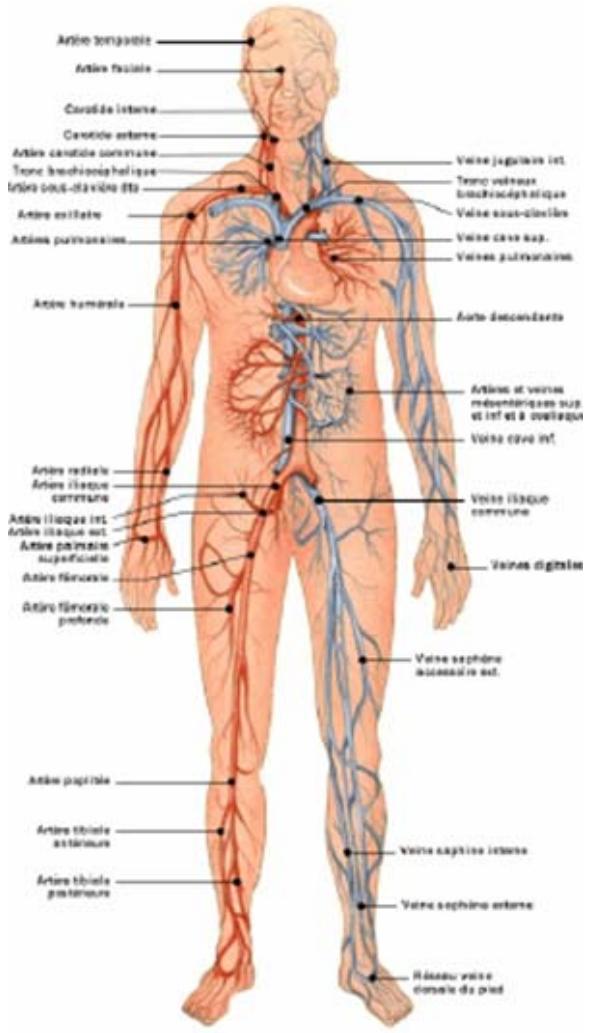
- To maintain the composition and fluidity of the blood within the blood vessels,
- To seal leaks in the blood vessels or stop blood loss,
- To restore normal vascular structure or effect repair by scar tissue.

The three cornerstones of hemostasis are:

- Vascular system
- Coagulation system
- Fibrinolytic system

Hemostasis

- About 3 to 5 litres of blood
- Over 100000 km of
  - Arteries
  - Veins
  - Capillares
- About 1000 m^2 endothelial surface



Hemostasis tests

- Procoagulant status: thrombosis & embolia
- Anticoagulant status: bleeding

•The aim of tests:

- Workup of acute bleeding / acute clots
- Identification of bleeding disorders (preoperative workup)
- Risk assessment (thrombosis)
- Monitoring of antioagulation
- DIC workup

Hemostasis tests

COMMON (screening tests)

- Platelet count
- PT / INR
- aPTT
- Fibrinogen
- D-dimer

SPECIFIC TESTS

identification of factor deficiency, antiphospholipid antibodies, platelet function testing, HIT tests, genetic tests

Hemostasis

4 steps

- **Vasoconstriction** - decreasing blood flow (within seconds)
- **Thrombocyta-plug formation**

  interaction between vessel wall, platelets and adhesive proteins

  → platelet clot

  primer hemostasis (3-5 minutes)
- **Coagulation - plug extended**

  consolidation of the platelet thrombus → insoluble fibrin net

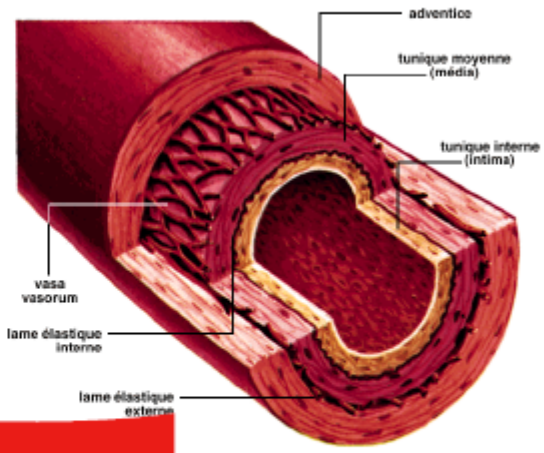
  coagulation factors and inhibitors

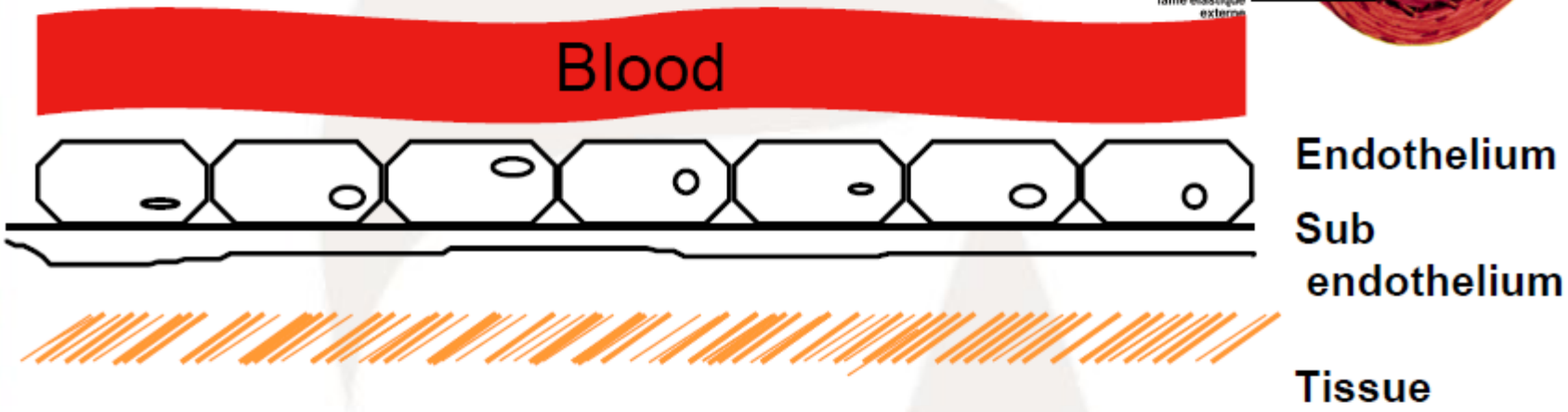
  secunder hemostasis (10-30 minutes)
- **Fibrinolysis**

  clot lysis → clot is dissolved (with repair process: days to weeks)

  fibrinolytic activators and inhibitors

Vessel wall





- Intact endothelium is non thrombogenic
  - Synthesis of vasodilatators (prostacyclin)
  - Limitation of thrombin generation
  - Regulation of fibrinolysis
  - No reaction either with platelets or with factors

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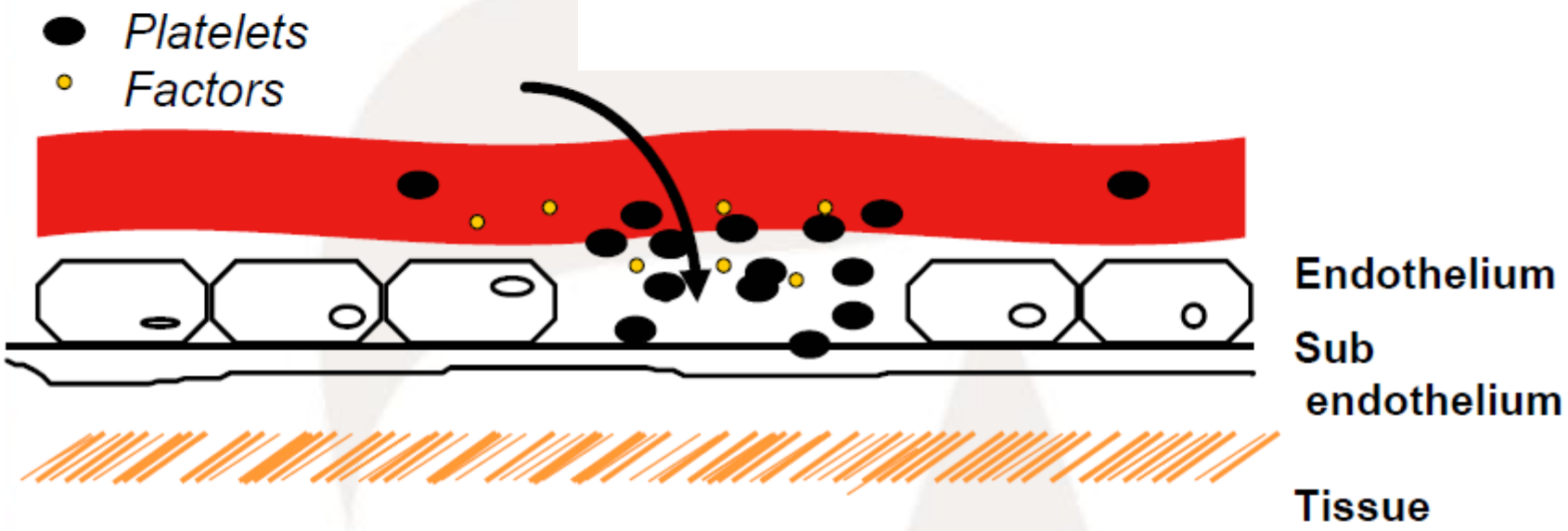
Vessel wall damage



- When a vessel wall is damaged
  - Exposure of the subendothelium
  - Platelet adhesion
  - Initiation of the mechanisms of coagulation and fibrinolysis

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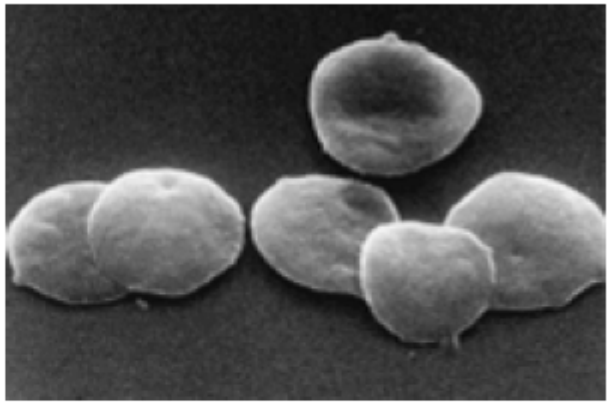
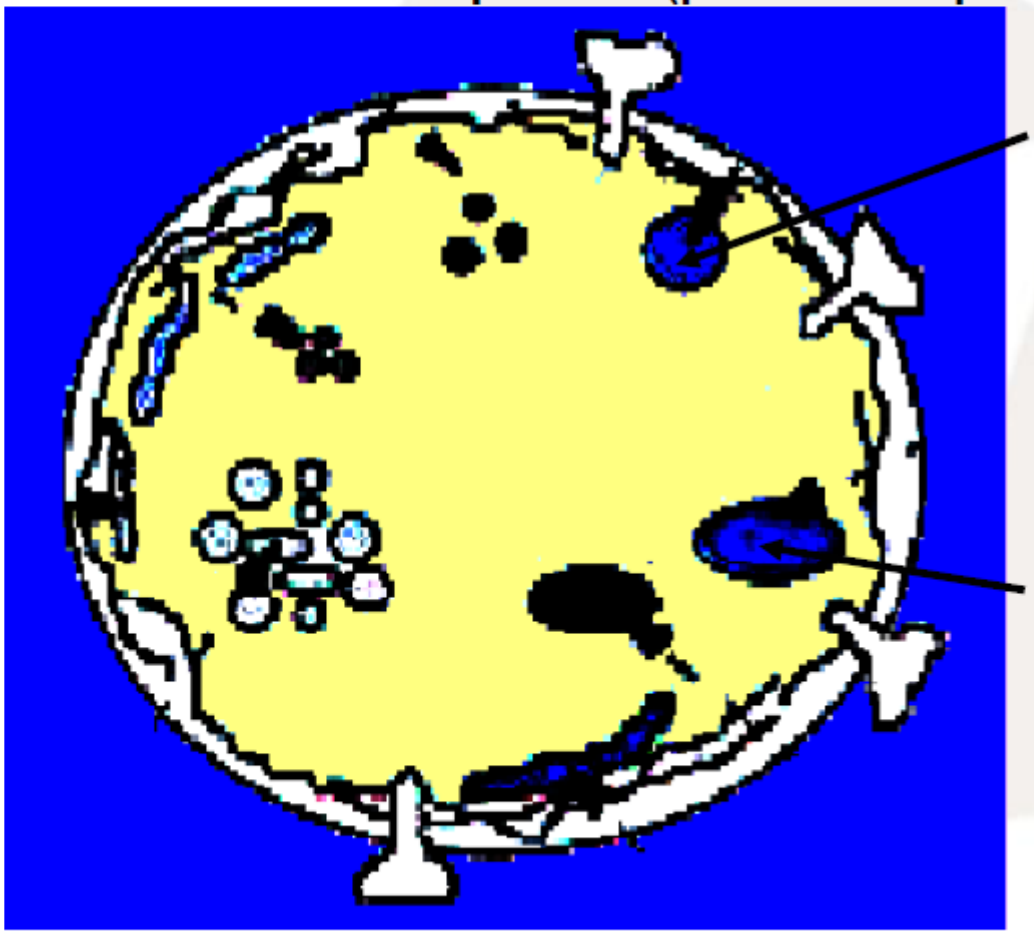
Platelet



GpIIb-IIIa (platelet receptor)



α granules
(PF4, β-TG, Fibrinogen, VWF, Factor V, PAI-1, …)

dense granules
(ATP, ADP, Serotonin, Ca^{2+}, Mg^{2+}, P, …)

GpIb-IX-V (platelet receptor)

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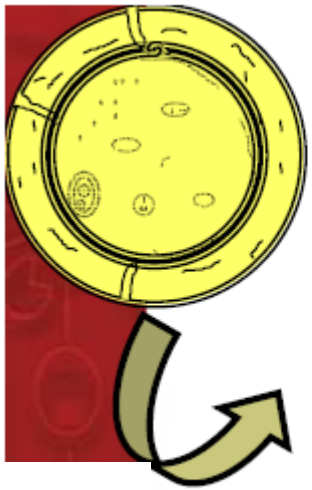
Primary hemostasis



