Cliniques universitaires SAINT-LUC UCLouvain BRUXELLES

# Laboratory tests in bleeding disorders

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# Summary

Introduction

- Pre-analytical phase
- General laboratory approach to bleeding

Coagulation cascade

- Basic principles of chronometric methods
- Routine laboratory tests in hemostasis
- Fibrinogen
- Coagulation factors

Primary hemostasis

- Platelet function analyzer
- Von Willebrand factor

Clinical case to conclude

# Introduction

# A. Pre-analytical phase

The “Pre-analytical phase” describes all actions and laboratory procedures that occur prior to the analytical phase.

The pre-analytical phase is implicated in **60-70% of laboratory errors**!

Important to control this phase to provide qualitative laboratory results.

# A. Pre-analytical phase

## Three broad categories

**Sample collection**

- Correct patient identification and sample labelling
- Phlebotomy technique
- Sample volume
- Sample collection tube

**Sample handling**

- Storage
- Mixing and centrifugation
- Transport conditions
- Delays in transport

**Patient-specific factors**

- Physiological variables
- Pathologies
- Stress, exercise,…
- Medication!

# A. Pre-analytical phase

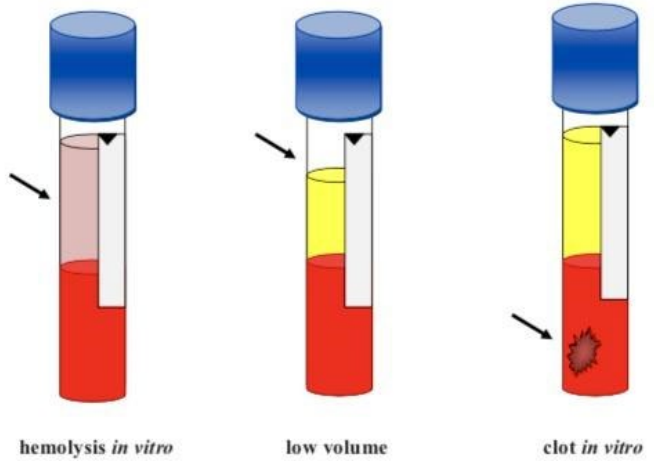
## Sample collection

- Conventional straight needle
- Butterfly device: ok if discard tube is taken
- 19-22 gauge needle
- Tourniquet <1 minute
- Citrate tube (3,2%): 1 volume of citrate for 9 volumes of blood
- Citrate tube in second position
  - ✓ After blood cultures
  - ✓ After discard tube (neutral without additif)
  - ✓ After serum tube (without clot activator)

# A. Pre-analytical phase

## Sample collection

- Adequate filling of tubes
  - underfilling: too much citrate → insufficient recalcification
  - overfilling: not enough citrate/difficult to homogenise (clot formation risk)
- Homogenization
- Transport at room temperature (exception: some fibrinolysis tests)
- Platelets are easily activated: system permitting slow manual drawing is preferred over vacuum system



# A. Pre-analytical phase

## Unacceptable samples

- Insufficiently filled tubes (or overfilled)
- Pre-analytical conditions not respected (anticoagulant, delay)
- Sampling on same side as heparin infusion
- Grossly hemolyzed, lipemic samples

# B. General approach to laboratory testing

Not possible to screen the entirety of hemostasis with a single test.

No single test can reliably exclude/confirm presence of bleeding disorder.

Typical evaluation:

- CBC (including platelet count and platelet morphology)
- PT, APTT, (TT)
- Fibrinogen activity, (D-dimer)
- Mucocutaneous bleeding: PFA-100/200, von Willebrand factor

Additional testing depending on results…

Repeat testing is appropriate given that results can be impacted by pre-analytical variables (especially when results are unexpected)

# B. General approach to laboratory testing

## Available tests for investigation of bleeding disorders

### Coagulation cascade

- PT, APTT, TT, fibrinogen
- Specific clotting factor assays
  - Typically performed when one or more of screening tests are abnormal
  - Mostly clot-based but chromogenic FVIII sometimes appropriate

### Primary hemostasis

- VWF antigen, VWF activity, (with multimer testing/RIPA when abnormalities found)
- PFA-100/200; platelet aggregation studies (light transmission/impedance aggregometry)

