BASICS OF COAGULATION METHOD

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Basics of Coagulation

- Hemostasis:-Hemostasis is the body's natural reaction to an injury that stops bleeding and repairs the damage. It culminates in formation of blood cot.

- The coagulation pathway is a cascade of events that leads to hemostasis. Mainly three components involved:

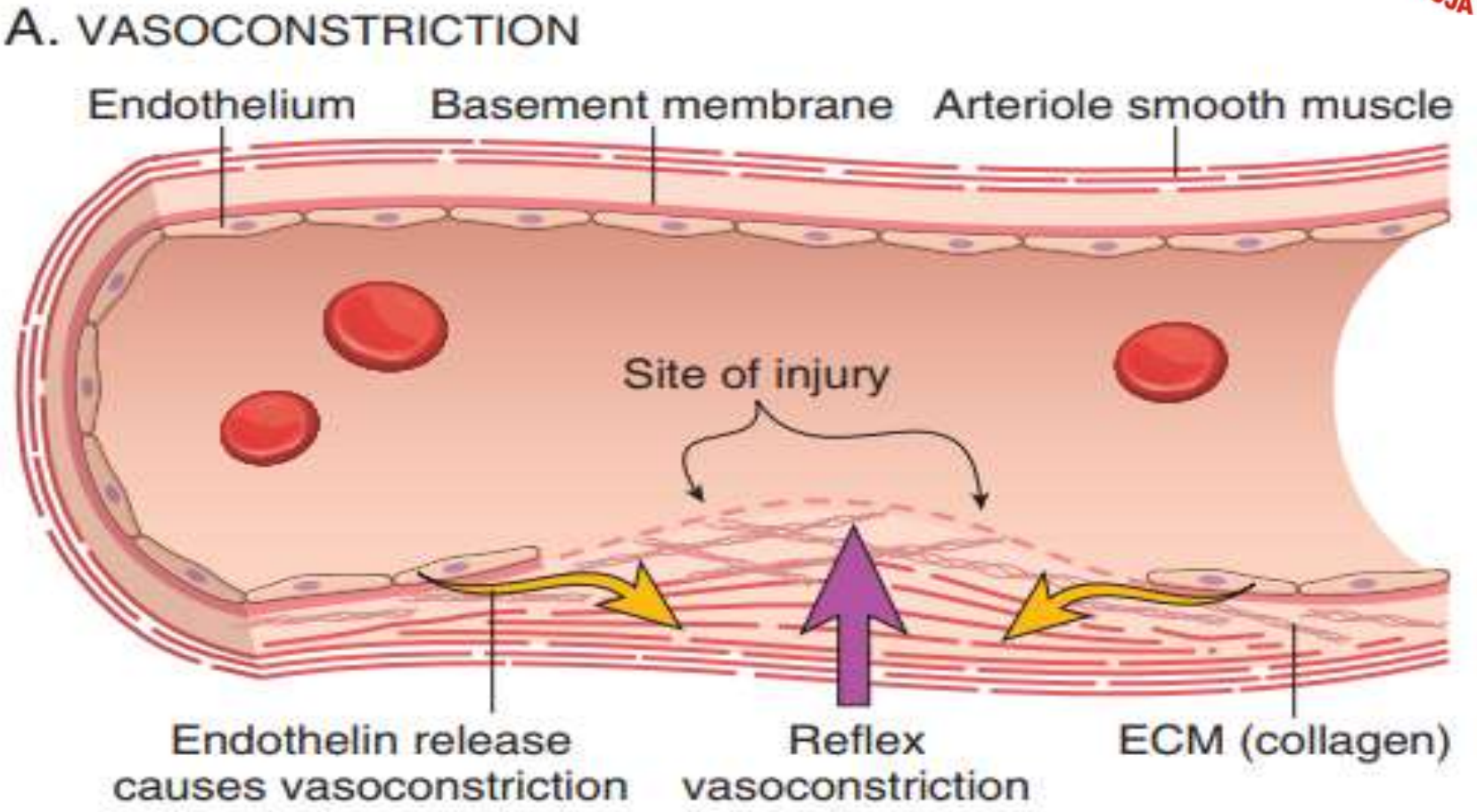
1) Platelets,

2) Vascular endothelium and

3) Coagulation factors

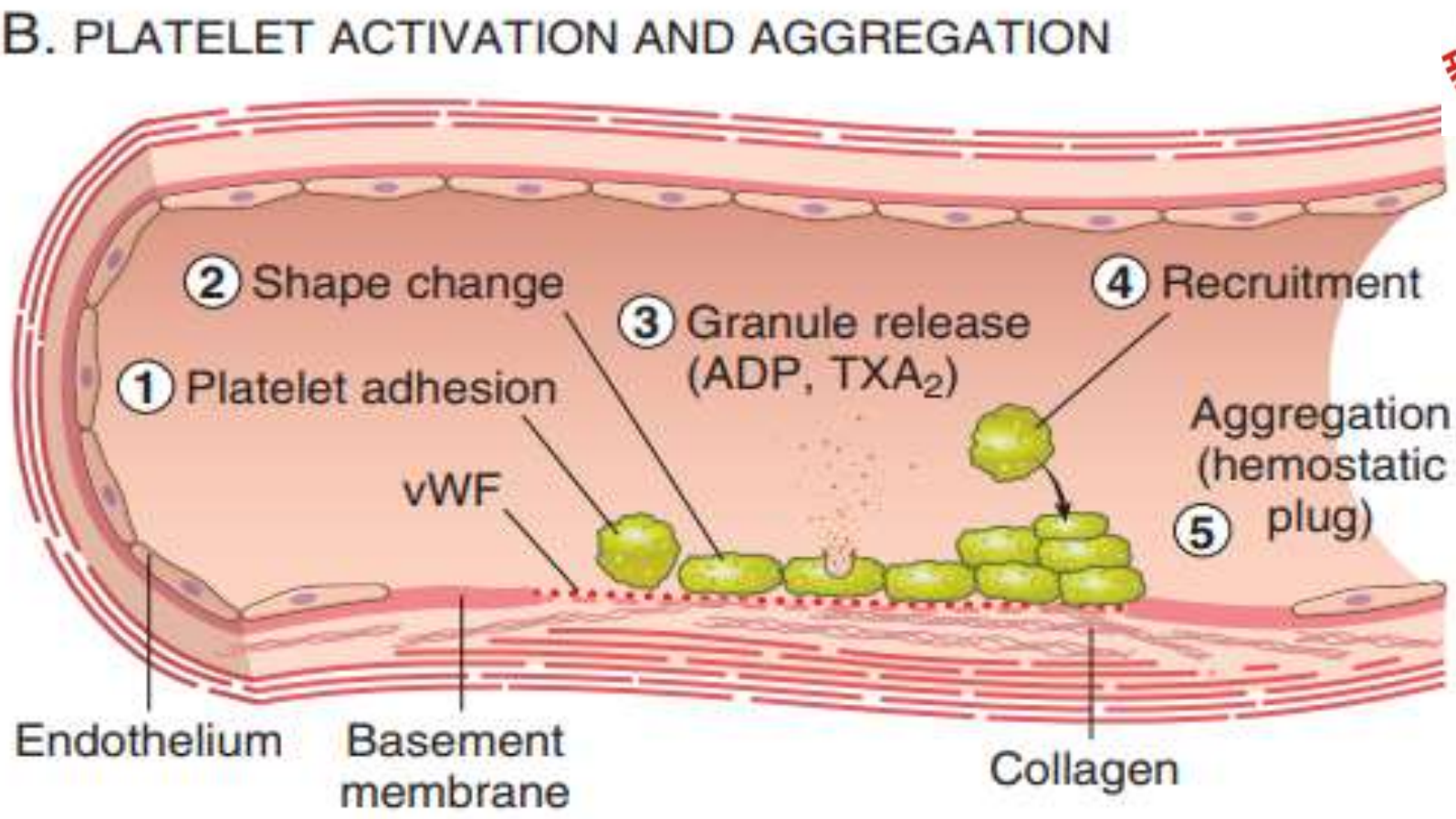
- The mechanism of hemostasis can divide into four stages:

1) Constriction of the blood vessel.
2) Formation of a temporary “platelet plug.“
3) Activation of the coagulation cascade.
4) Formation of “fibrin plug” or the final clot.