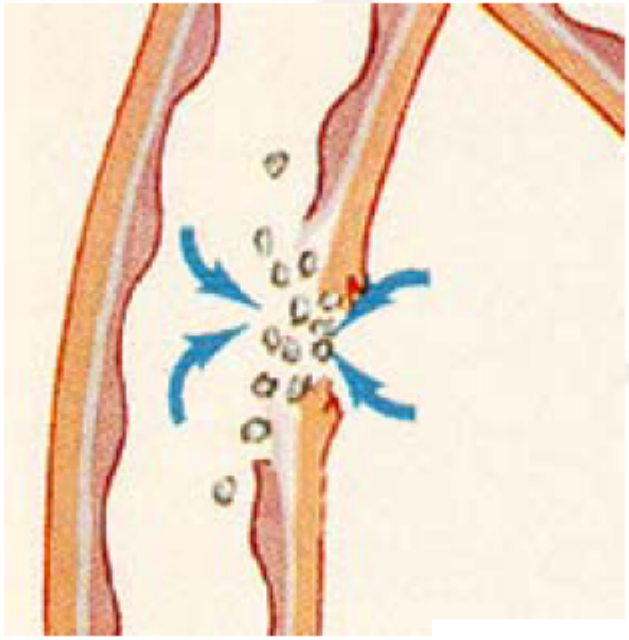
Platelet

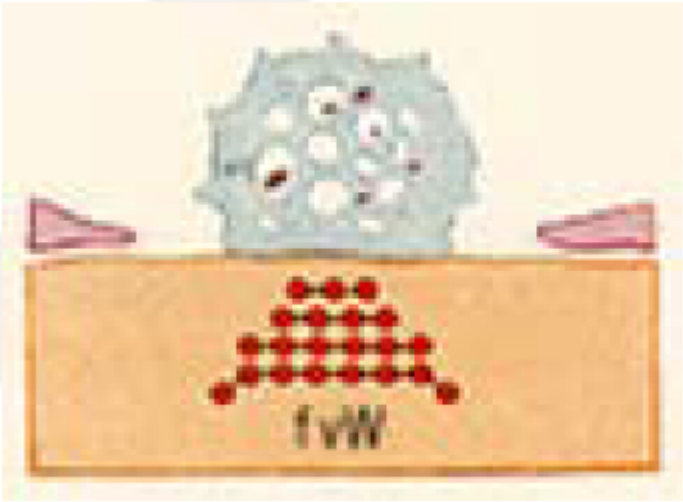
Inert platelet

Initiation of primary haemostasis

Vascular constriction

1. Platelet adhesion





vWF: Von Willebrand Factor

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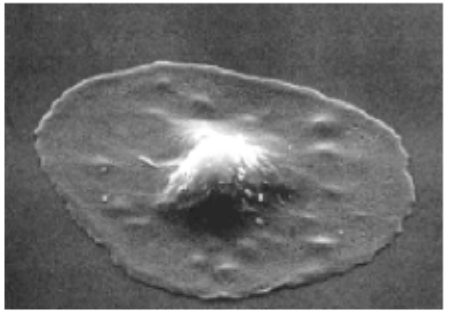
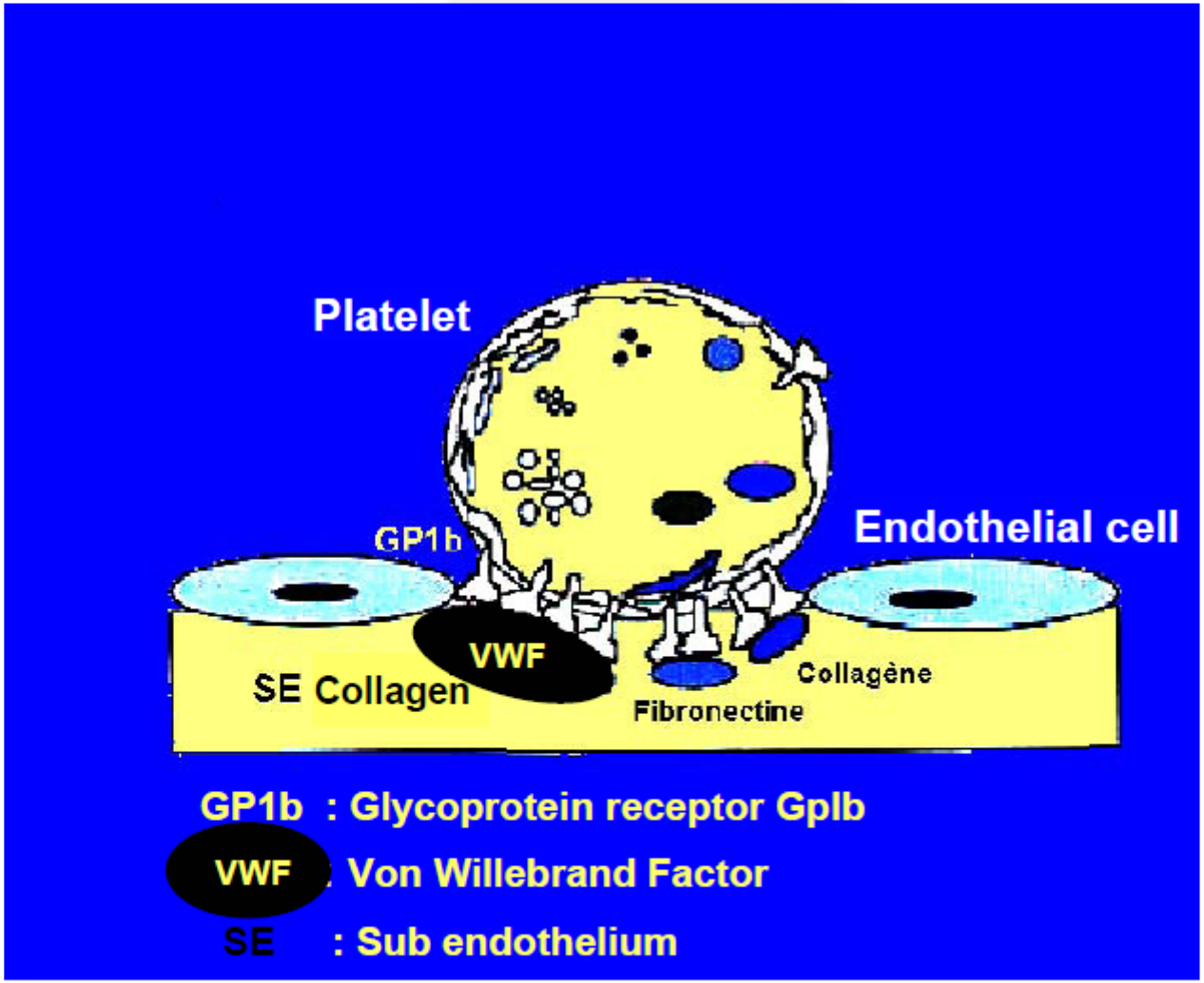
Platelet adhesion (1)





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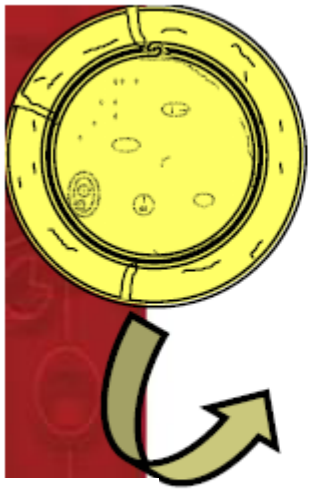
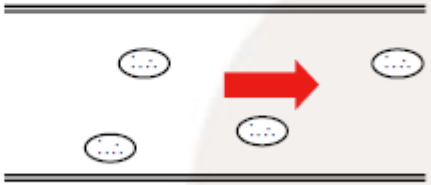
Primary hemostasis

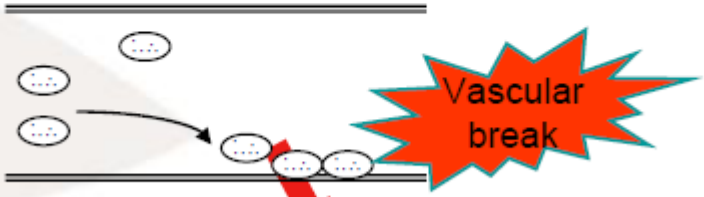


Platelet



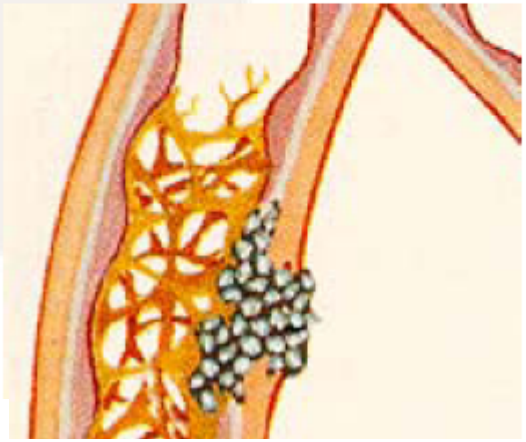
Inert platelet

2. Activation
Shape change



Vascular contraction
1. Platelet adhesion

2. Activation
Release



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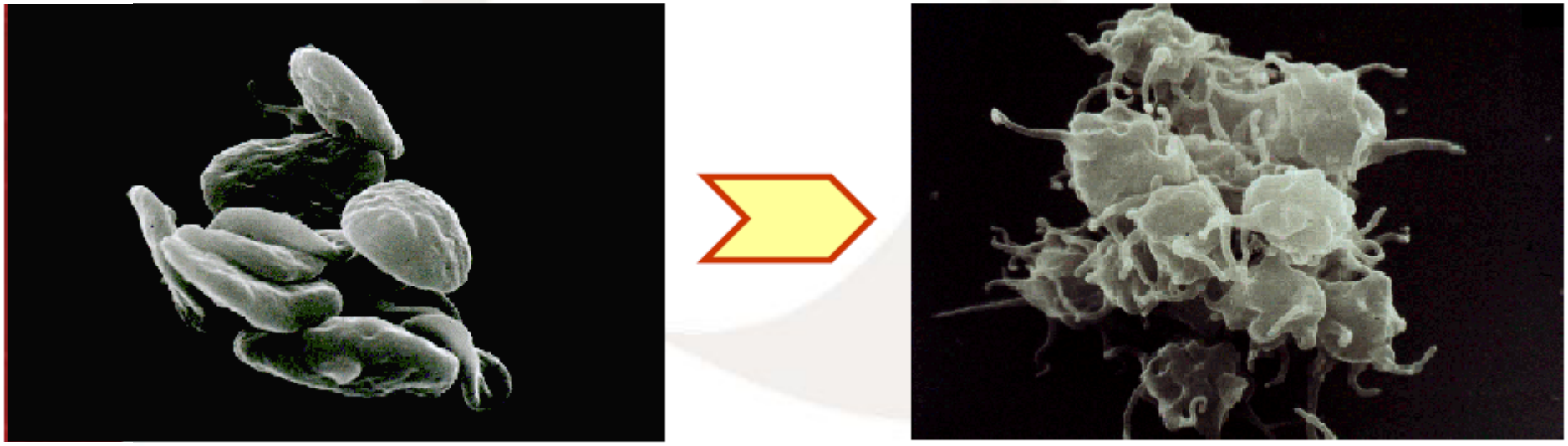
Platelet activation (2)

- The interaction of VWF results in platelet activation
  - Shape change



Platelet activation (2)

- The interaction of VWF results in platelet activation
  - Shape change
  - Release of platelet contents

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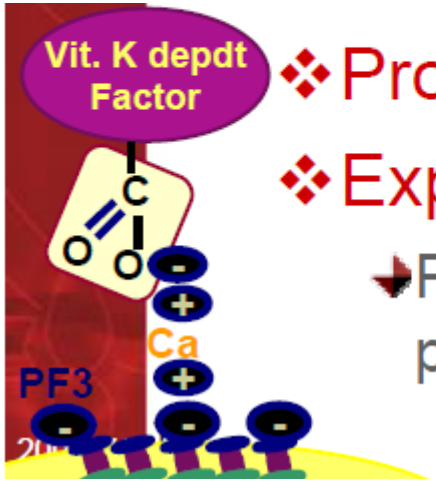
Table 1. Platelet granule contents

Alpha granules[a]	Dense granules	Lysosomal granules[b]
Albumin	Serotonin	Cathepsin D
Fibrinogen	ATP	Cathepsin E
Fibronectin	ADP	Carboxypeptidase A
Vitronectin	Calcium	Carboxypeptidase B
Osteonectin	Pyrophosphate	Proline carboxypeptidase
von Willebrand factor		β-*N*-acetyl-D-hexosaminidase
von Willebrand antigen II		β-D-glucuronidase
Thrombospondin		β-D-galactosidase
Platelet factor 4		α-D-mannosidase
IgG, IgA, IgM		α-L-arabinofuranosidase
C1 inhibitor		α-D-galactosidase
Plasminogen		α-L-fucosidase
Plasminogen activator inhibitor-1		β-D-fucosidase
Platelet-derived collagenase inhibitor		β-D-glucosidase
High molecular weight kininogen		α-D-glucosidase
Protein S		Acid phosphatase
α_2-antitrypsin		Arylsulphatase
α_2-macroglobulin		
α_2-antiplasmin		
Multimerin		
Platelet basic protein		
β-thromboglobulin		
Histidine-rich glycoprotein		
Connective tissue-activating protein III		
Neutrophil-activating protein II		
Platelet-derived growth factor		
Transforming growth factor β-		
Endothelial cell growth factor		
Coagulation factor V		
Coagulation factor VIII		

[a]From reference [5].

Platelet activation (2)

- The interaction of VWF results in platelet activation
  - Shape change
  - Release of platelet contents
    - Alpha granules: PF4, β-TG, …
    - Dense granules: ADP (induces aggregation)
  - Production of thromboxane A2 (TX A2)
  - Exposure of membrane phospholipids
    - Platelet Factor 3 (PF3), support for the coagulation process



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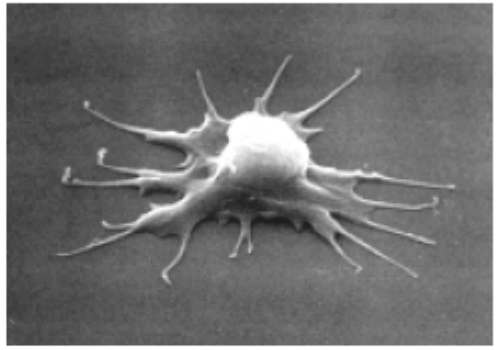
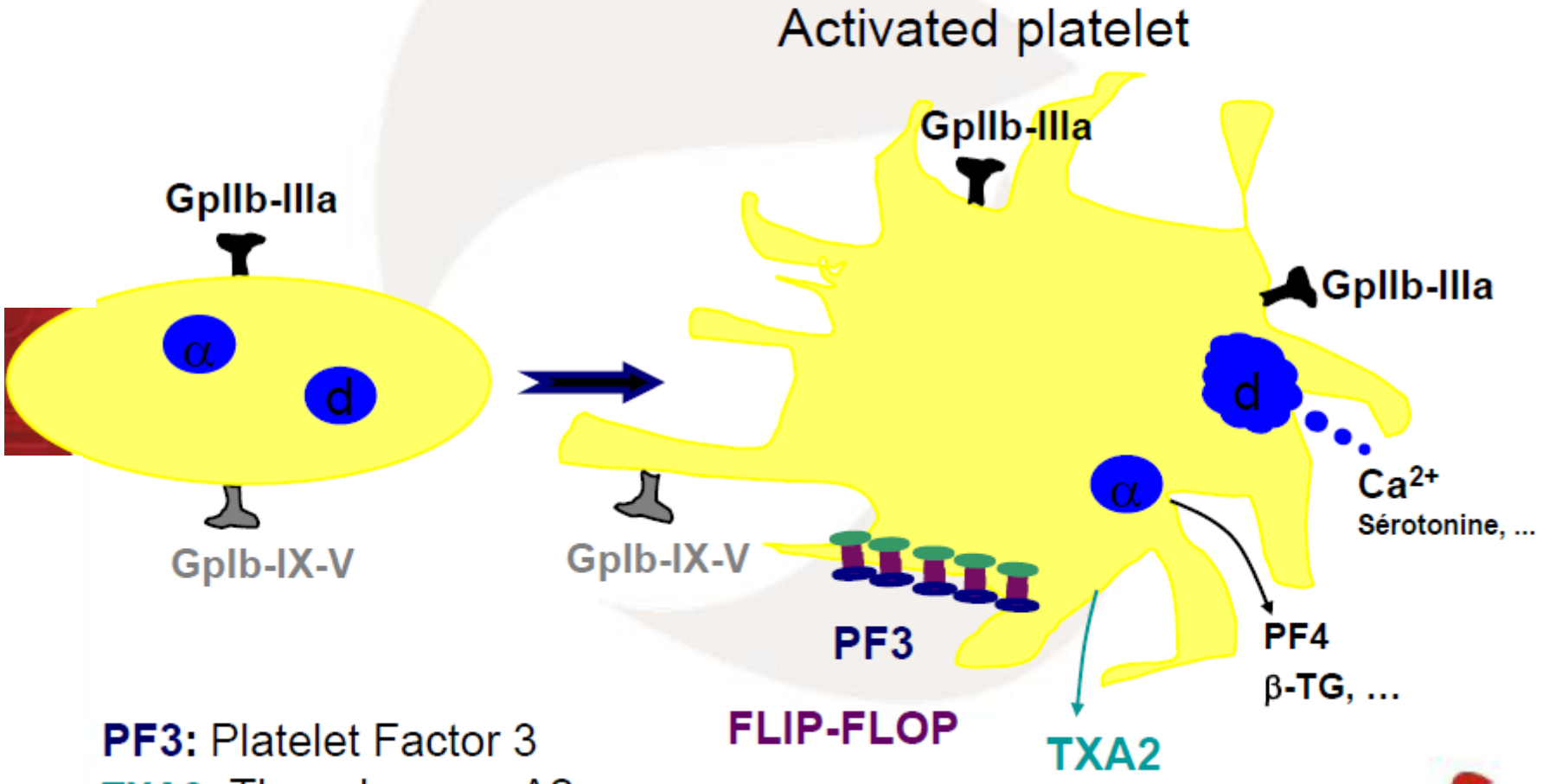
Platelet activation (2)