### Fibrinolysis

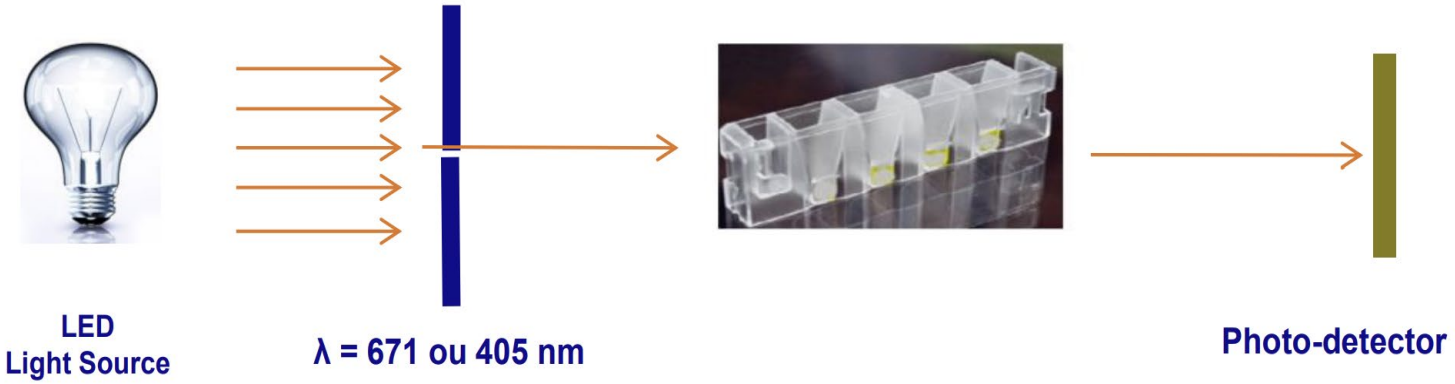
- FXIII activity and antigen assays (FXIII deficiency is very rare!)
- D-dimer, euglobulin lysis time

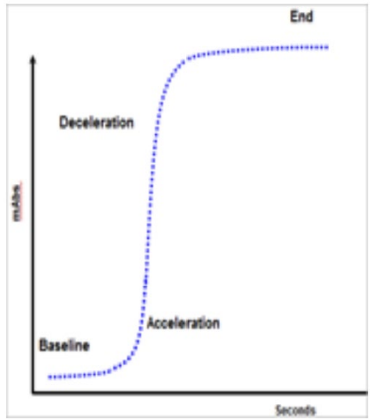
# Coagulation cascade

# A. Basic principles of chronometric methods

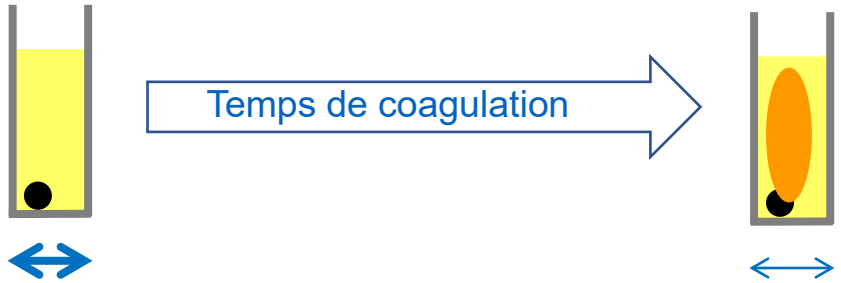
The principal of chronometric clot detection is used to measure and register the time needed for a plasma sample to clot: **tranformation of fibrinogen in fibrin.**

✓ optical: change in optical density




✓ mechanical: change in movement of magnetic bead



# A. Basic principles of chronometric methods

## Laboratory hemostasis is automated

Optical and mechanical principles

Different techniques used:

- **Chronometric**
- Chromogenic
- Immunological

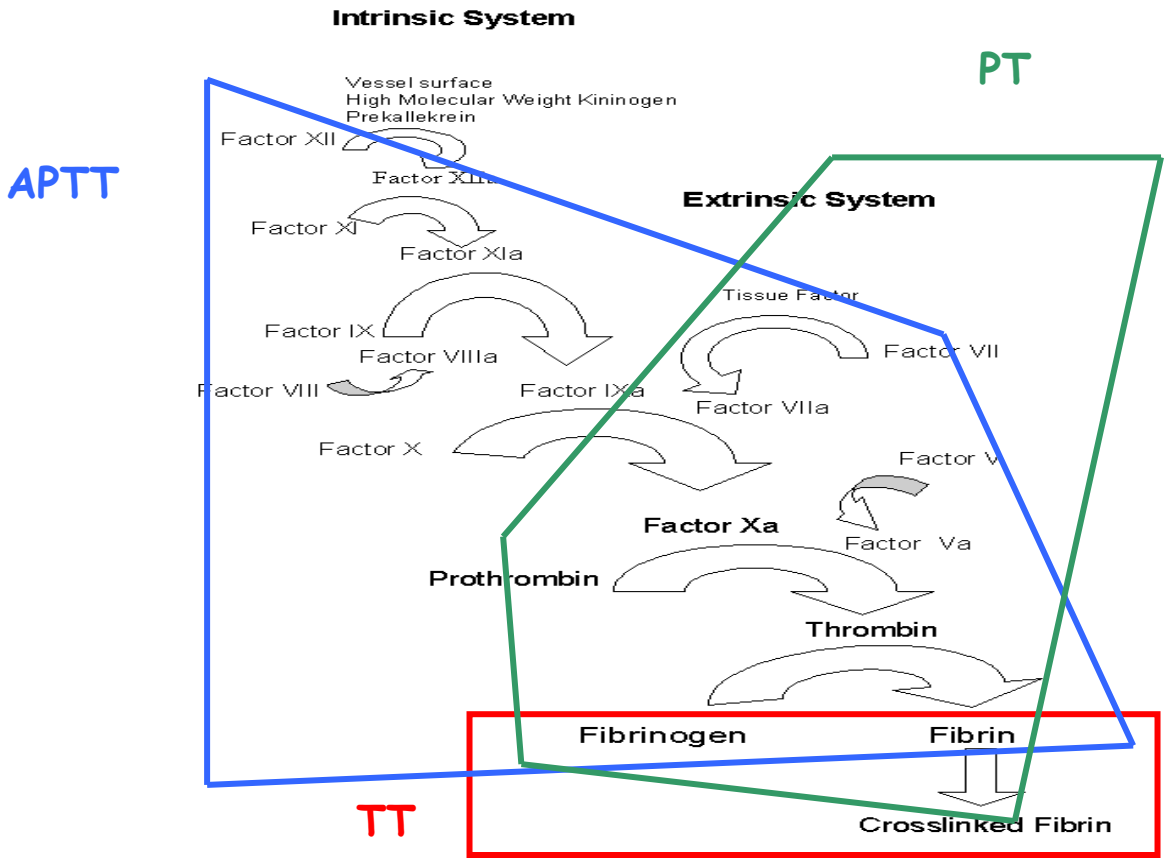
# B. Routine laboratory hemostasis

## Routine/basic coagulation tests

a) Prothrombine Time (PT)

b) Activated partial thromboplastin time (APTT)

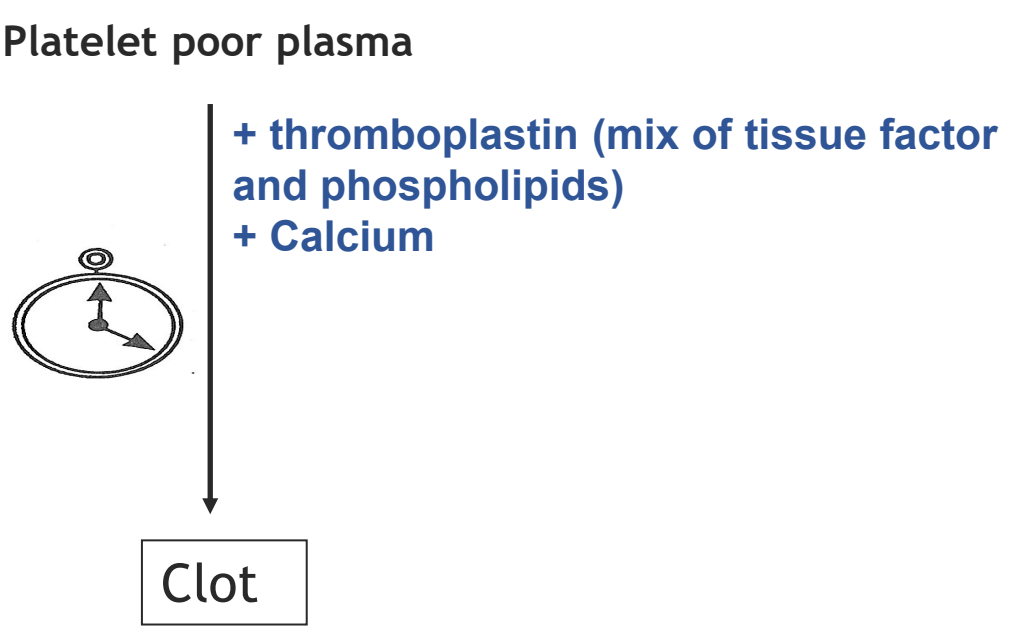
c) Thrombin time (TT)



# B. Routine laboratory hemostasis

## 1. Prothrombin time (PT)

- Monitors coagulation in extrinsic and common coagulation pathways.
- Application: factor deficiencies, monitoring of vitamin K antagonists,  liver disease,…
- Type of assay: Tissue factor and phospholipids are added to plasma and the clotting time is recorded.



# B. Routine laboratory hemostasis

## 1. Prothrombin time (PT)

The INR is a calculation generated from the PT and mean normal prothrombin time (MNPT).

$$\text{INR} = \left( \frac{\text{Patient PT in seconds}}{\text{MNPT in seconds}} \right)^{\text{ISI}}$$

INR = International Normalized Ratio

ISI = International Sensitivity Index

MNPT* = Mean Normal Prothrombin Time

* Mean PT of healthy volunteers (not on anticoagulants obtained using the same reagent as for the patient

# B. Routine laboratory hemostasis

## 1. Prothrombin time (PT)

International normalized ratio (INR) = PT ratio using a reference thromboplastin

Application: monitoring of vitamin K antagonists (VKA)