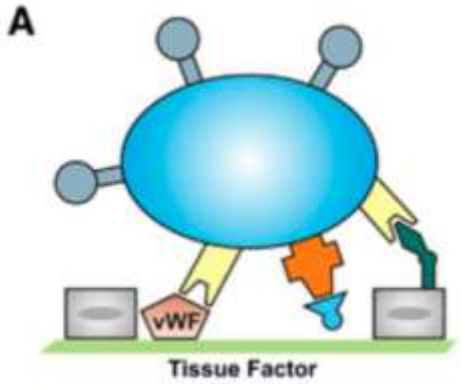


Vascular spasm ⟶ Vasoconstriction ⟶ Stop the blood flow

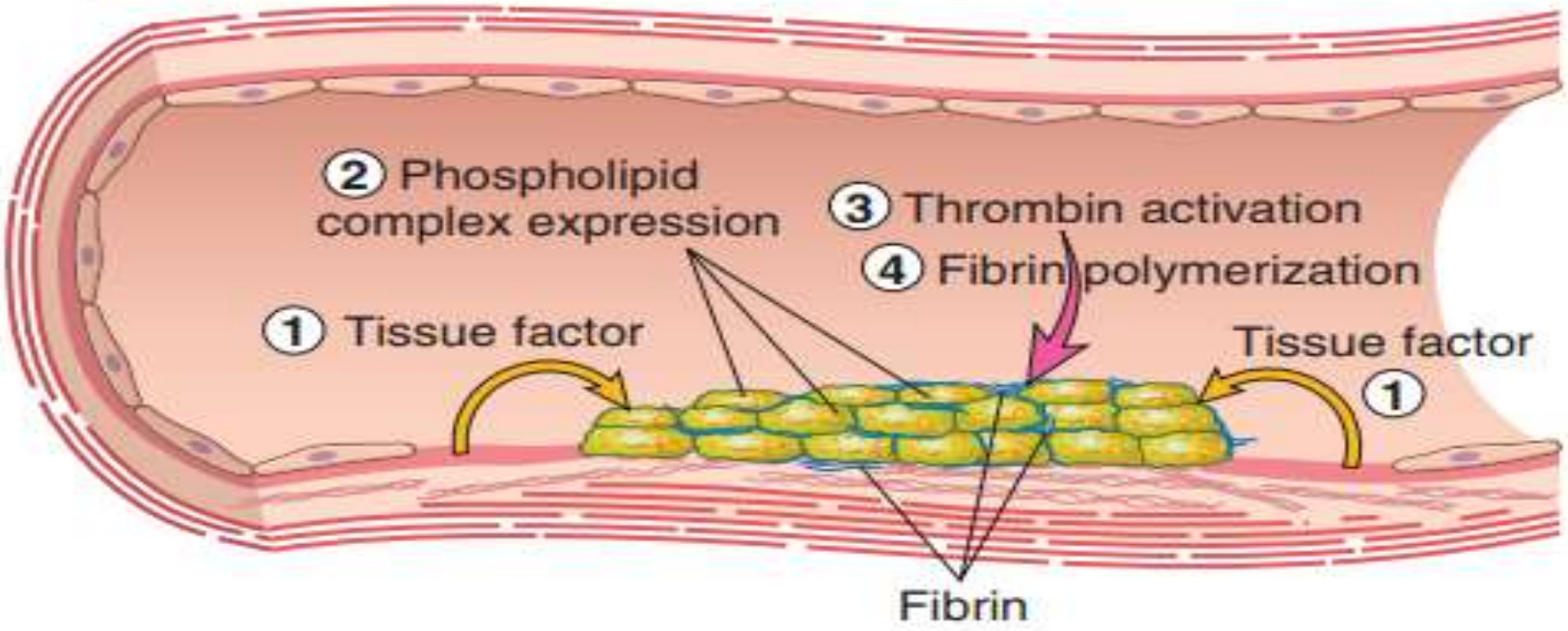
This reaction can be responded within 30 minutes, and is localized to the injured area.