PF3: Platelet Factor 3
TXA2: Thromboxane A2

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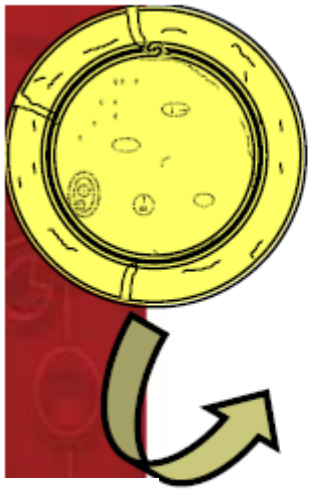
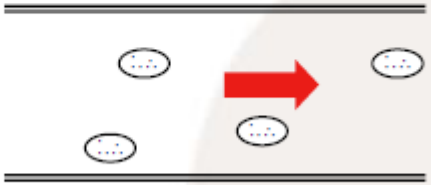
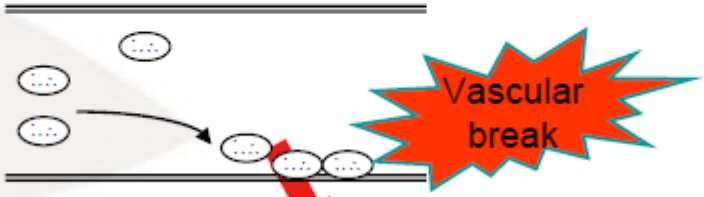
Primary hemostasis



Platelet



Inert platelet



Vascular contraction

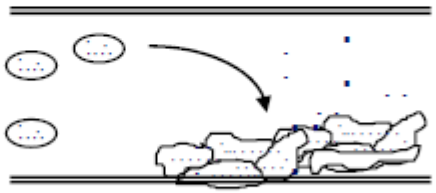
1. Platelet adhesion

2. Activation

Shape change

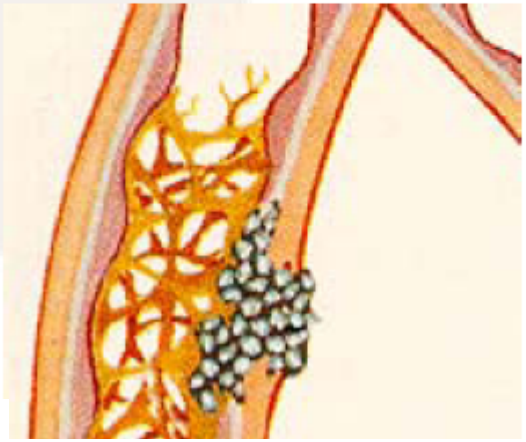
2. Activation

Release



3. Aggregation

⇨ PLATELET CLOT



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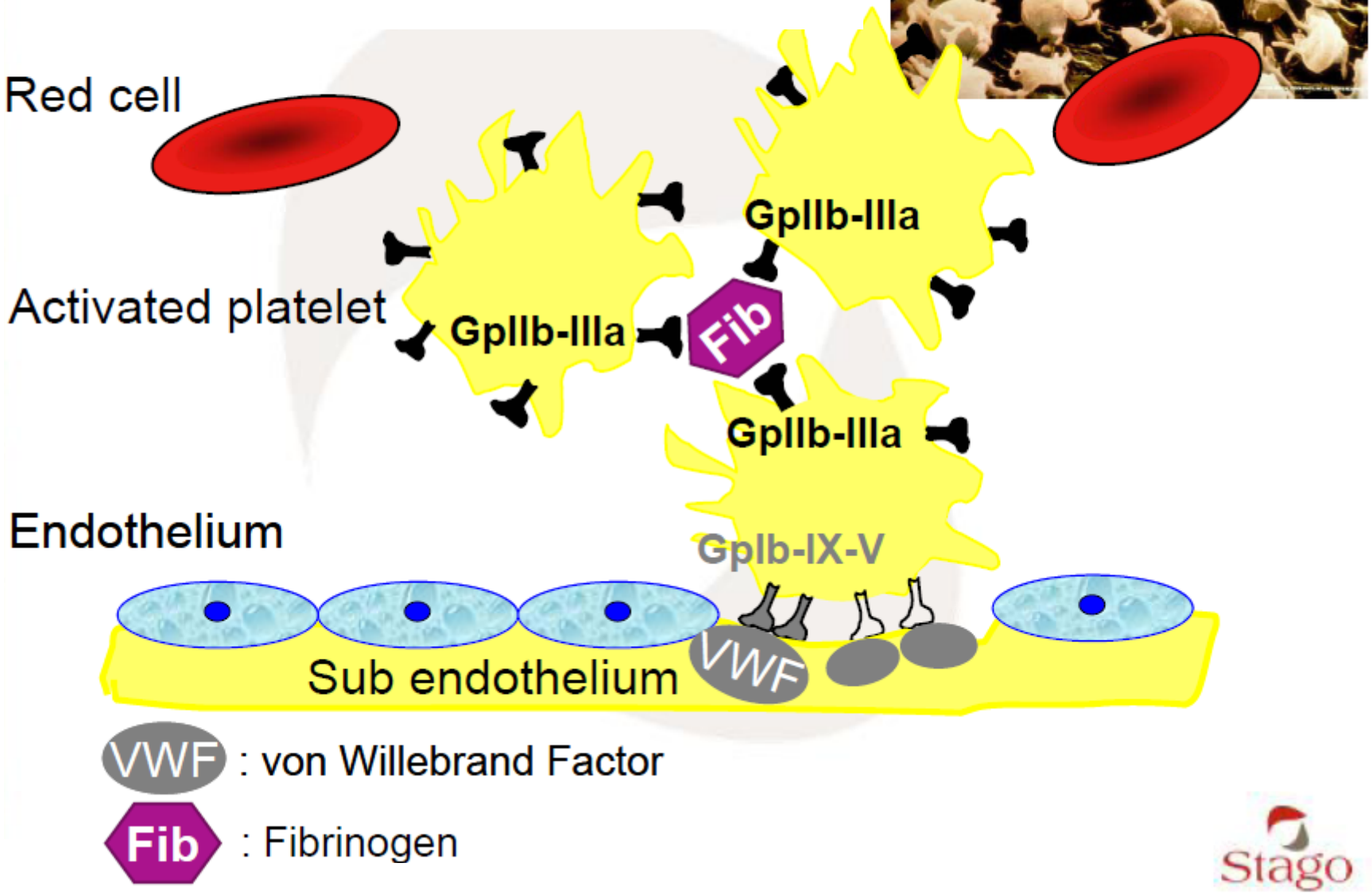
Platelet aggregation (3)



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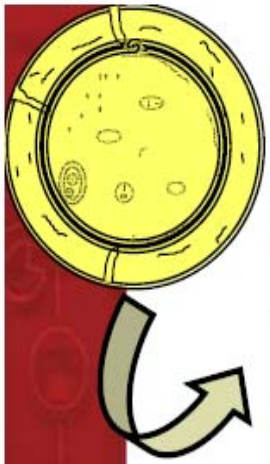
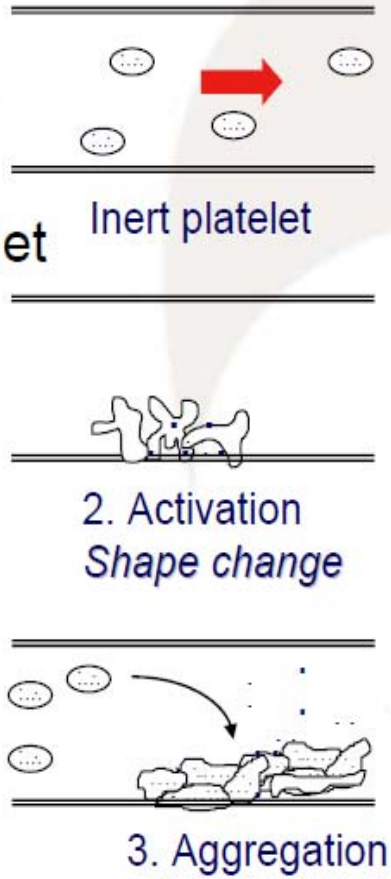
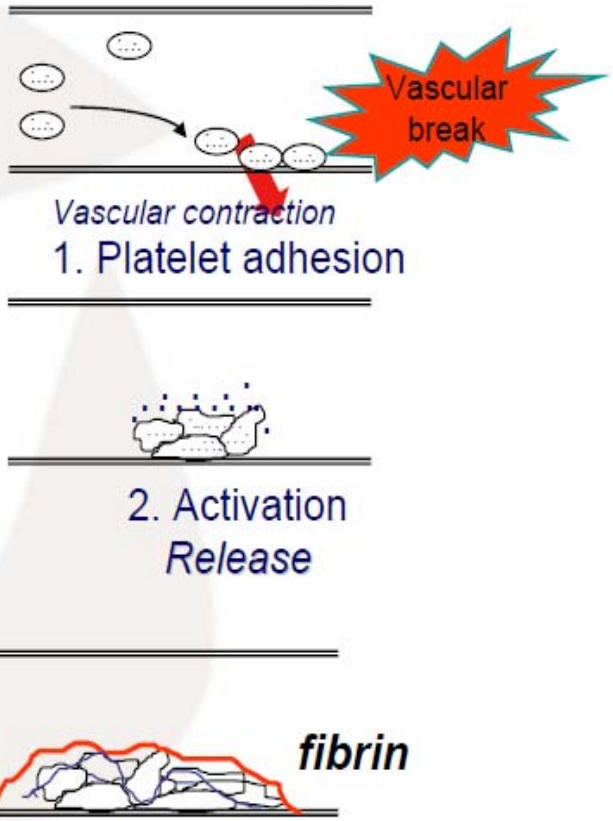
Primary Haemostasis summary



Platelet



PLATELET CLOT



COAGULATION

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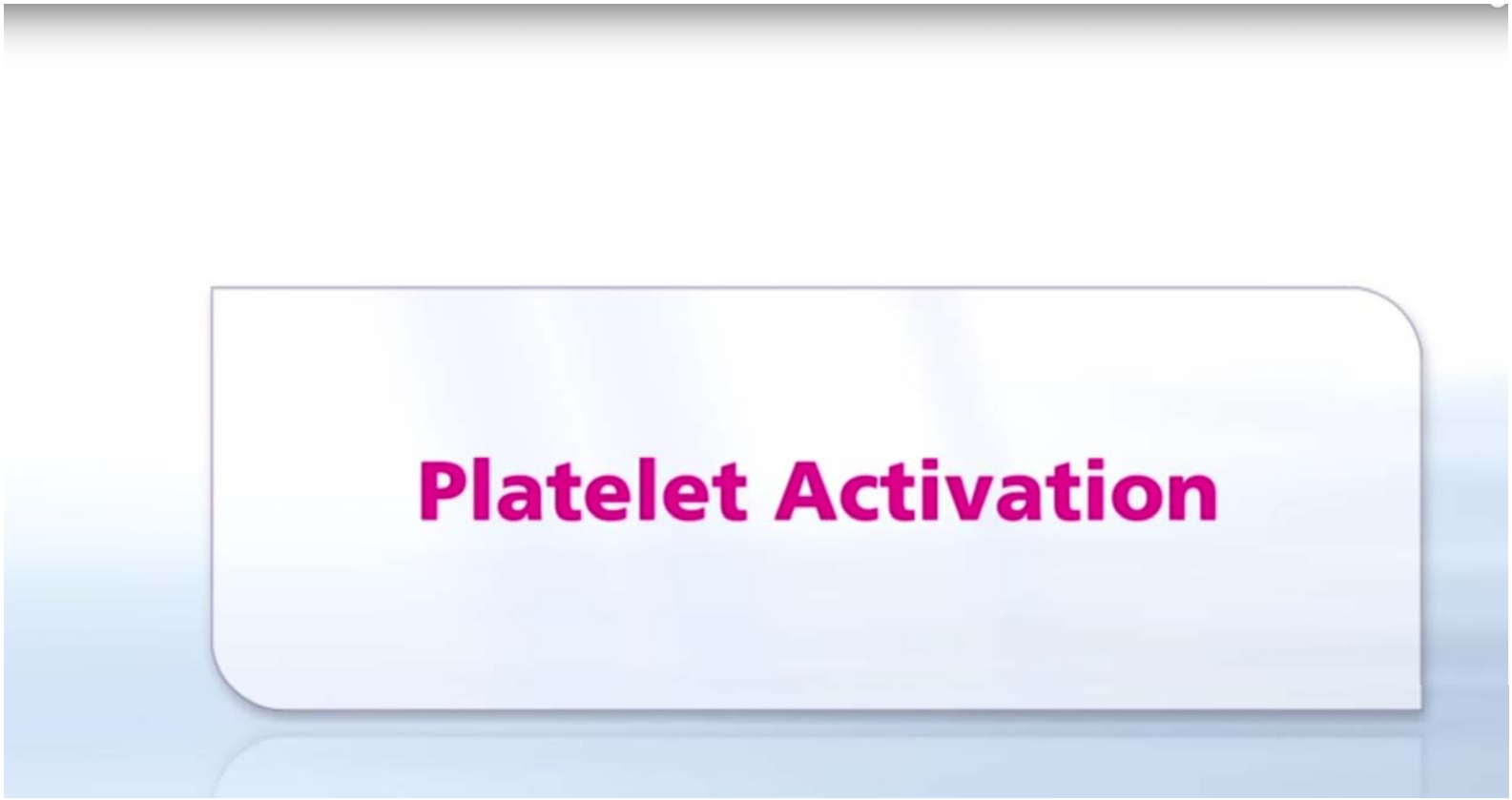

https://www.youtube.com/watch?v=R8JMfbYW2p4

Assays for primary Haemostasis

- Bleeding time
- Von Willebrand Factor
  - Antigen determination
  - Activity
- Platelet count
- Platelet aggregation
- Activation markers (β-TG, PF4, GPV)
- Specialised tests for platelet function

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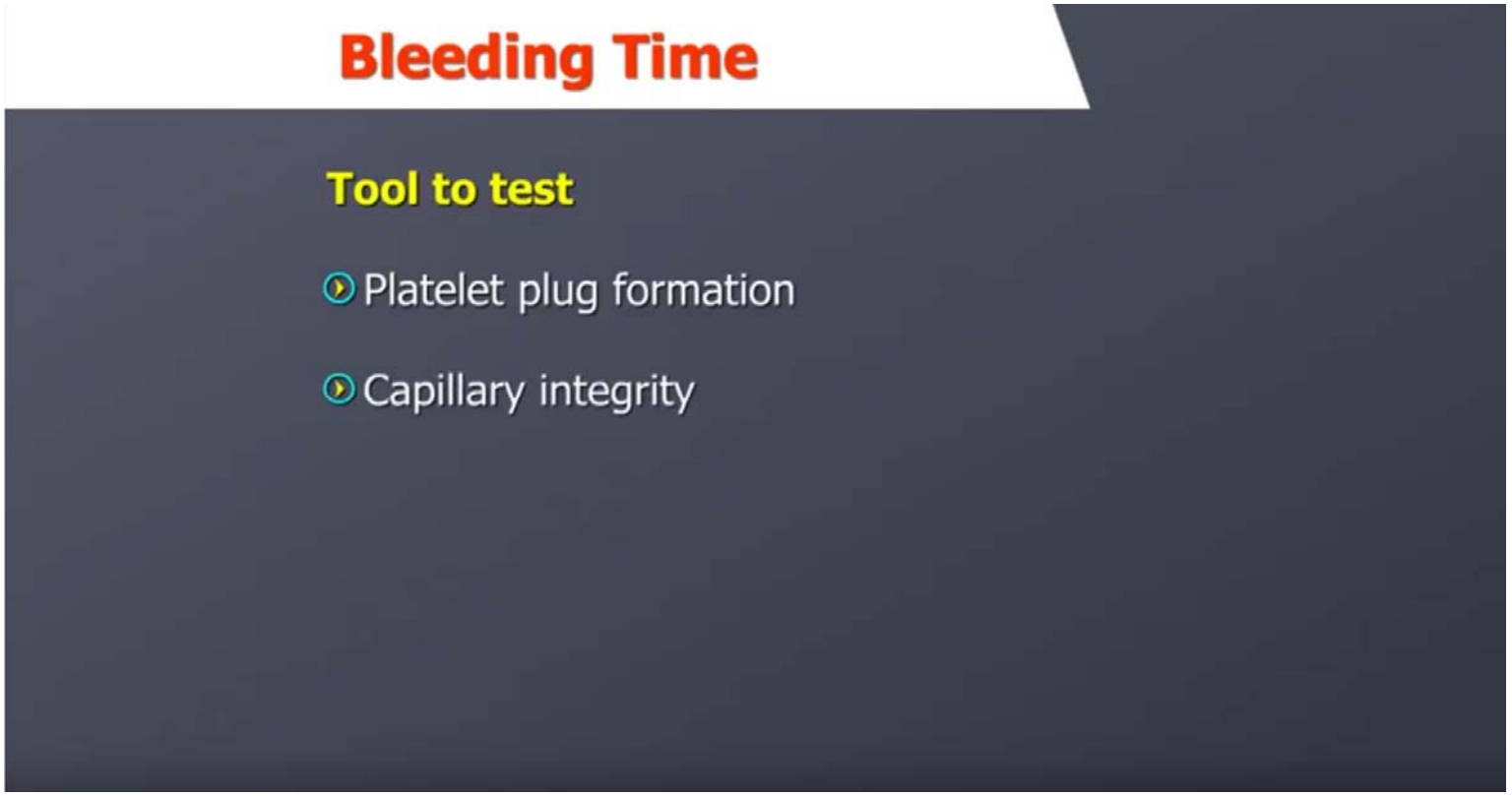
Bleeding time

Ivy method

utilizing an incision on the ventral side of the forearm

Duke method

the patient is pricked with a special needle or lancet, preferably on the earlobe or fingertip



https://www.youtube.com/watch?v=bMVy6pCWhRk

Platelet count

- Part of CBC
- EDTA-anticoagulated

LOW

Increased destruction:
- Medications
- Autoimmune response
- DIC

Low production:
- Bone marrow disease
- Dietary

Hypersplenism

HIGH

Reactive:
- Infection
- Postoperative
- Cancer
- Acute blood loss

Myeloproliferative

Platelet count

- Falsely low PLT count
  - Microclots in tube
  - EDTA-dependent agglutinins

  Blood smear should be reviewed.