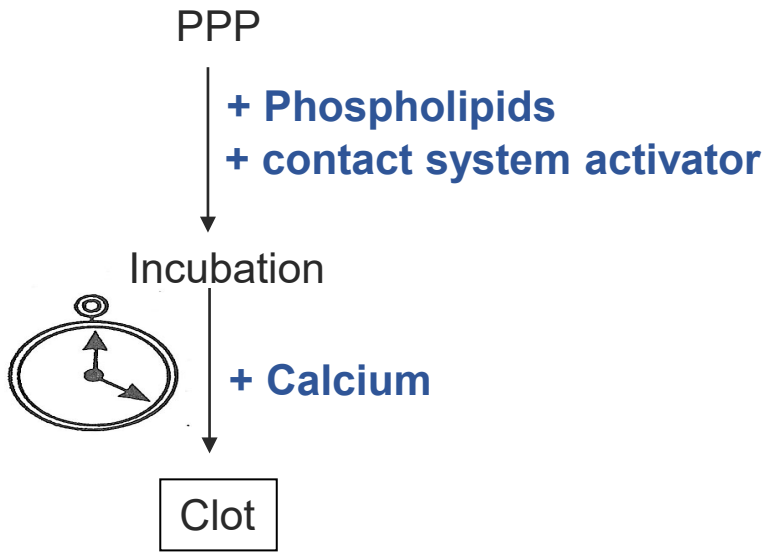
| Thromboplastine | PT patient (sec) | Mean normal PT (sec) | PT ratio | ISI | INR |
|---|---|---|---|---|---|
| A | 19 | 12 | 1.3 | 3.2 | 2.6 |
| B | 18 | 12 | 1.5 | 2.4 | 2.6 |
| C | 21 | 13 | 1.6 | 2.0 | 2.6 |
| D | 24 | 11 | 2.2 | 1.2 | 2.6 |
| E | 38 | 14.5 | 2.6 | 1.0 | 2.6 |

Enables comparing results between different laboratories and a standardized follow-up of a patients treated with VKA.

# B. Routine laboratory hemostasis

## 2. Activated partial thromboplastin time (APTT)

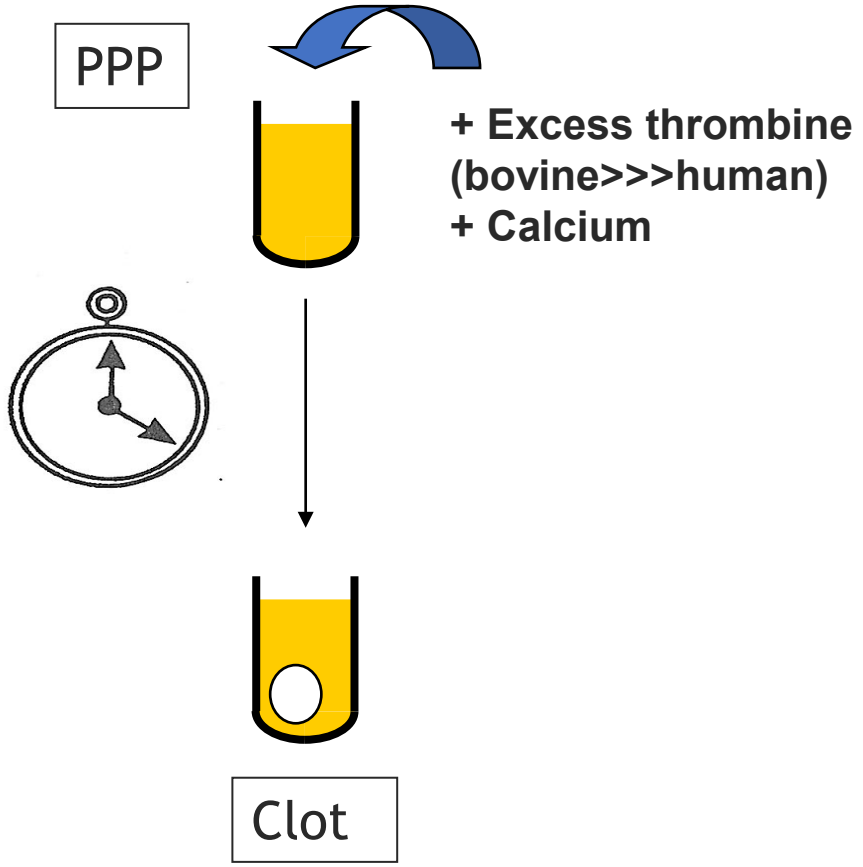
- Monitors coagulation in intrinsic and common coagulation pathways.
- Type of assay: Activators (such as micronized silica, ellagic acid, kaolin) plus phospholipids and calcium are added to plasma and the clotting time is recorded. Incubation phase is needed (activation of the contact phase is very slow)
- Choice of reagent: sensitive to factor deficiencies; weak/modest sensitivity to lupus anticoagulans



# B. Routine laboratory hemostasis

## 3. Thrombin time (TT)

- Monitors the formation of fibrin.
- Application: it is a simple and rapid test, which can be used for evaluating the presence of UFH and dabigatran in patient plasma due to its high sensitivity. The TT is also sensitive to deficiencies and defects of fibrinogen.
- Fibrinogen/fibrin degradation products (including D-dimers) can also prolong TT.
- Excess thrombins and calcium are added to plasma and the clotting time recorded



# B. Routine laboratory hemostasis

## 4) Interpretation

### Prolonged PT with normal APTT

Factor VII deficiency

- Congenital = rare; acquired inhibitor = very rare
- Acquired = Start of VKA treatment, vitamin K deficiency, liver disease, … (short half-life)