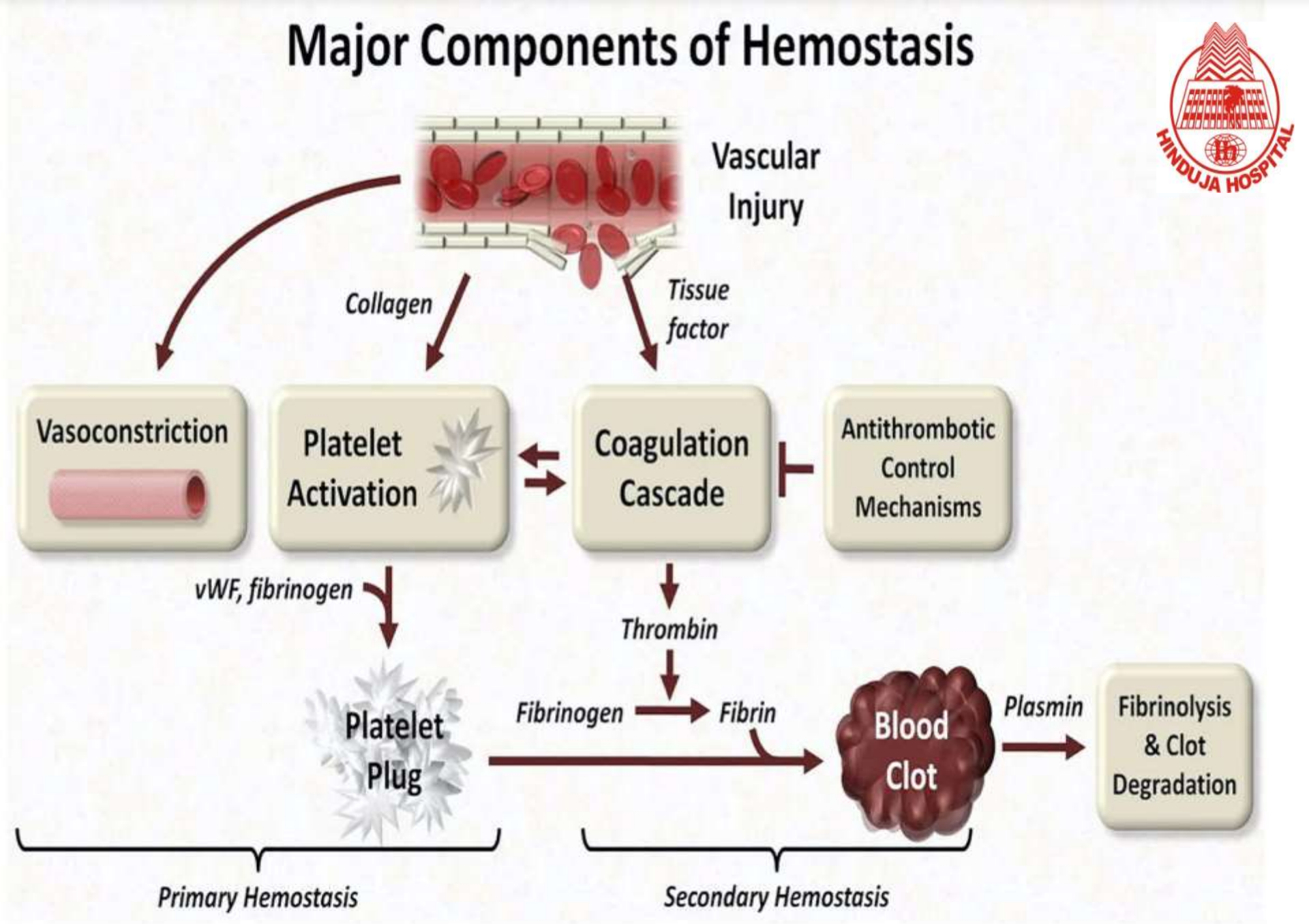
Platelet **adhesion** is mediated by von Willebrand Factor (vWF) that binds to Gp Ib-IX in the platelet membrane.