  PLT histogram may be informative

Less commonly ordered tests

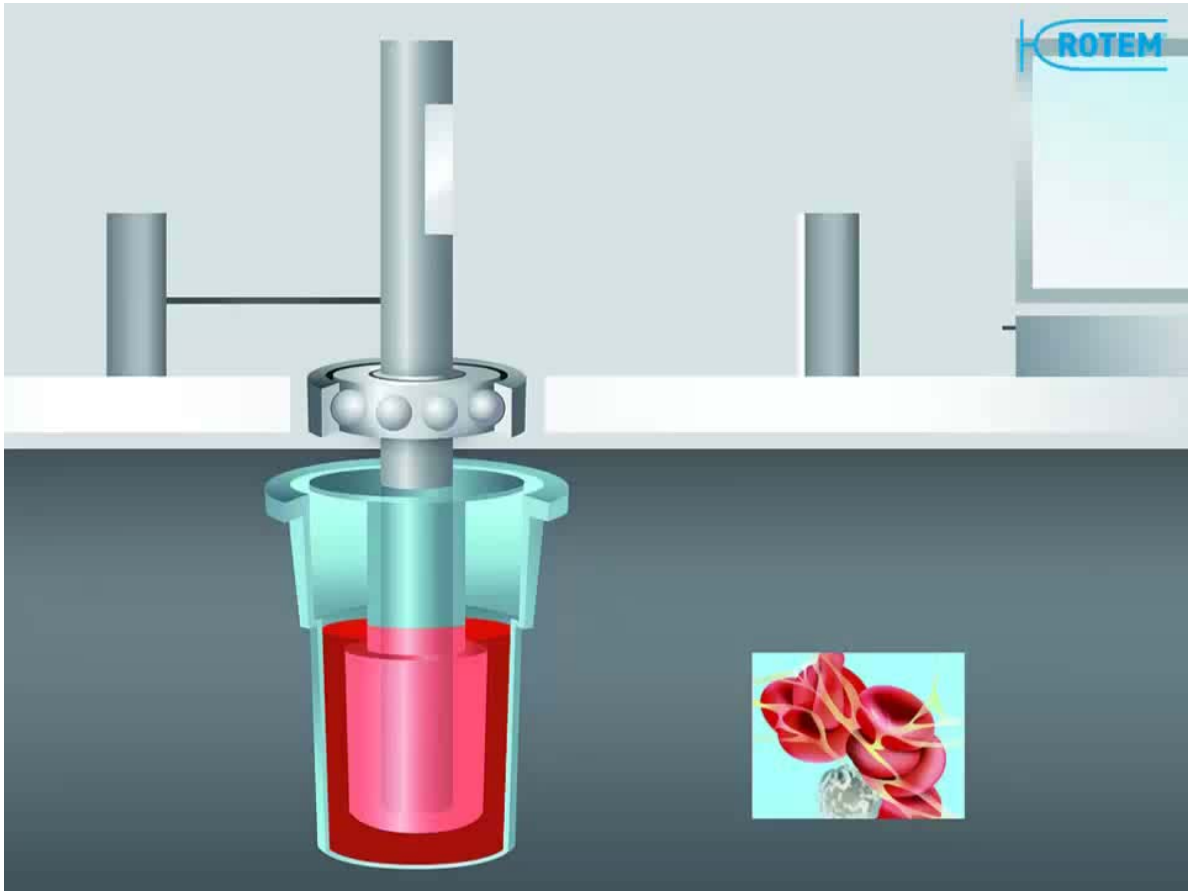
- Platelet aggregation test
- PFA-100
- ROTEM / Thrombelastography
- Thrombin generation assays

Platelet aggregation

- Addition of a platelet agonist to the PRP leads to platelet activation, a change in their shape from discoid to spiny spheres which is associated with a transient increase in optical density.
- Platelets will only aggregate (although they may agglutinate) if fibrinogen is present and so it is important to check fibrinogen levels before undertaking platelet aggregation testing.

https://www.youtube.com/watch?v=q1BK8ks_HsE

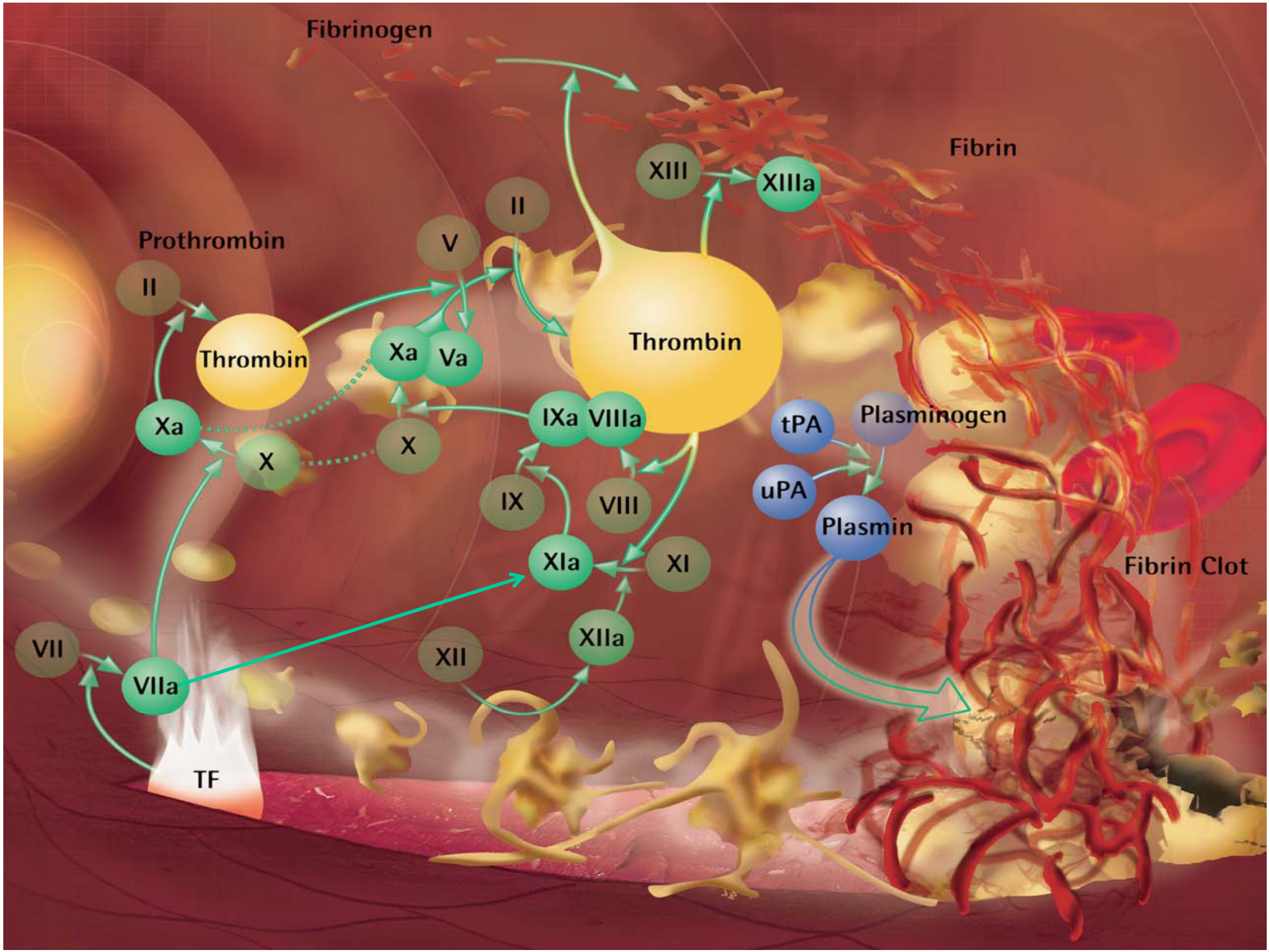
Thromboelastography

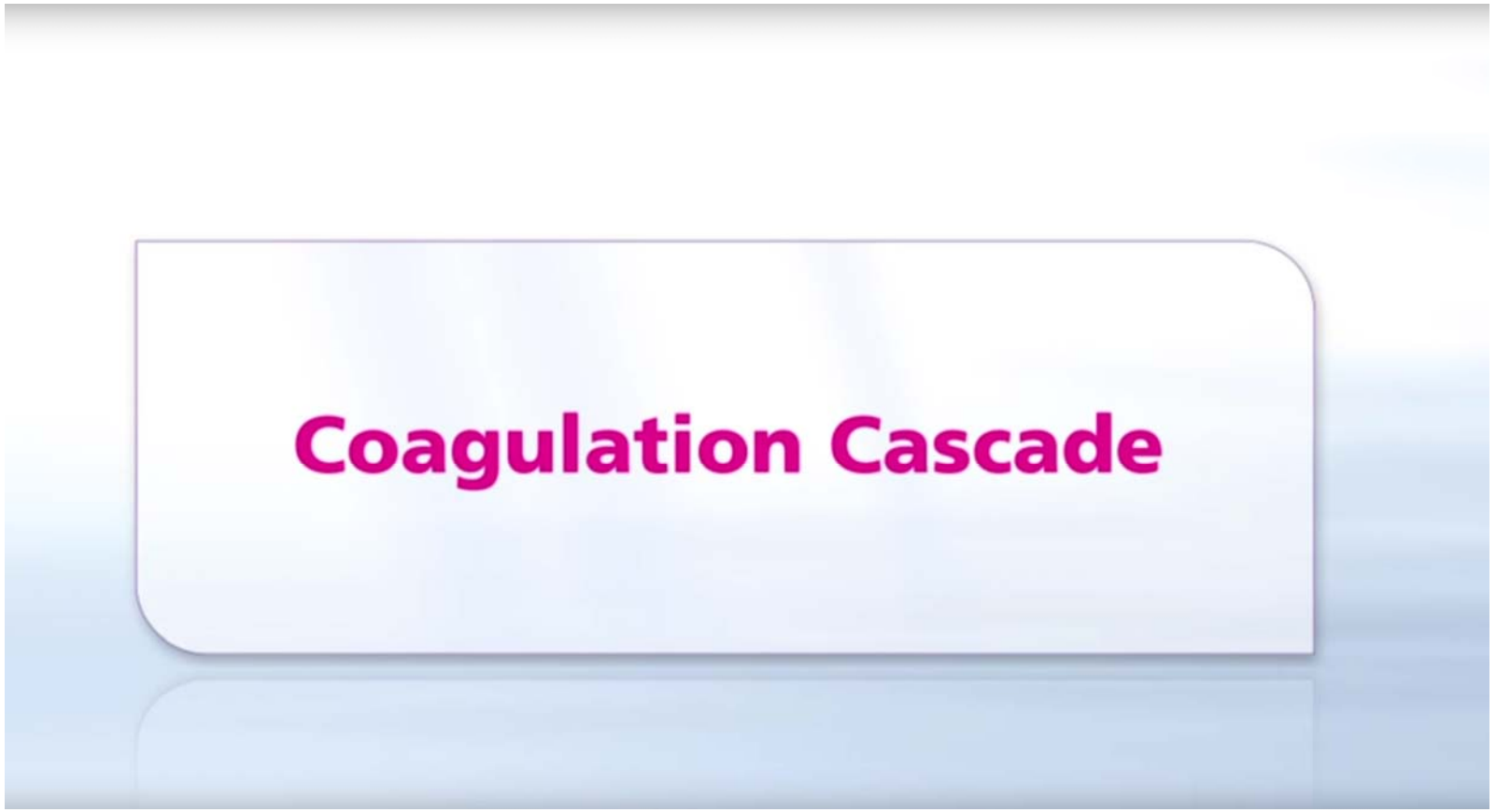


https://www.youtube.com/watch?v=FTUgIgeIjrI

https://www.youtube.com/watch?v=zEFszp9DKLE

OVER the platelets


Fibrinogen
Fibrin
XIII
XIIIa
II
Prothrombin
V
II
Thrombin
Xa
Va
Thrombin
Xa
X
X
IXa
VIIIa
tPA
Plasminogen
uPA
Plasmin
IX
VIII
XIa
XI
Fibrin Clot
VII
VIIa
XII
XIIa
TF



https://www.youtube.com/watch?v=cy3a__OOa2M

The *initiation of coagulation*

- Begins after an injury on cell surfaces (monocytes, fibroblasts).
- Tissue Factor (TF) released during an injury binding to these surfaces, forms a complex with FVIIa (extrinsic pathway).
- TF-VIIa complex activates FX to FXa and FIX to FIXa
- FXa forms a complex with the FVa →small thrombin.

The *amplification* of coagulation

- Small amounts of thrombin formed in the initiation phase
- Activates adhered platelets and other factors (V, VIII, XI)
- In this phase the coagulation process moves from the TF-carrying cells to the platelets.

The *propagation of coagulation*

- On the surface of the activated platelets by attachments of the FIXa (formed by the TF-VIIa complex in the initial phase) and of FVIIIa, Va and XIa (formed in the amplification phase)
- The resulting VIIIa/IXa complex (tenase complex of the intrinsic pathway) activates further FX
- FXa with the help of FVa forms an activator complex (prothrombinase complex) and large amounts of thrombin are formed
- Thrombin induced fibrin formation completes the coagulation process.