A lot of causes of prolonged PT

# B. Routine laboratory hemostasis

## 4) Interpretation

### Prolonged PT and prolonged APTT

- Congenital factor deficiency or acquired inhibitors in common pathway: X, V, II, I (fibrinogen)
- Acquired FX deficiency (amyloidosis)
- Liver disease
- Vitamin K deficiency
- DIC
- Anticoagulant treatment: VKA, some DOAC, Curative doses of heparin

# B. Routine laboratory hemostasis

## 4) Interpretation

### Normal PT and prolonged APTT

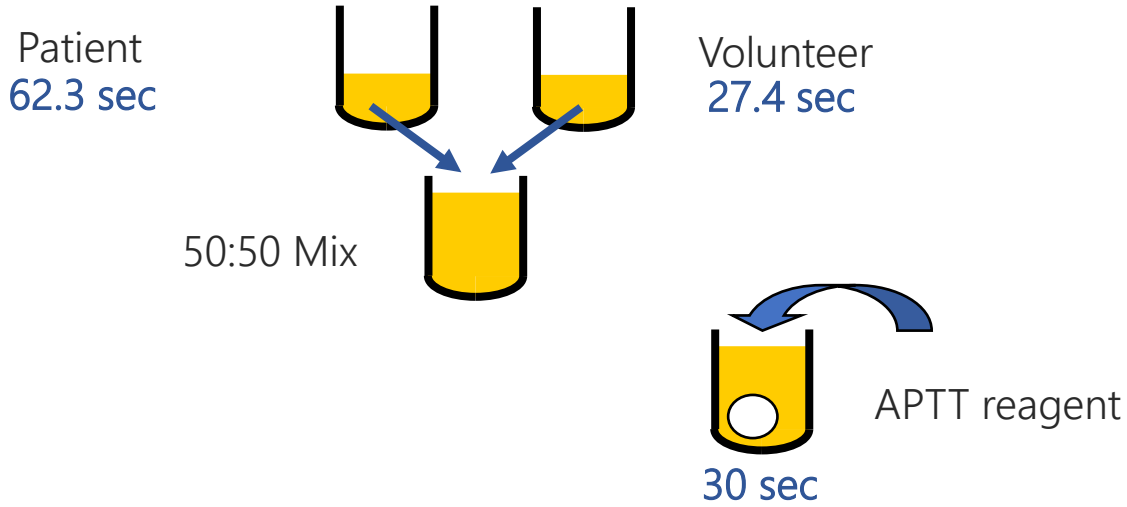
- von Willebrand disease (if concurrent low FVIII)
- Congenital factor deficiency: VIII, IX, XI, XII and contact factors
- Acquired factor deficiency (eg. FVIII)
- Heparin treatment, DOAC,…
- Lupus anticoagulant

# B. Routine laboratory hemostasis

## 4) Interpretation

### Isolated prolonged APTT

Mixing study

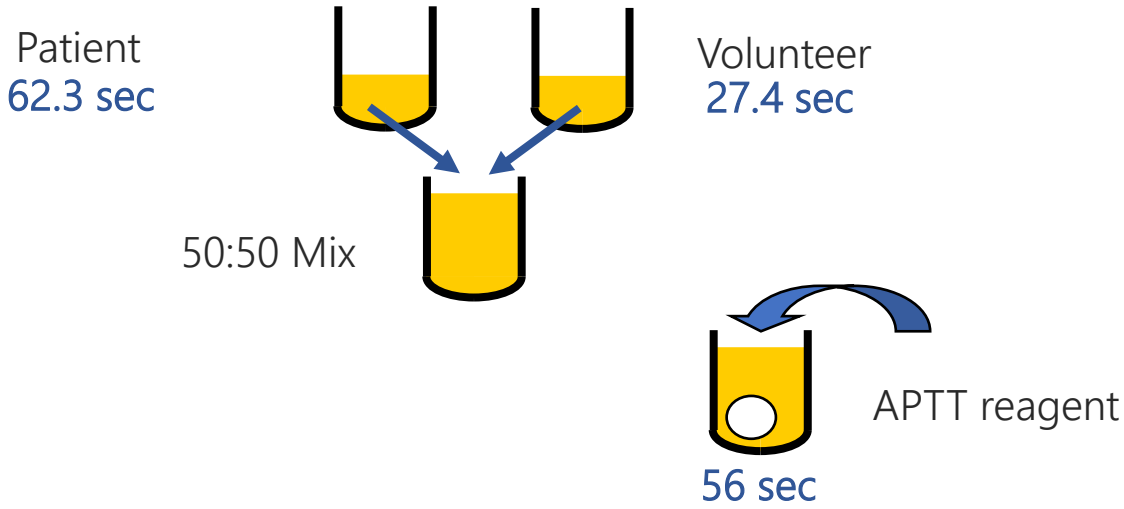


# B. Routine laboratory hemostasis

## 4) Interpretation

### Isolated prolonged APTT

Mixing study



# C. Fibrinogen testing

Type of assay

- Quantitatif assay: fibrinogen antigen

- Qualitatif assay: Clauss fibrinogen, (PT-derived fibrinogen)

Application:

- Acquired hypofibrinogenemia (DIC, liver disease, medication,…)
- Acquired dysfibrinogenemia (liver disease, malignancies,…)
- Congenital hypo/dysfibrinogenemia

# D. Coagulation factors

## Technical aspects

### One stage APTT-based assay

Widely used to measure factor levels for the purpose of detecting a deficiency (patients with history of bleeding or prolonged APTT), or to monitor replacement therapy (e.g. hemophilia).

"Intrinsic" coagulation pathway factors (VIII, IX, XI, XII) are all measured in test systems based on the correction of the APTT.

Factor activity is assessed by measuring the ability of an unknown sample to correct the prolonged APTT of factor-deficient plasma (deficient in the factor of interest), relative to the effect of a known calibrator.

Important to understand the impact of method variations, limitations, and result interpretation.

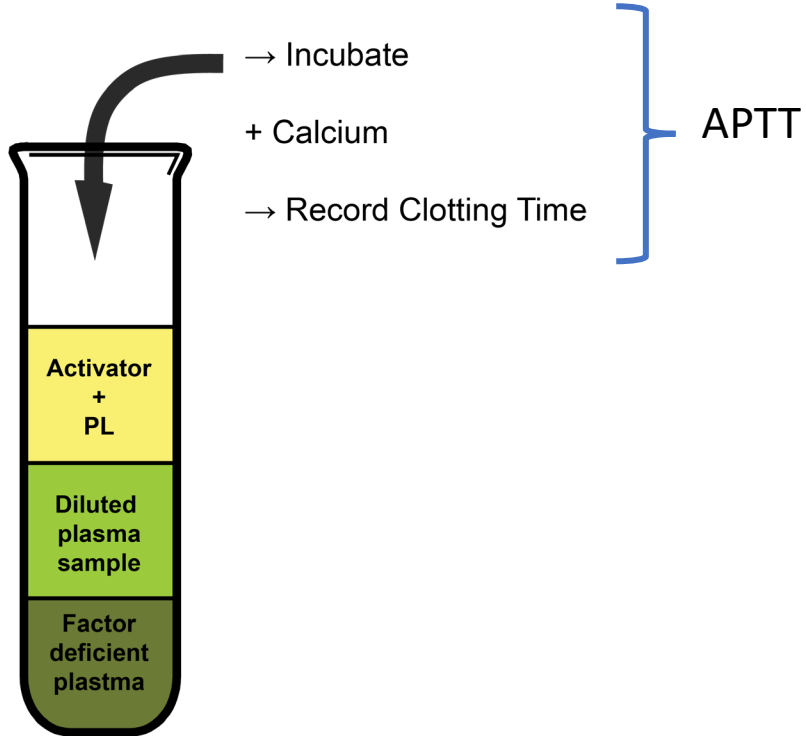
- Duncan et al. Methods in Molecular Biology 2017.

# D. Coagulation factors

## Technical aspects

### One stage APTT-based assay (example FVIII)

- Sample dilution 1/10
- 1:1 mix diluted patient sample/factor VIII deficient plasma
- APPT reagents (phospholipids (PL) + contact phase activator)
- Record time and interpret on calibration curve