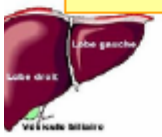
The *stabilization* of clotting

- Thrombin activated FXIIIa cross-links the soluble fibrin-network between two D subunits

- D-Ds resist to fibrinolysis

Coagulation factors

N°	Name	Vit. K dep.	Function	Minimum level*
II	Prothrombin	Yes	Proenzyme	40-50 %
V	Proaccelerin	No	Proenzyme Cofactor	25 %
VII	Proconvertin	Yes	Proenzyme	20 %
X	Stuart	Yes	Proenzyme	10 % 40-50 % Surgery
I	Fibrinogen	No	Substrate	0.5-1 g/l
XIII	Fibrin Stabilising Factor	No	Proenzyme	10 %
XII	Hageman	No	Proenzyme	-
XI	Rosenthal	No	Proenzyme	10-15 % 20-40 % surgery
IX	Anti haemophilic B	Yes	Proenzyme	10-25 % 60 % surgery
VIII	Anti haemophilic A	No	Proenzyme Cofactor	30 % 60 % surgery



Synthesised by the liver

* for a normal haemostasis

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Activated factors

N°	Name	Vit. K dep.	Function
IIa	thrombin	Yes	enzyme
Va	accelerin	No	enzyme Cofactor
VIIa	convertin	Yes	enzyme
Xa	-	Yes	enzyme
I	Fibrinogen	No	Substrate
XIIIa	-	No	enzyme
XIIa		No	enzyme
XIa		No	enzyme
IXa		Yes	enzyme
VIIIa		No	enzyme Cofactor

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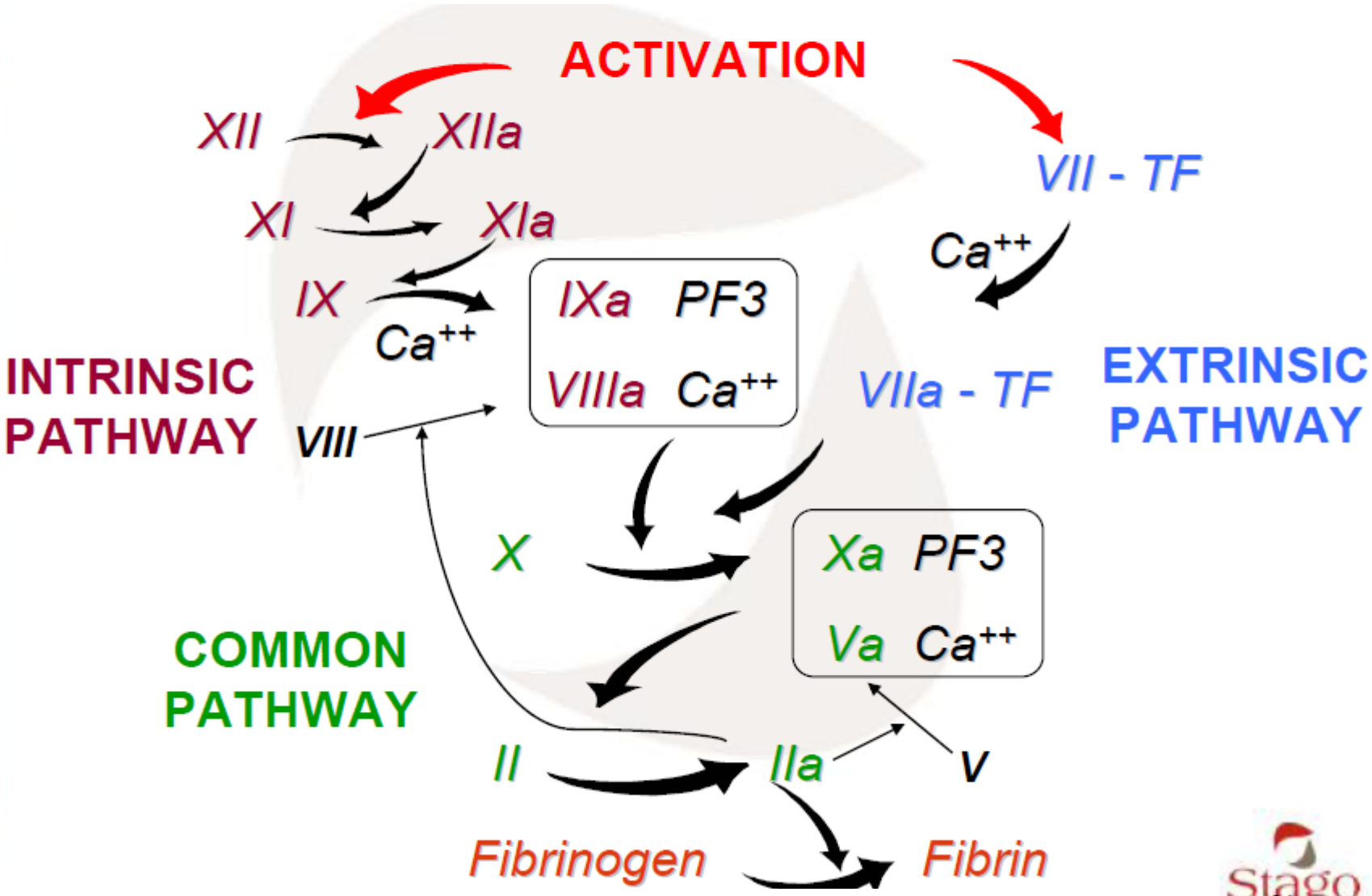
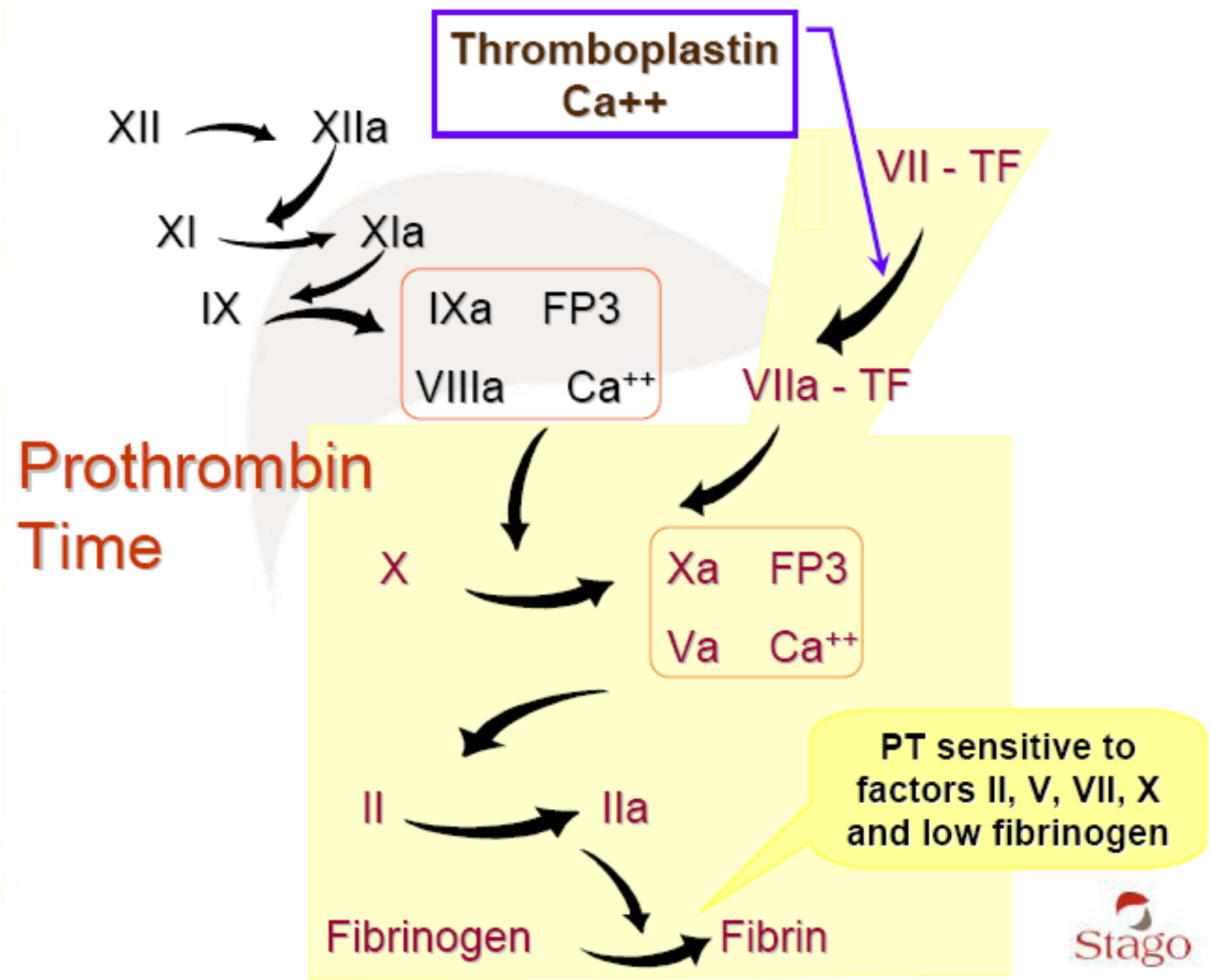
Coagulation




Thromboplastin
Ca++
XII
XIIa
XI
XIa
IX
IXa FP3
VIIIa Ca++
VII - TF
VIIa - TF
Prothrombin Time
X
Xa FP3
Va Ca++
II
IIa
Fibrinogen
Fibrin
PT sensitive to factors II, V, VII, X and low fibrinogen
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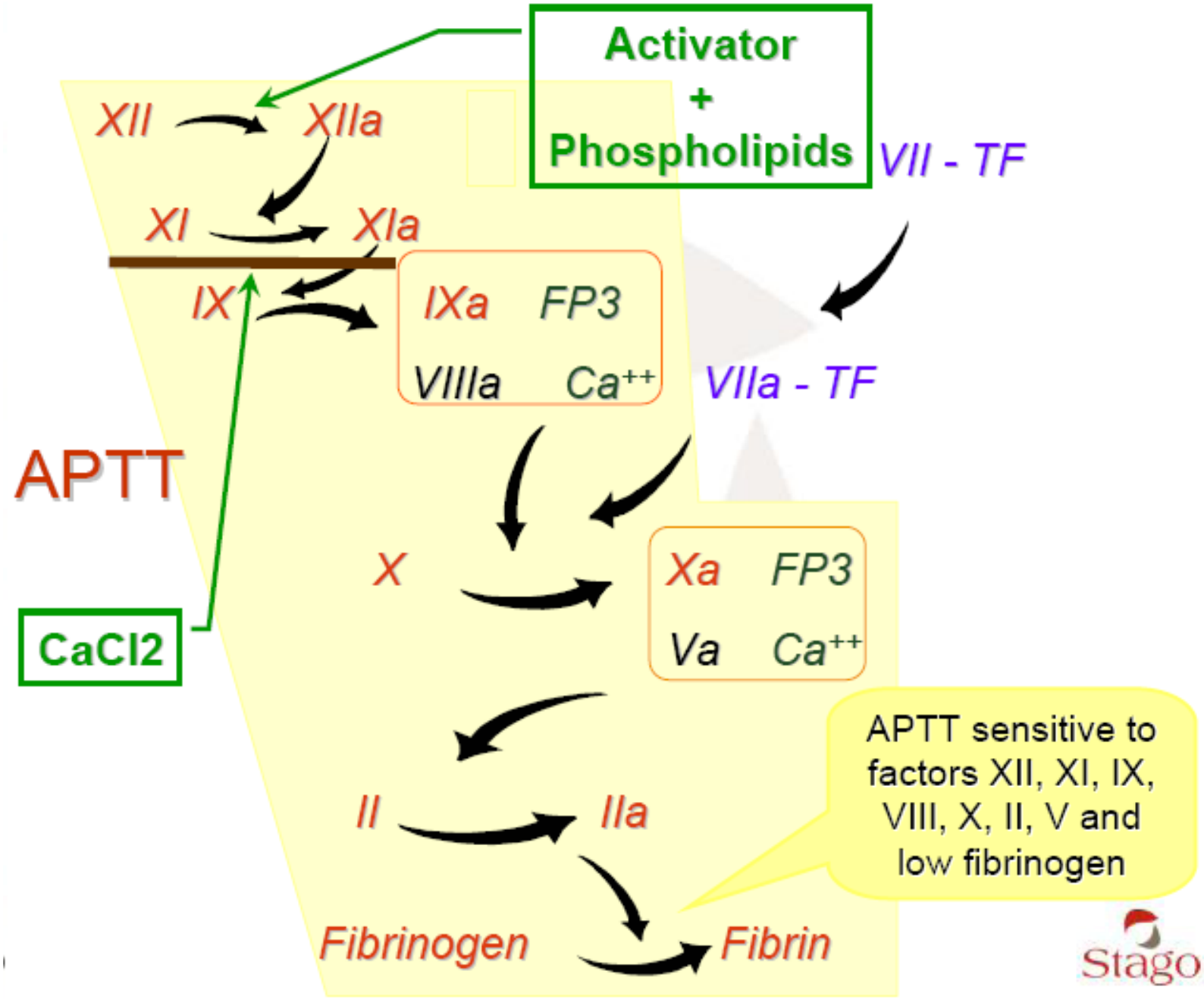
Activator
+
Phospholipids
XII
XIIa
XI
XIa
IX
IXa FP3
VIIIa Ca++
VII - TF
VIIa - TF
APTT
CaCl2
X
Xa FP3
Va Ca++
II
IIa
Fibrinogen
Fibrin
APTT sensitive to factors XII, XI, IX, VIII, X, II, V and low fibrinogen
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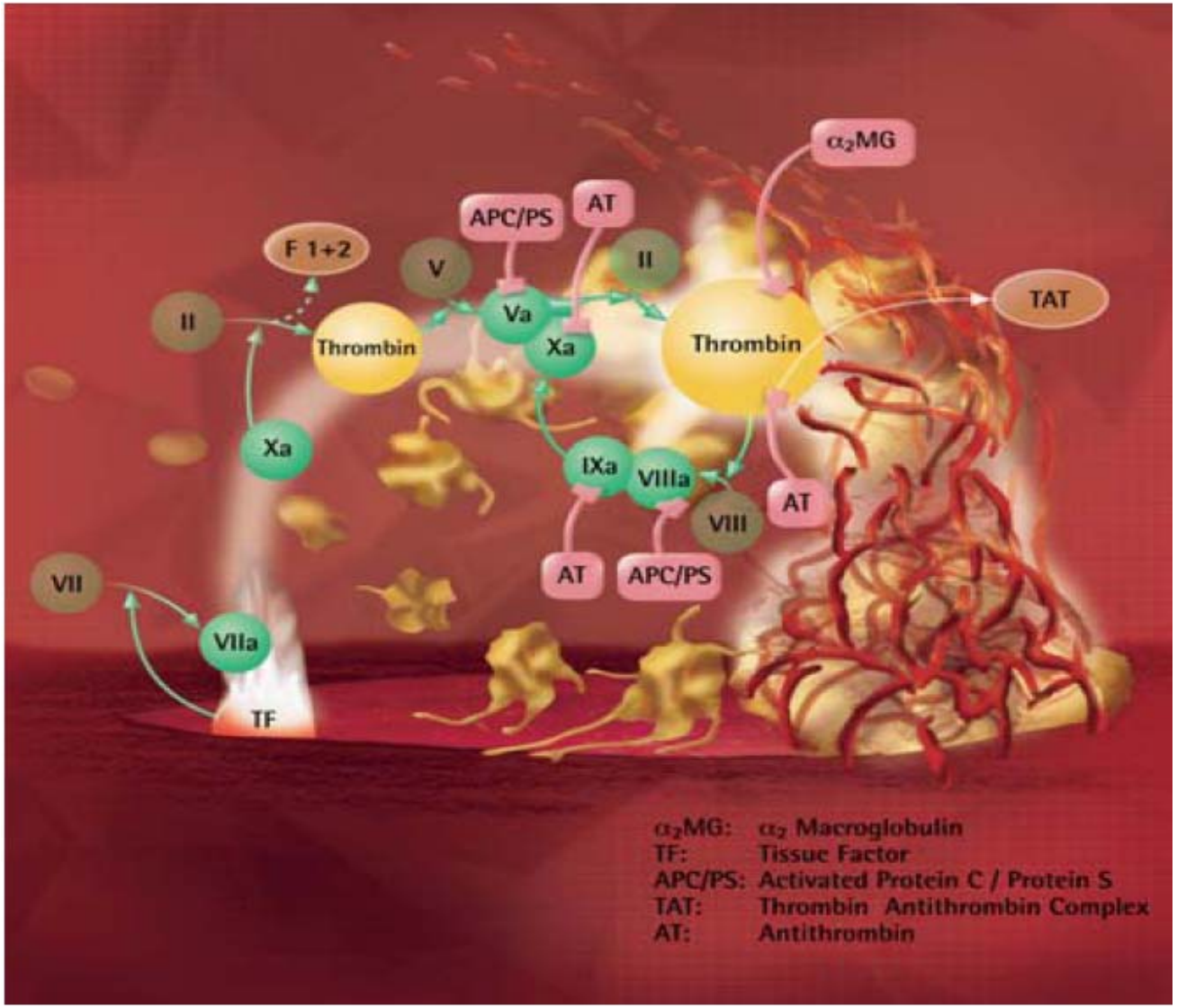
Natural inhibitors



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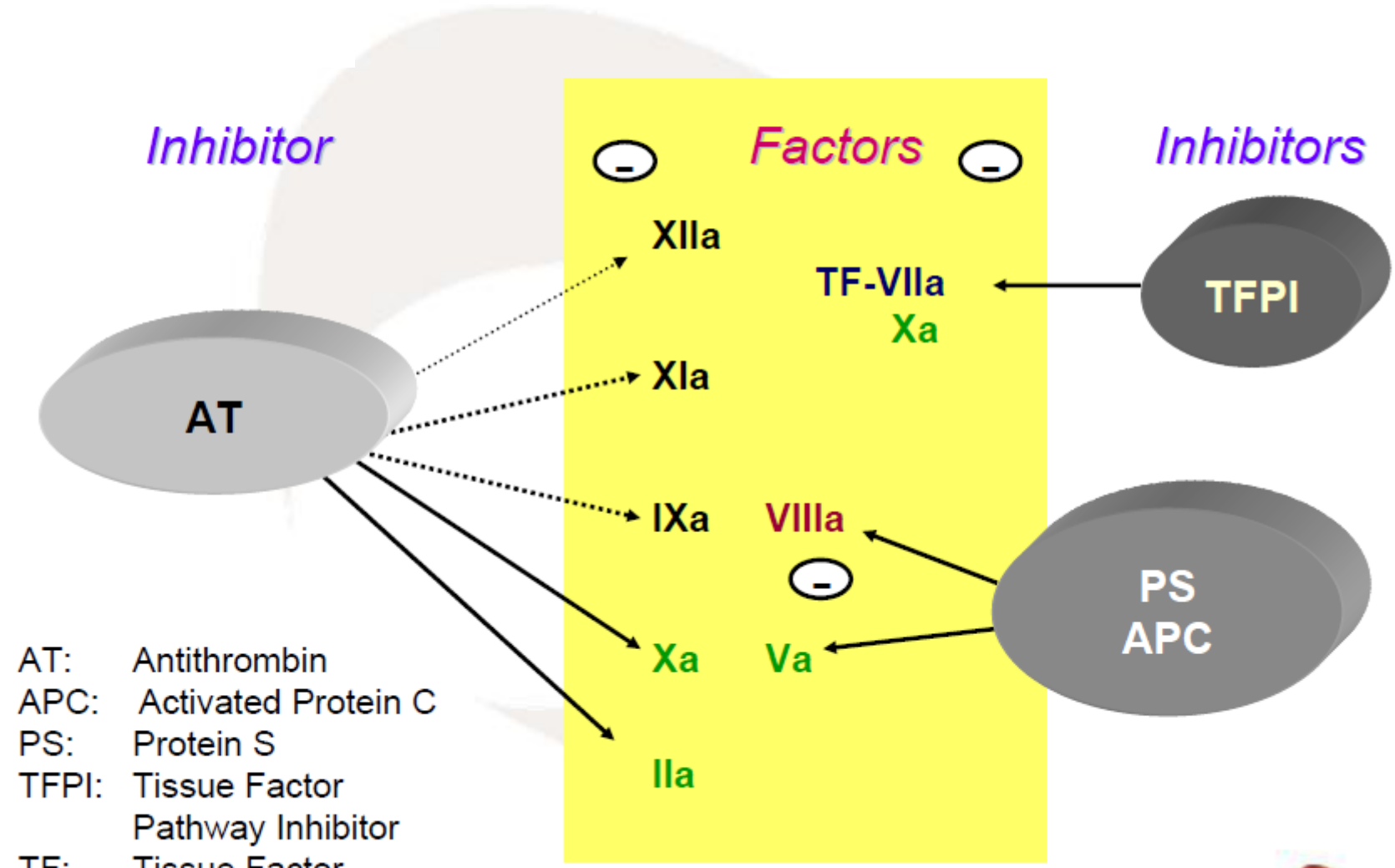
Inhibitors



AT: Antithrombin
APC: Activated Protein C
PS: Protein S
TFPI: Tissue Factor Pathway Inhibitor
TF: Tissue Factor

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Main assays for coagulation

- Screening tests: PT, APTT, (Thrombin time)
- Fibrinogen
- Factor assays (II, V, VII, VIII, IX, X, XI, XII)
- Inhibitors (AT, Protein C, Protein S)
- APC-Resistance
- Lupus Anticoagulant

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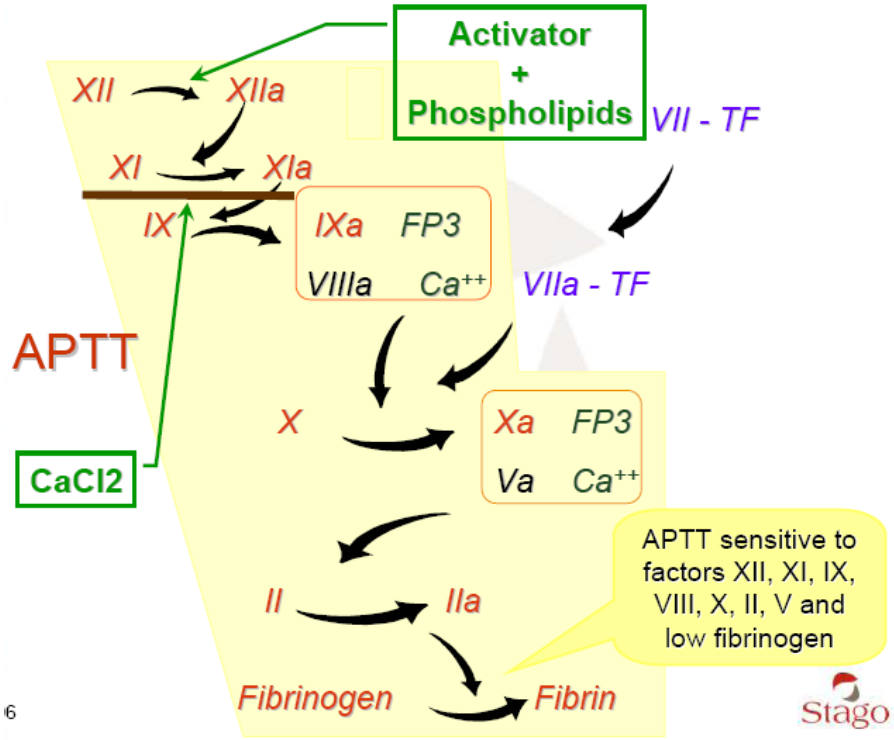
Activator
+
Phospholipids
XII
XIIa
XI
XIa
IX
IXa FP3
VIIIa Ca++
VII - TF
VIIa - TF
APTT
CaCl2
X
Xa FP3
Va Ca++
II
IIa
Fibrinogen
Fibrin
APTT sensitive to factors XII, XI, IX, VIII, X, II, V and low fibrinogen
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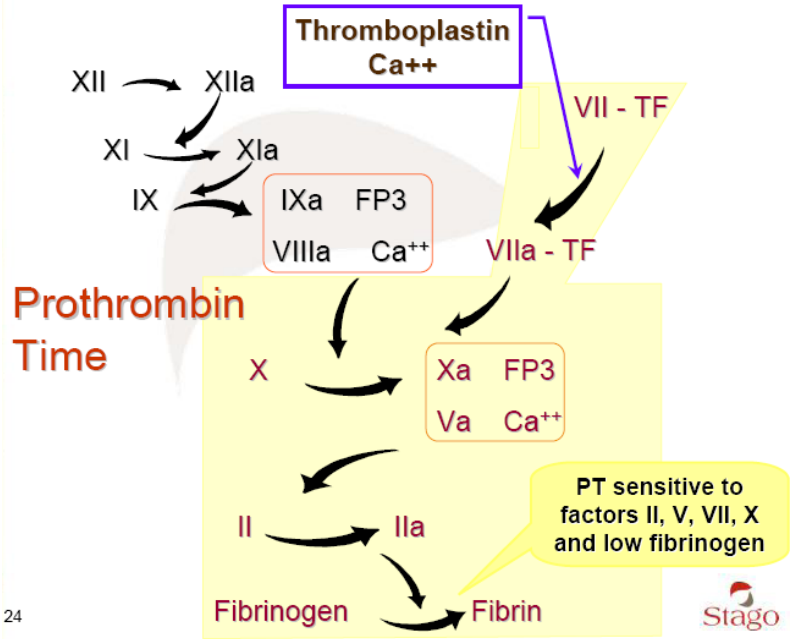
6


Thromboplastin
Ca++
XII
XIIa
XI
XIa
IX
IXa FP3
VIIIa Ca++
VII - TF
VIIa - TF
Prothrombin Time
X
Xa FP3
Va Ca++
II
IIa
Fibrinogen
Fibrin
PT sensitive to factors II, V, VII, X and low fibrinogen
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24

aPTT

PT

Preanalytical process

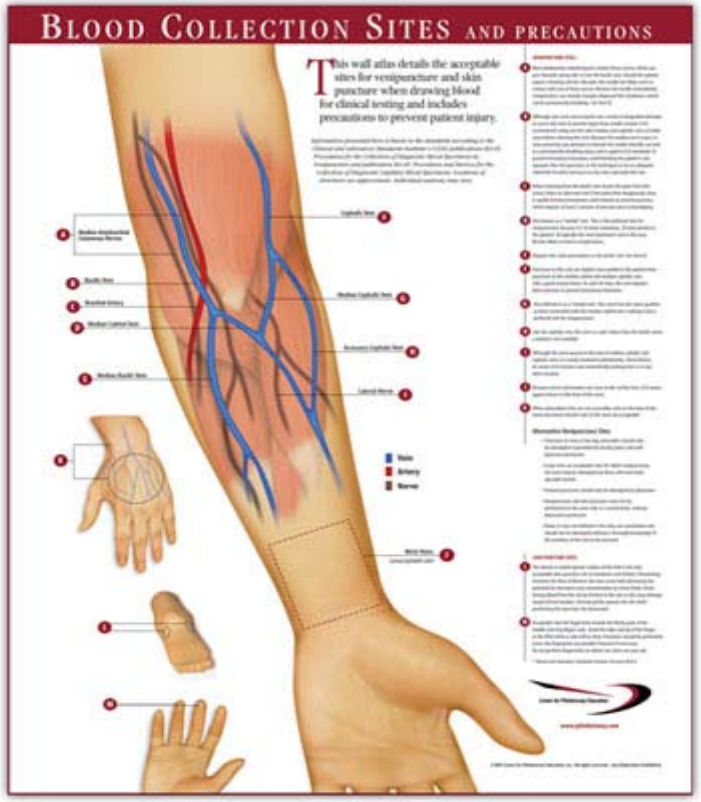
From the blood collection, through procession, until analysis:

- ***Blood sampling*** (7-9 am, relaxed position, good sampling technique, adequate puncture site, appropriate tube, proper order of sampling, correct blood-anticoagulant rate),
- ***Transporting*** (method, temperature, storage time)***,***
- ***Preparation*** (centrifugation, preparation of aliquot, freezing/thawing)

Blood sampling technique

- **Puncture site:** peripheral vein (vena cubitalis)

  From cannula: 5-10 ml blood must be discarded preventing contamination by fluid from the line.

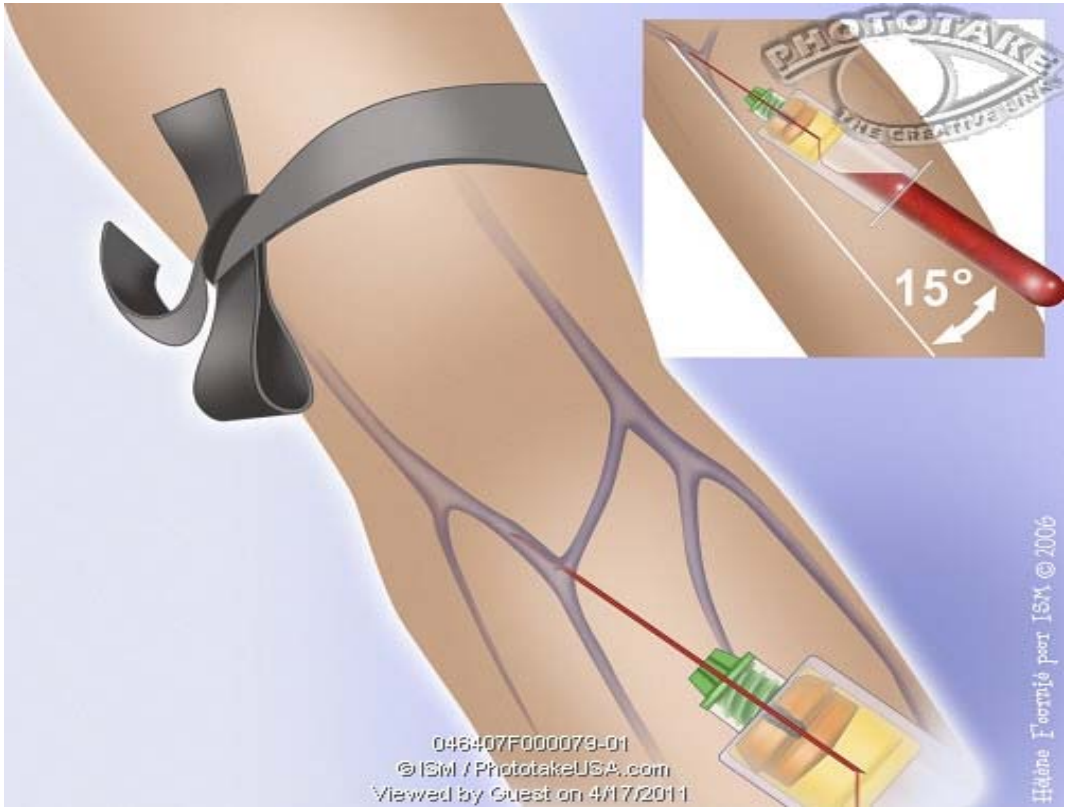


- **Tourniquet:** minimal stasis (<1 min) (release of fibrinolytic components).

  Long stasis:

  ❖PT, APTT and TT are shortened

  ❖AT, fibrinogen-level increase by cc. 10% .



- **Sequencing:**

  blood culture – native tube – **citrate tube** – EDTA-tube - others.