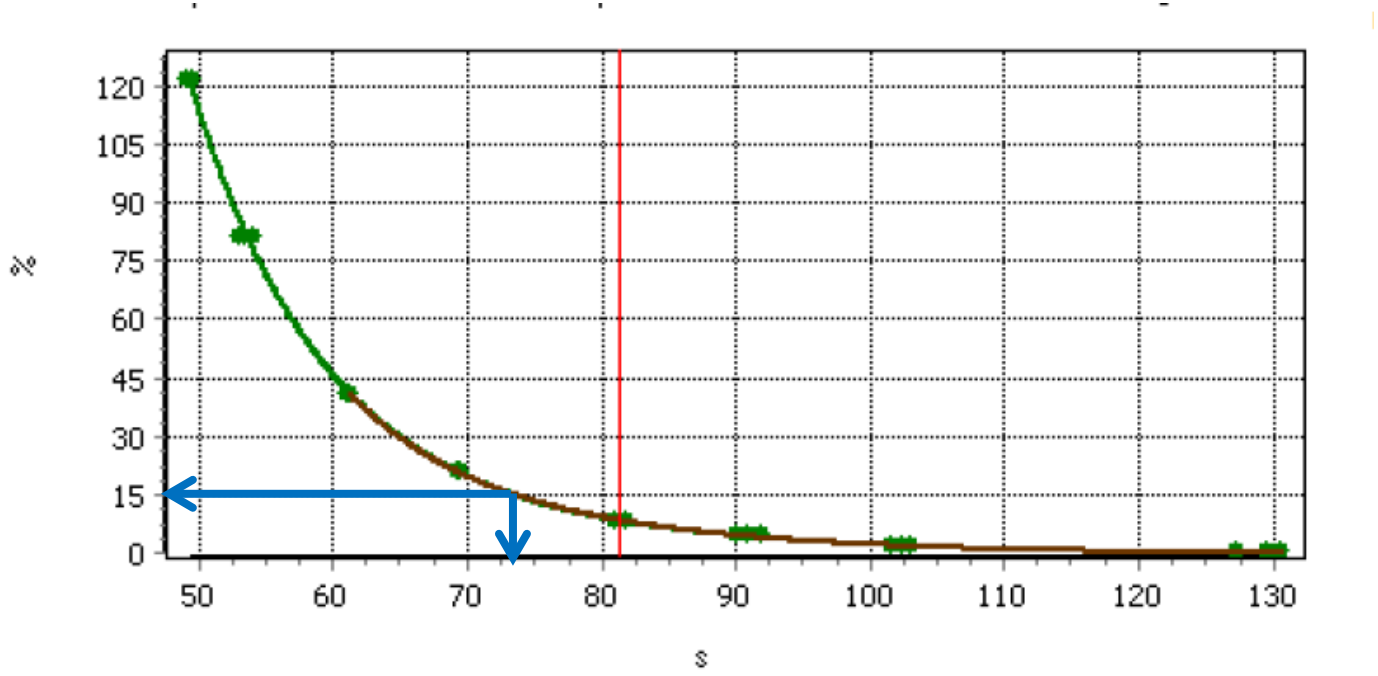

- Duncan et al. Methods in Molecular Biology 2017.

# D. Coagulation factors

## Technical aspects

### One stage APTT-based assay (example FVIII)

- Record time and interpret on calibration curve (traceable to WHO standard)




- Duncan et al. Methods in Molecular Biology 2017.

# D. Coagulation factors

## Technical aspects

### Chromogenic FVIII assay

- Two stage assay with cleavage of a chromogenic substrate
- Human or bovine reagents
- Diagnosis mild hemophilia A (discrepancy between one-stage and chromogenic assay)
- Follow-up treatment in hemophilia A

# D. Coagulation factors

## Interpretation

### Intrinsic factors (FVIII, FIX, XI, XII)

Deficiency in all factors

- inhibitor (LAC?)
- repeat measurement after dilution of plasma

Isolated deficiency

- congenital : rare !
- acquired (auto-immune disease): very rare!! (testing for coagulation inhibitors, usually FVIII)
- FVIII deficiency in context of von Willebrand disease

**Deficiency in factor XII : no bleeding risk**

# D. Coagulation factors

## Technical aspects

### One-stage PT based assay

"Extrinsic/common" coagulation pathway factors (II, V, VII, X) are all measured in test systems based on the correction of the PT.

Factor activity is assessed by measuring the ability of an unknown/patient sample to correct the prolonged PT of factor-deficient plasma (deficient in the factor of interest), relative to the effect of a known calibrator.

Important to understand the impact of method variations, limitations, and result interpretation.

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# D. Coagulation factors

## Interpretation

### Extrinsic/common pathway factors

Deficiency in all factors

- DIC?
- liver disease? (normal to high FVIII)

Deficiency in factors II, VII, X (and IX)

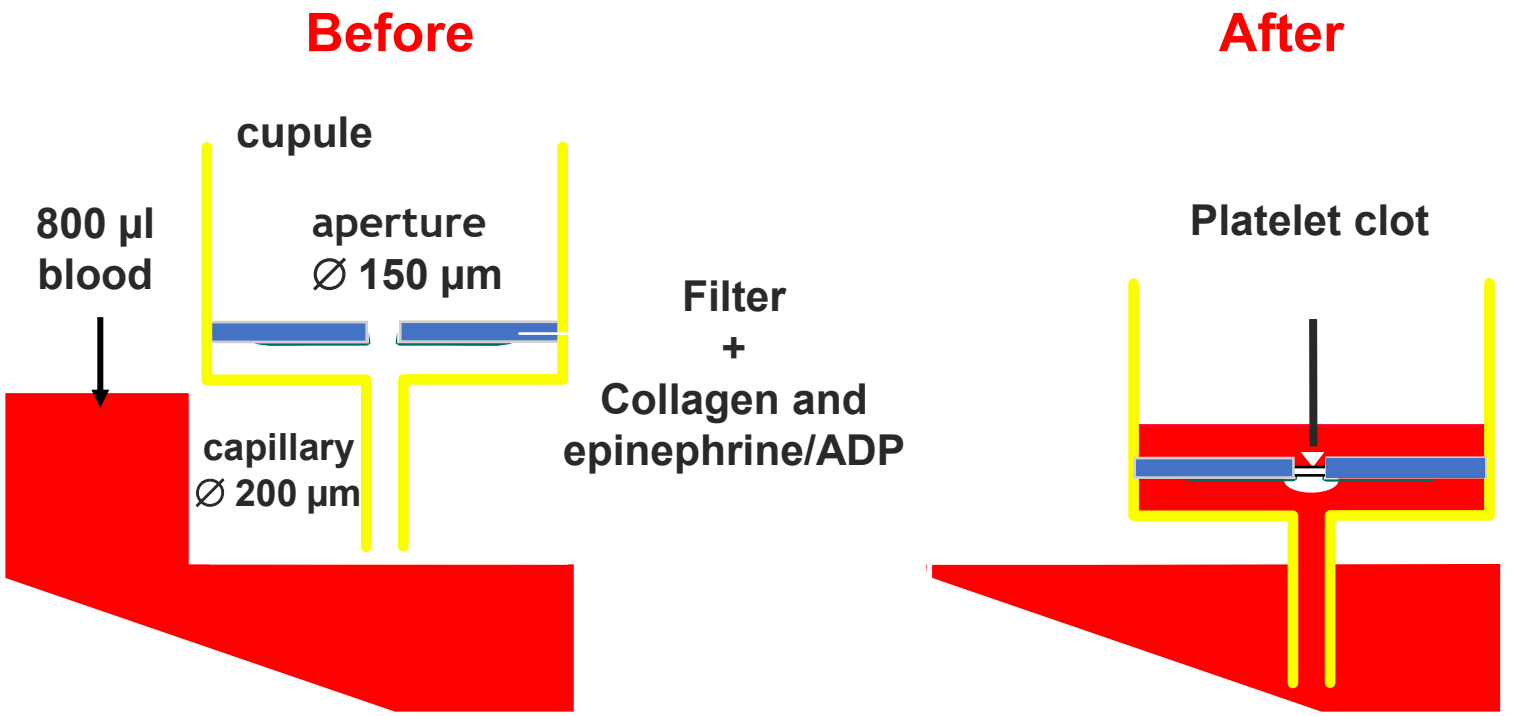
- vitamin K deficiency? treatment with VKA? malabsorption?