Remember before the analysis

- Patient identifiers,
- Quantity (<10% inaccuracy in rutine tests), **LOOK at expiry date**
- Clot,
- Ht estimation after centrifugation (>0,55 and <0,25: corrected

  blood sampling:

  9 x Na-citrate (ml) x 0,55/ 1- patient Ht,

  i.e. patient Ht: 0,8

  9 x 1ml x 0,55/0,2 = 24.75 ml venous blood)
- Hemolysis

In vivo preanalytical variations

- gender
- age
- Body weight
- Biorhytm (PAI: plasminogen activator inhibitor, tPA: tissue-Plasminogen activator)
- Nutrition (animal fat, fish, fruits, gatlich, vitamine-C)
- Drugs
- Illness, **FEVER**
- Ethnic, geographic factors
- pregnancy
- Physical and mental stress (hypercoagulability)
- Recreation drugs, stimulants (alcohol, coffee, tobacco)
- operation

PTT

- PT measures factors I (fibrinogen), II (prothrombin), V, VII, and X.
- An excess of calcium (in a phospholipid suspension) is added to the test tube, thereby reversing the effects of citrate and enabling the blood to clot again.
- In order to activate the extrinsic / tissue factor clotting cascade pathway, tissue factor and phosphilids are added and the time the sample takes to clot is measured optically or mehanically.
- Normal range: 11-14 seconds
- Reagent and system dependent

INR

- International Normalised Ratio

$$INR = \left\{ \frac{\text{patient's PT}}{\text{MNPT}} \right\}^{\text{power ISI}}$$

- With

  MNPT : Mean Normal Prothrombin Time

  ISI : International Sensitivity Index

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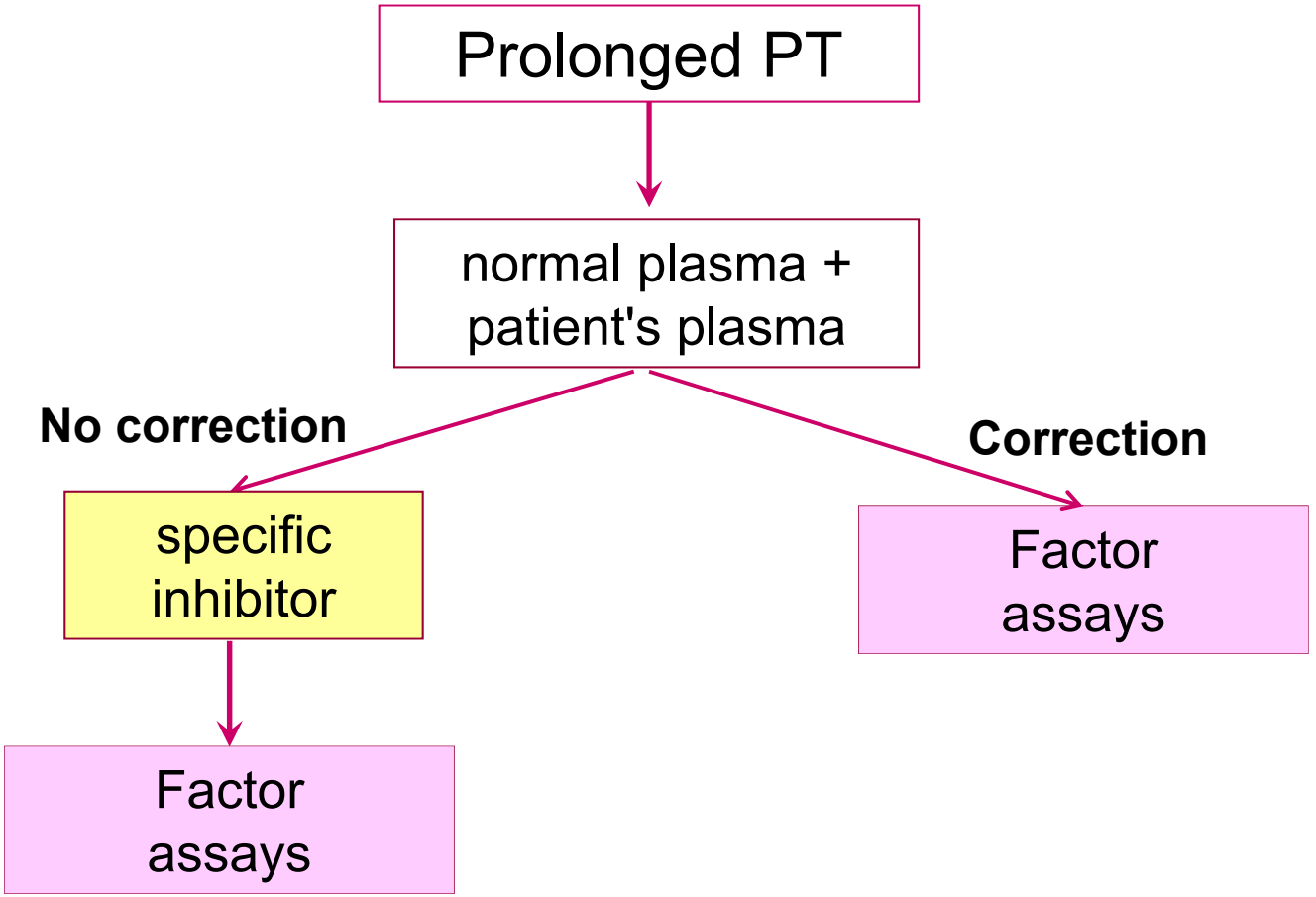
HIGH INR

- Decreased synthesis of factors
  - Vitamin K deficiency
  - Hepatic damage
- Anticoagulants
- Increased consumption (DIC)

Investigation of prolonged PT



aPTT

- The partial thromboplastin time (PTT) or activated partial thromboplastin time (aPTT or APTT) is a performance indicator measuring the efficacy of both the "intrinsic" (now referred to as the contact activation pathway) and the common coagulation pathways.
- It is also used to monitor the treatment effects with heparin.
- A sample of the plasma is extracted from the test tube and placed into a measuring test tube.
- Next, an excess of calcium (in a phospholipid suspension) is mixed into the plasma sample (to reverse the anticoagulant effect of the additive)
- With phospholipids WITHOUT tissue factor
- In order to activate the intrinsic pathway of coagulation, an activator (such as silica, celite, kaolin, ellagic acid) is added
- The time the sample takes to clot is measured optically or mechanically.

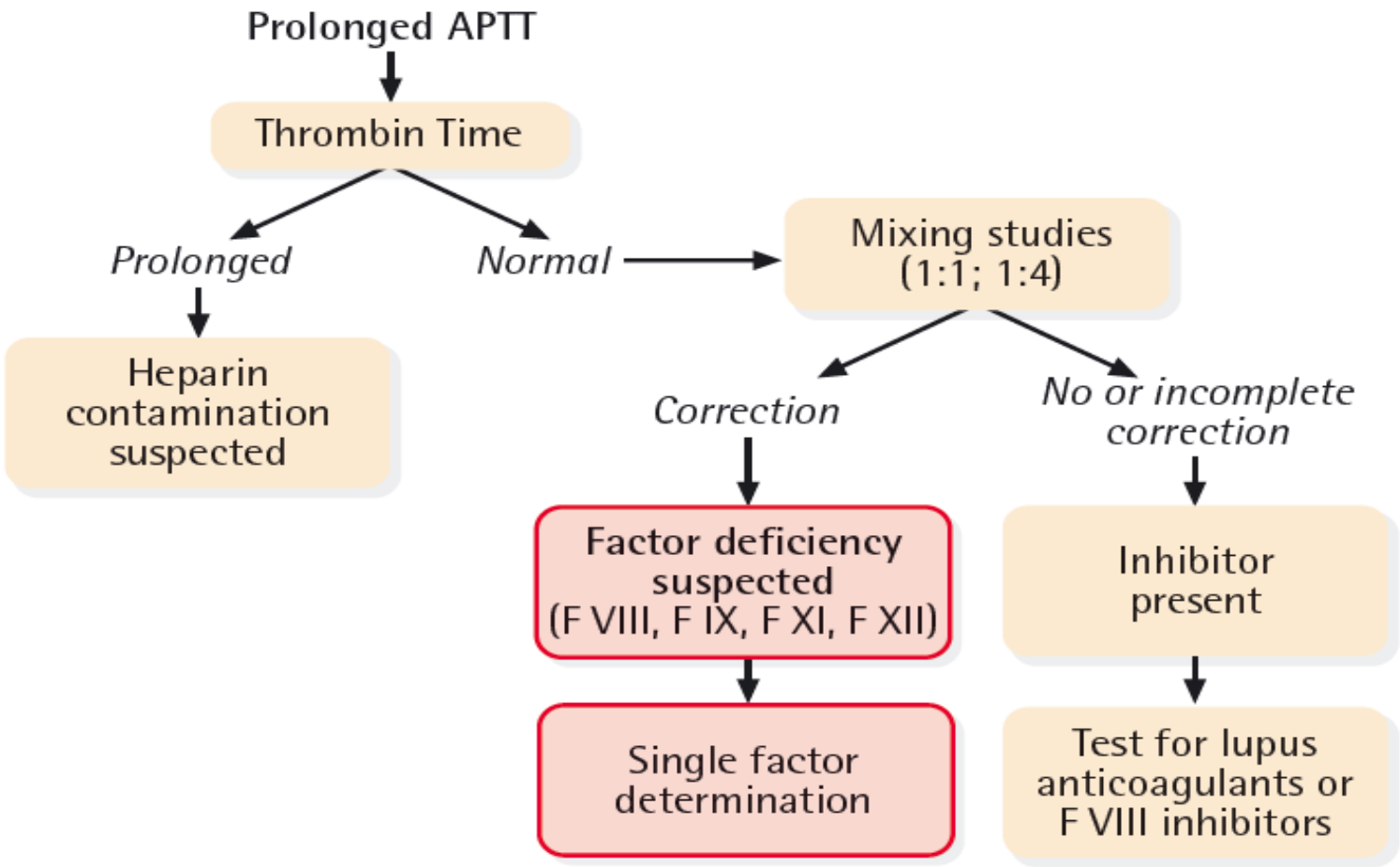
aPTT

- Reference range: 25 – 40 sec
- No standardization
- Own normal range should be established

High values:

- Some anticoagulants
- vWF disease
- Hemophilias
- Antiphospholipid antibodies
- Sepsis / DIC

Algorithm of prolonged APTT



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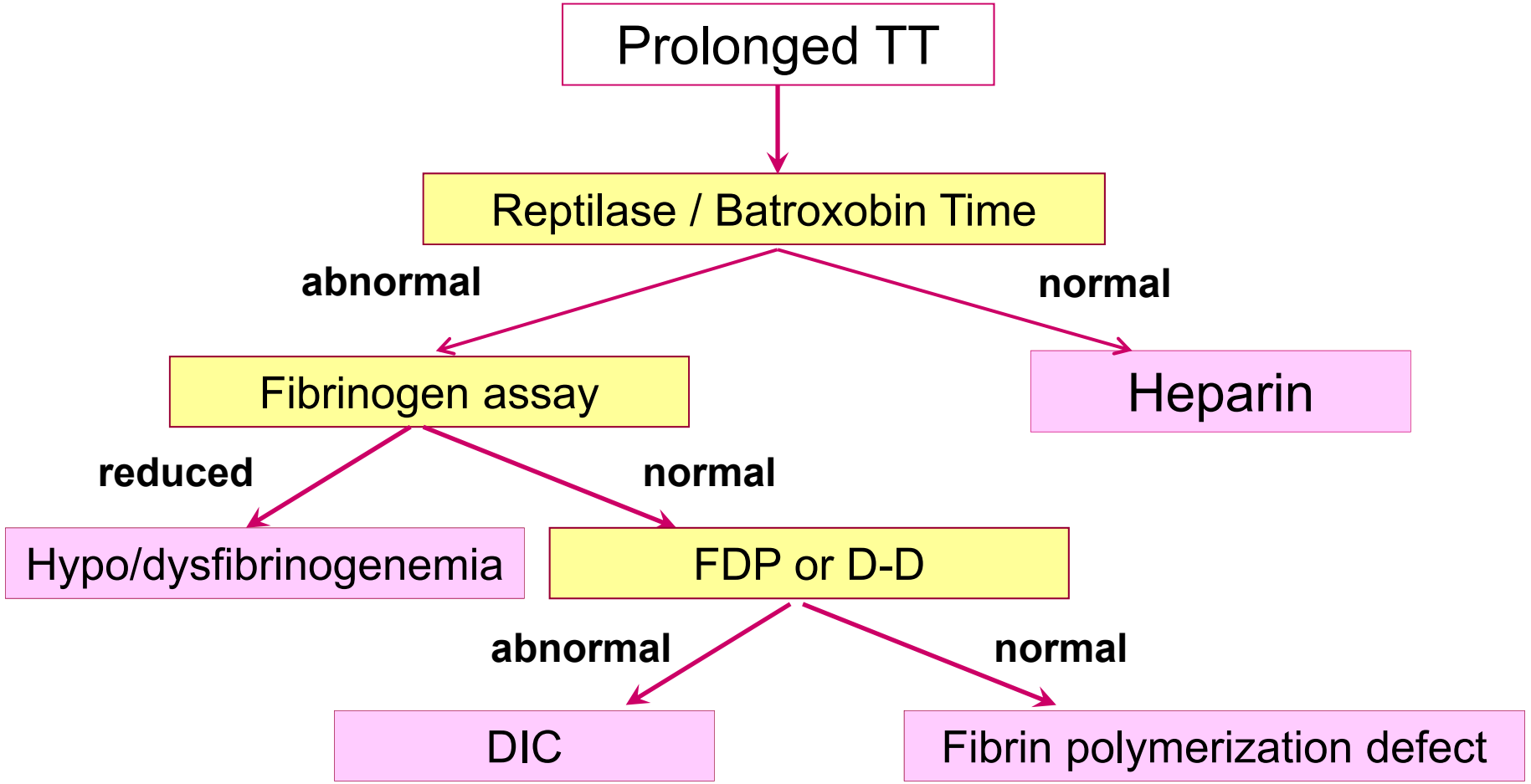
Thrombin time

- The thrombin time compares the rate of clot formation to that of a sample of normal pooled plasma.
- Bovine thrombin is added to the samples of plasma. If the time it takes for the plasma to clot is prolonged, a quantitative (fibrinogen deficiency) or qualitative (dysfunctional fibrinogen) defect is present.
- Normal values for thrombin time are 12 to 14 seconds
- Thrombin time can be prolonged by heparin, fibrin degradation products, and fibrinogen deficiency or abnormality.

Investigation of prolonged TT



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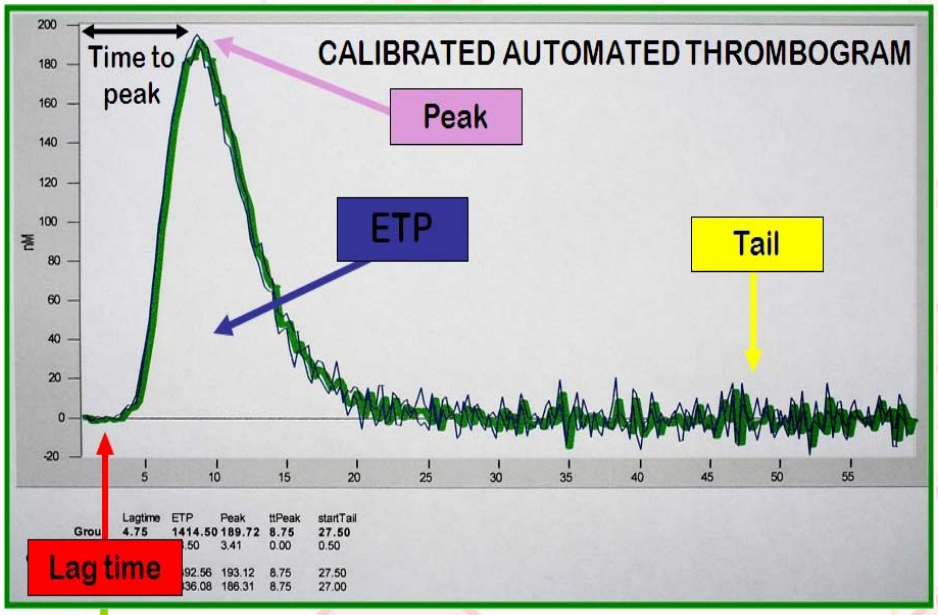
METHOD - Fluorogenic

TECHNOTHROMBIN® TGA
Thrombin Generation Assay



Thrombin generation detects the whole kinetic of Thrombin generation

- lag phase
- peaktime
- slope / velocity index

$$\text{velocity index} = \frac{\text{peak thrombin}}{\text{peaktime} - \text{lag phase}}$$

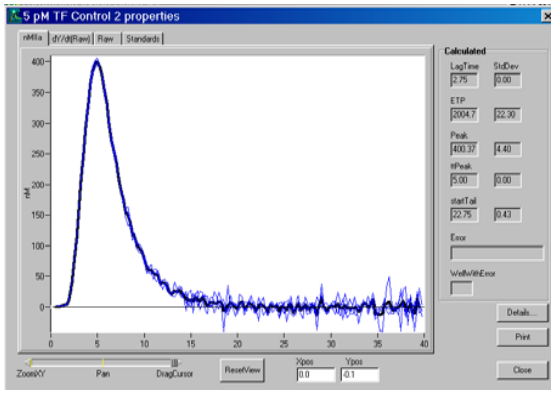
- peak thrombin
- area under the curve (AUC)

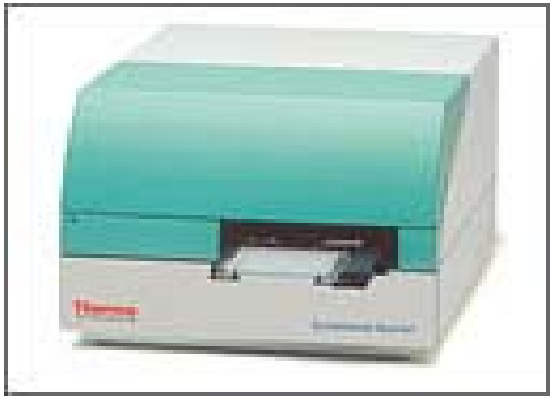
Using conventional coagulation tests only detect the initial phase of thrombin generation with endpoint “generation of first fibrin”

technoclone

CAT® System

- **Continuous** fluorescent measurement of Thrombin Generation
  - Measurement possible on **PPP** and **PRP**





Dedicated Software

Fluoroska n Ascent

+ a complete range of dedicated reagents

Fibrinogen

Liver produces

Normal range: 2-4 g/L

Absent in serum

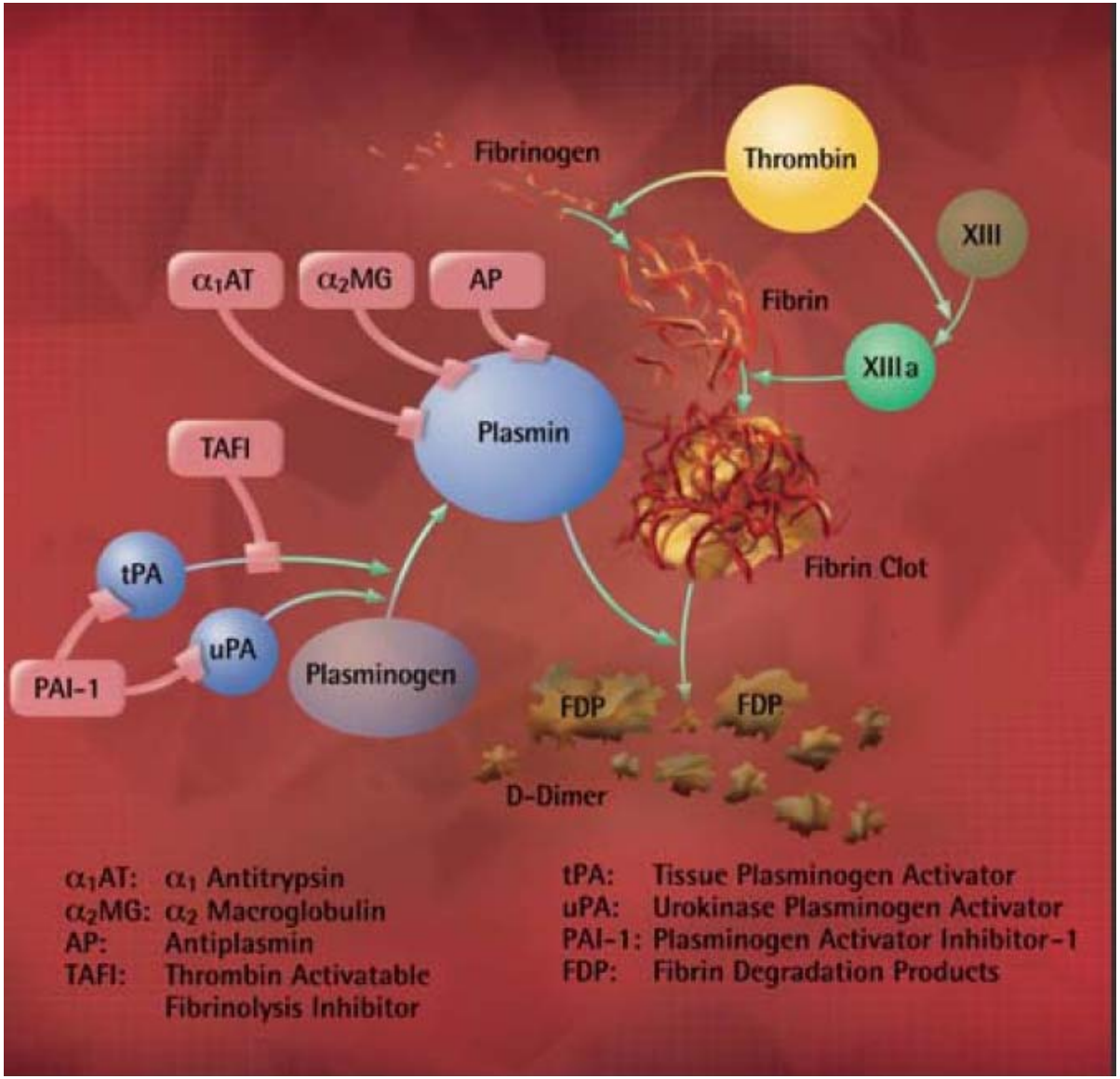
Acute phase reactant

(its diagnostic information is limited)

High levels: fever, infection

Low levels: liver failure and DIC

Fibrinolysis



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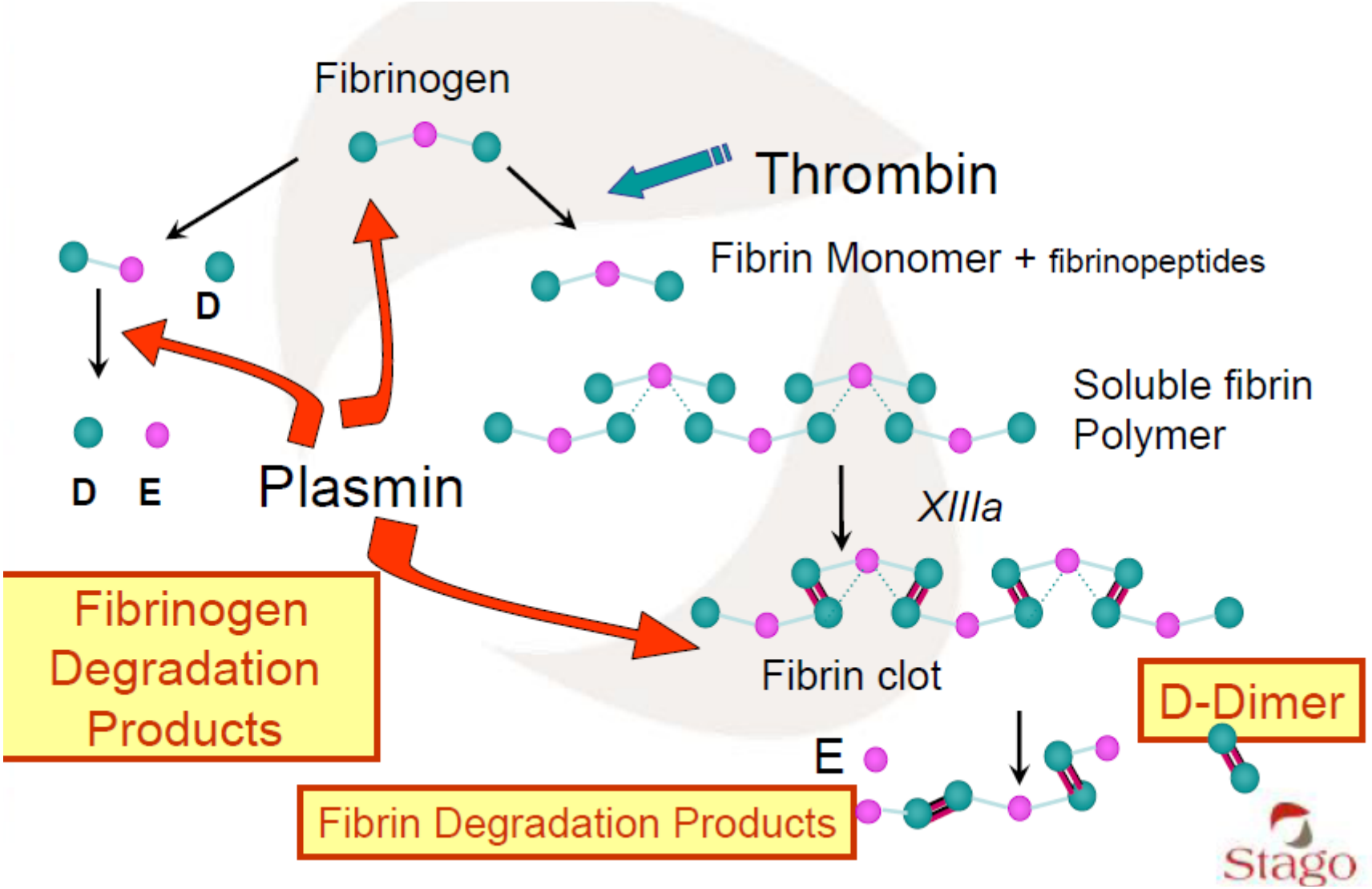
Coagulation and fibrinolysis



Main assays for fibrinolysis

- Fibrinogen Degradation Products
- D-Dimer
- Plasminogen
- Antiplasmin
- Plasminogen Activator
- Plasminogen Activator Inhibitor

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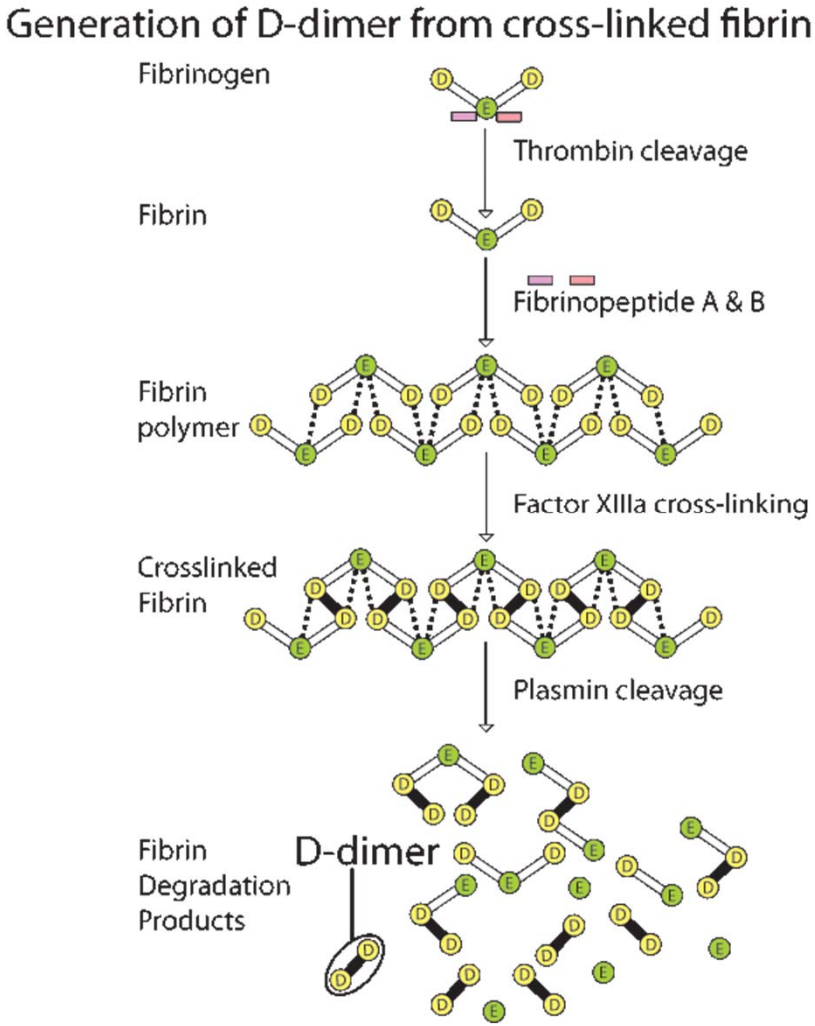
D-dimer



- Fibrin degradation product
- Ref range: <0,5 mg/L
- Intravascular coagulation

D-dimer is increased

- Venous thrombosis
- Arterial thrombosis
- DIC
- Infection / sepsis
- Surgery / trauma
- Pregnancy
- Chronic disease

D-dimer

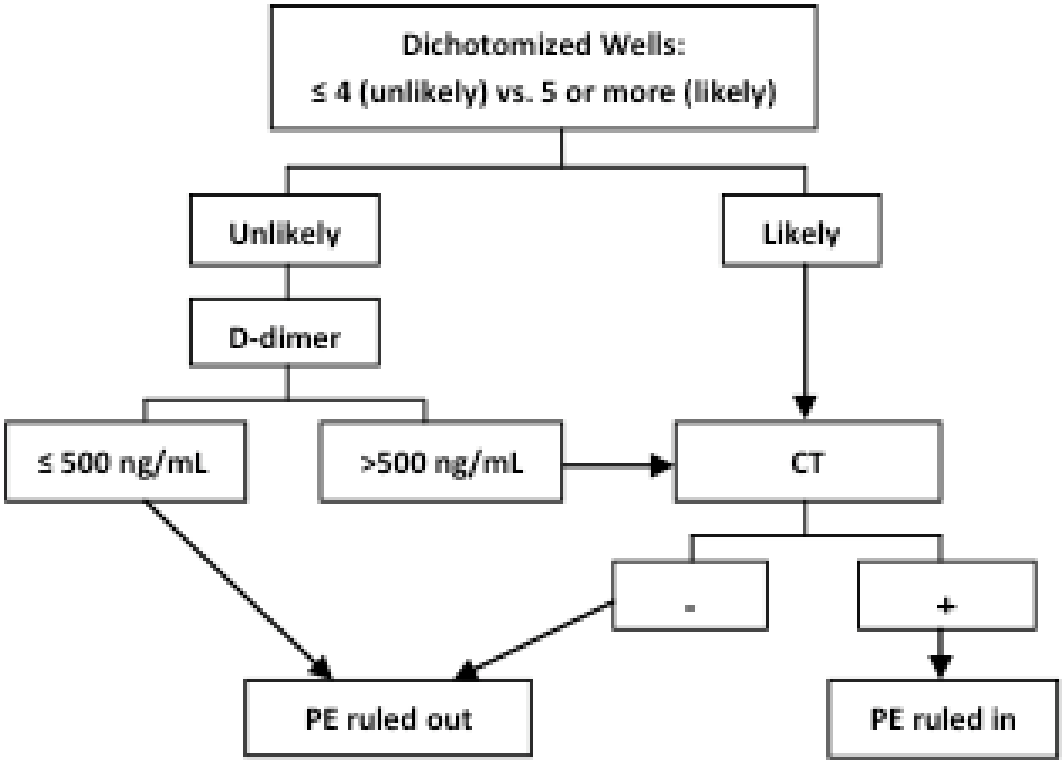
- Suitable for ruling out venous thrombosis in low-risk outpatients
- NOT appropriate for verification of PE / DVT
- Well’s criteria & D-dimer

Wells score

Criteria	Points
Clinical signs/symptoms of DVT	3
PE is most likely diagnosis	3

Wells' score	Original	Simplified
Clinical signs of DVT	3	1
Alternative diagnosis less likely than PE	3	1
Previous PE or DVT	1.5	1
Heart rate >100 bpm	1.5	1
Surgery or immobilisation within 4 weeks	1.5	1
Haemoptysis	1	1
Active cancer	1	1
Clinical probability		
PE unlikely	≤4	≤1
PE likely	>4	>1

Use of D-dimer in thrombosis assessment



Not suitable for hospitalized patients' assessment

DIC
(disseminated intravascular coagulation)

- characterized by systemic activation of blood coagulation
- generation and deposition of fibrin
- systemic microvascular thrombi; MODS
- accelerated fibrinolysis may cause severe bleeding.
- a patient with DIC can present with a simultaneously occurring thrombotic and bleeding problem

Risk conditions for DIC

- Sepsis
- Trauma (neurotrauma)
- Organ destruction
- Malignancies
- Severe transfusion reactions
- Obstetric complications
- Severe hepatic failure
- Severe toxic reactions
- Hyperthermia
- Etc.

Lab tests for DIC assessment

- Platelet count (<50 G/L)
- Global clotting times (aPTT and PT) usually increased
- Assay for D-dimer or FDPs
- One or two clotting factors and inhibitors (eg, antithrombin) – test availability depends on clinical site
- Fibrinogen – not recommended as acute phase reactant