Isolated deficiency

- congenital: rare!
- acquired: rare (FX deficiency in amyloidosis)

# Primary hemostasis

# A. Platelet function analyzer (PFA)




Highly dependent on hematocrit, platelet count and von Willebrand factor

No interpretation when:

- Hct <25%
- Platelet count <100.000/µL

Pathological when prolonged: severe platelet dysfunction (Glanzmann, Bernard-Soulier), von Willebrand disease and anti-platelet treatment

# A. Platelet function analyzer (PFA)

Two types of cartridges: collagen +

- Epinephrine (von Willebrand disease, platelet disorder, aspirin)
- ADP (von Willebrand disease, platelet disorder)

| PFA : epinephrine | PFA : ADP | Think of… |
|---|---|---|
| ↗ | Normal | Aspirin<br>Platelet disorder |
| ↗ | ↗ | vW disease<br>Platelet disorder |

# B. von Willebrand factor

Quantitatif (antigen) :

Automated immunological assays

Qualitative (activity) :

Ristocetin cofactor activity
→ platelet affinity

Collagen binding assay
→ collagen affinity

→ If abnormalities: add RIPA, multimer analysis

Results

- Reference : 50 – 150 % (variable in function of blood group)
- Activity/antigen ratio= +/- 1
- If ratio < 0.7 : Qualitatif problem

# Clinical case

# Clinical case

28 y old female

Iron deficiency anemia

Nose bleeds, heavy menstrual period

Familiy history: sister, mother, maternal aunt: heavy periods

# Clinical case

GP suspects a bleeding disorder.

Results:

- Hypochromic, microcytic anemia
- Normal platelet count and WBC
- PT = normal
- APTT = prolonged
- TT = normal
- Fibrinogen = normal

# Clinical case

GP orders the following tests:

FII

FV

FVII

FVIII

FIX

FX

FXI

FXII

The technologist comes to see you:

- there is not enough plasma
- are all the tests necessary?
- how do we proceed?

# Clinical case

You suspect a hereditary bleeding disorder

Only the APTT is elevated

Normal PT → extrinsic and common pathways

Prolonged APTT → deficienct factor most likely in intrinsic pathway

# Clinical case

Cancel:

- ~~FXII~~
- ~~FVIII~~
- ~~FIX~~

FVIII

FIX

- ~~FXI~~

FXI

- ~~FXII~~

FXII → no bleeding

FVIII and FIX deficiencies are X-linked: unlikely but not impossible

FXI deficiency is very rare

# Clinical case

Medical history

Recurrent nose bleeds

Heavy menstruation

Familiy history: sister, mother, maternal aunt: heavy periods

→ Mucocutaneous bleeding

→ Autosomal dominant inheritance

→ Primary hemostasis: platelet disorder or von Willebrand disease

# Clinical case

Laboratory testing changes to

- Von Willebrand antigen and activity
- FVIII

Why is the APTT prologned?

Von Willebrand factor is a carrier for FVIII

# Thank you very much for your attention!

Questions?

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# Extra slides

# A. Pre-analytical phase

## Sample handling

### Centrifugation

- Platelet poor plasma (PPP)
- 10 to 15 minutes
- 1500 to 2500 g
- T° between 15 and 20°C
- Double centrifugation
- Sometimes centrifugation 4°C (fibrinolysis)

### Storage of plasma

- Freezing within 4h after sampling
- Limited storage at -20°C
- Storage of PPP at -30°C when analysis within 15 days
- Storage at -80°C

### Unacceptable samples

- Insufficiently filled tubes (or overfilled)
- Pre-analytical conditions not respected (anticoagulant, delay)
- Sampling on same side as heparin infusion
- Grossly hemolyzed, lipemic samples