C. ACTIVATION OF CLOTTING FACTORS AND FORMATION OF FIBRIN



Major Components of Hemostasis



Blood clotting mechanism

- Vascular Spasm
- Platelet Plug Formation
- Coagulation factors activation

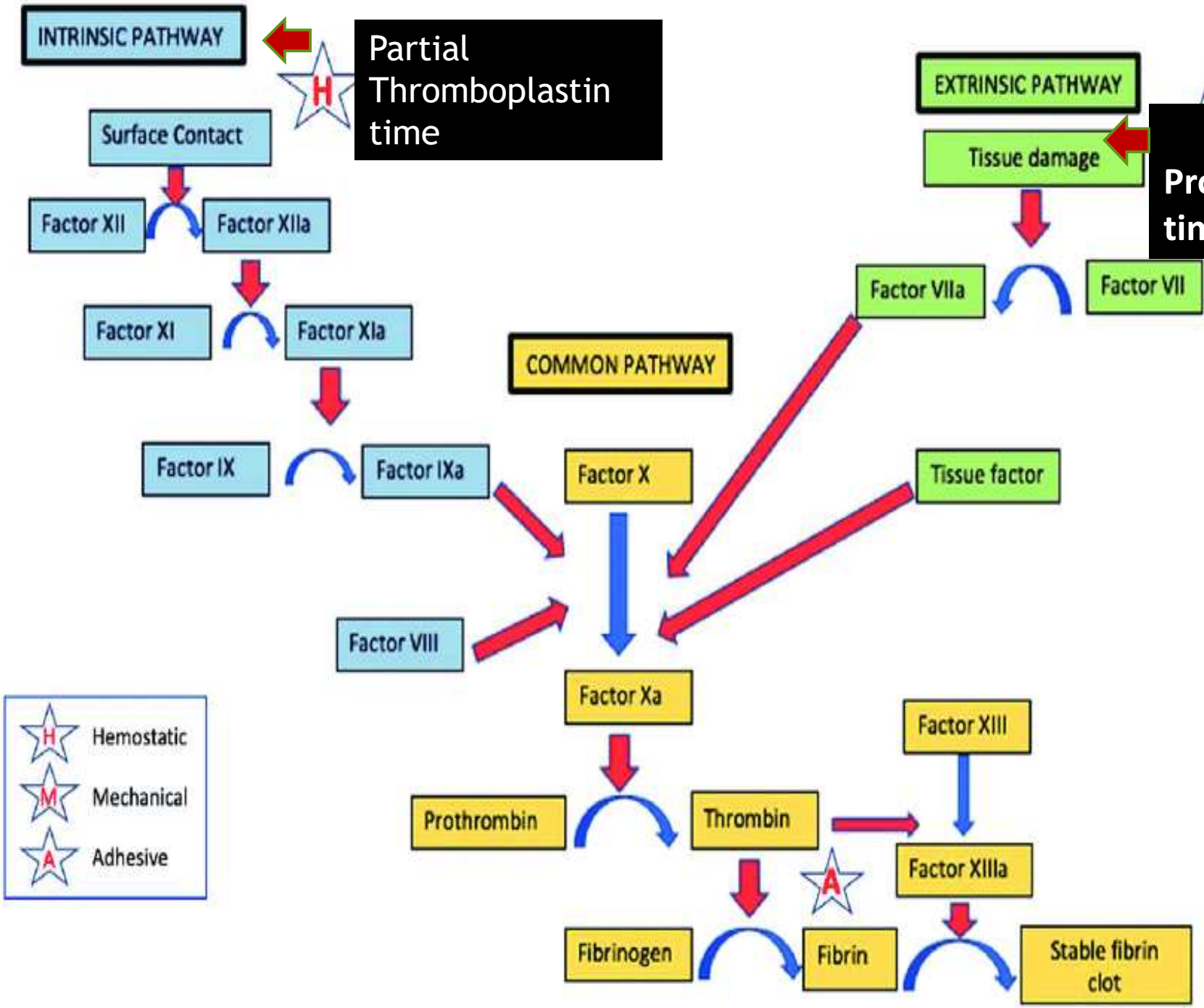
Stages of Blood coagulation

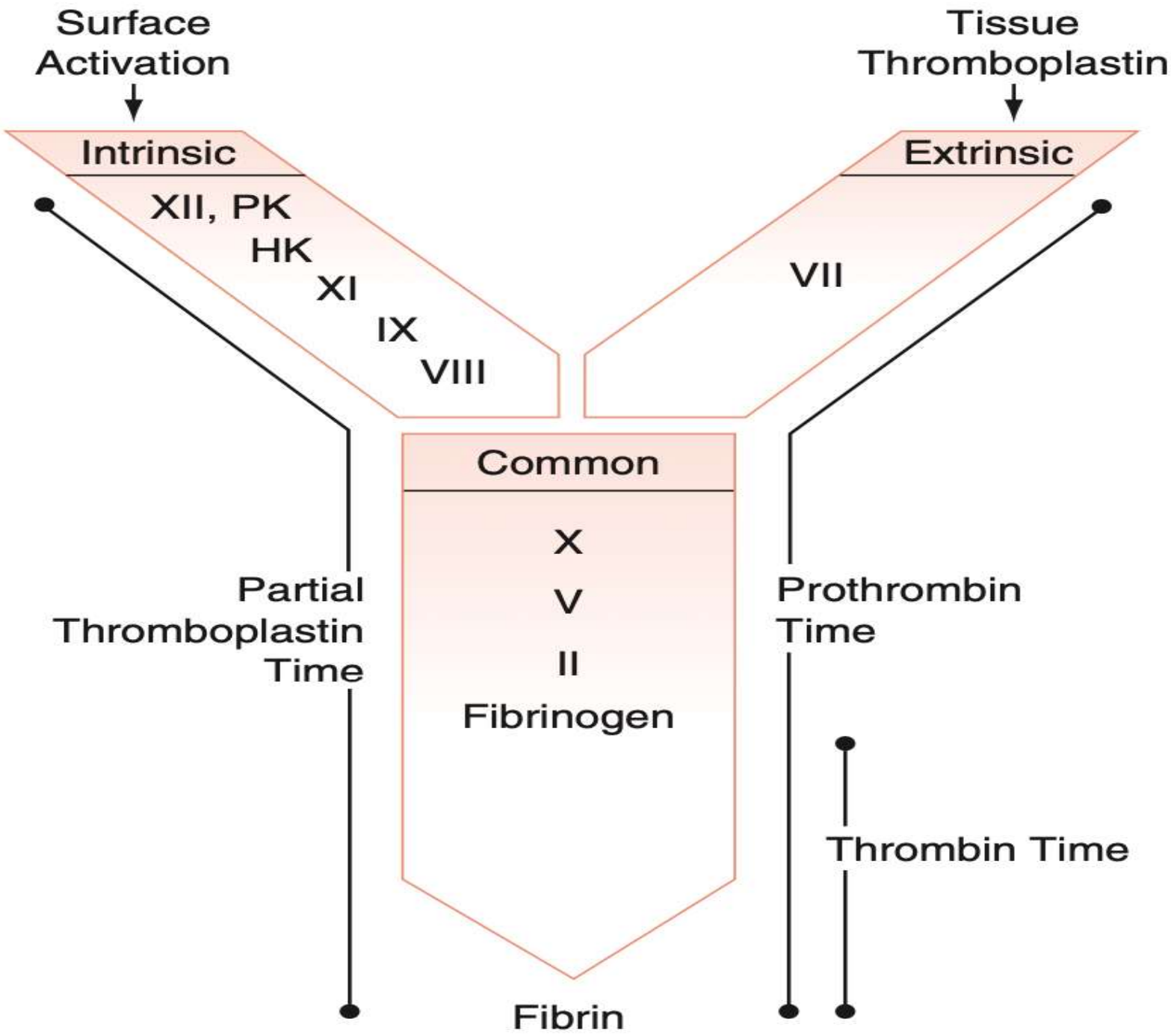
- Intrinsic Pathway
  Extrinsic Pathway
- Formation of Prothrombinase
  (Activated Factor X)

Coagulation factors

- I – Fibrinogen
- II – Prothrombin
- III – Tissue thromboplastin
- IV – Calcium
- V – Labile factor, proaccelerin
- VI –
- VII – Stable factor, proconvertin

- VIII – Antihemophilic factor
- IX – Christmas factor
- X – Stuart Prower factor
- XI – Plasma thromboplastin antecedant (PTA)
- XII – Hageman factor
- XIII – Fibrin stabilizing factor


INTRINSIC PATHWAY
Partial Thromboplastin time
Surface Contact
Factor XII
Factor XIIa
Factor XI
Factor XIa
Factor IX
Factor IXa
EXTRINSIC PATHWAY
Tissue damage
Prothrombin time
Factor VIIa
Factor VII
COMMON PATHWAY
Factor X
Tissue factor
Factor VIII
Factor Xa
Factor XIII
Prothrombin
Thrombin
Factor XIIIa
Fibrinogen
Fibrin
Stable fibrin clot
Hemostatic
Mechanical
Adhesive


Surface Activation
Tissue Thromboplastin
Intrinsic
XII, PK
HK
XI
IX
VIII
Extrinsic
VII
Common
X
V
II
Fibrinogen
Partial Thromboplastin Time
Prothrombin Time
Thrombin Time
Fibrin

Extrinsic pathway depends upon	Intrinsic Pathway depends on
Vessels injury	Vessel Injury
Tissue Factor	Collagen Contact
Tissue thromboplastin(III)	
Factor VII	Factor XII,XI,IX,VIII
Calcium	Calcium

Screening tests for hemostasis

Tests of primary hemostasis	Assessment
Bleeding time	Platelet and vascular defects
PFA-100 system	Platelet function
Platelet count	Quantification of platelets
Blood smear	-Quantification and morphological abnormalities of platelets -Detection of underlying hematological disorder.

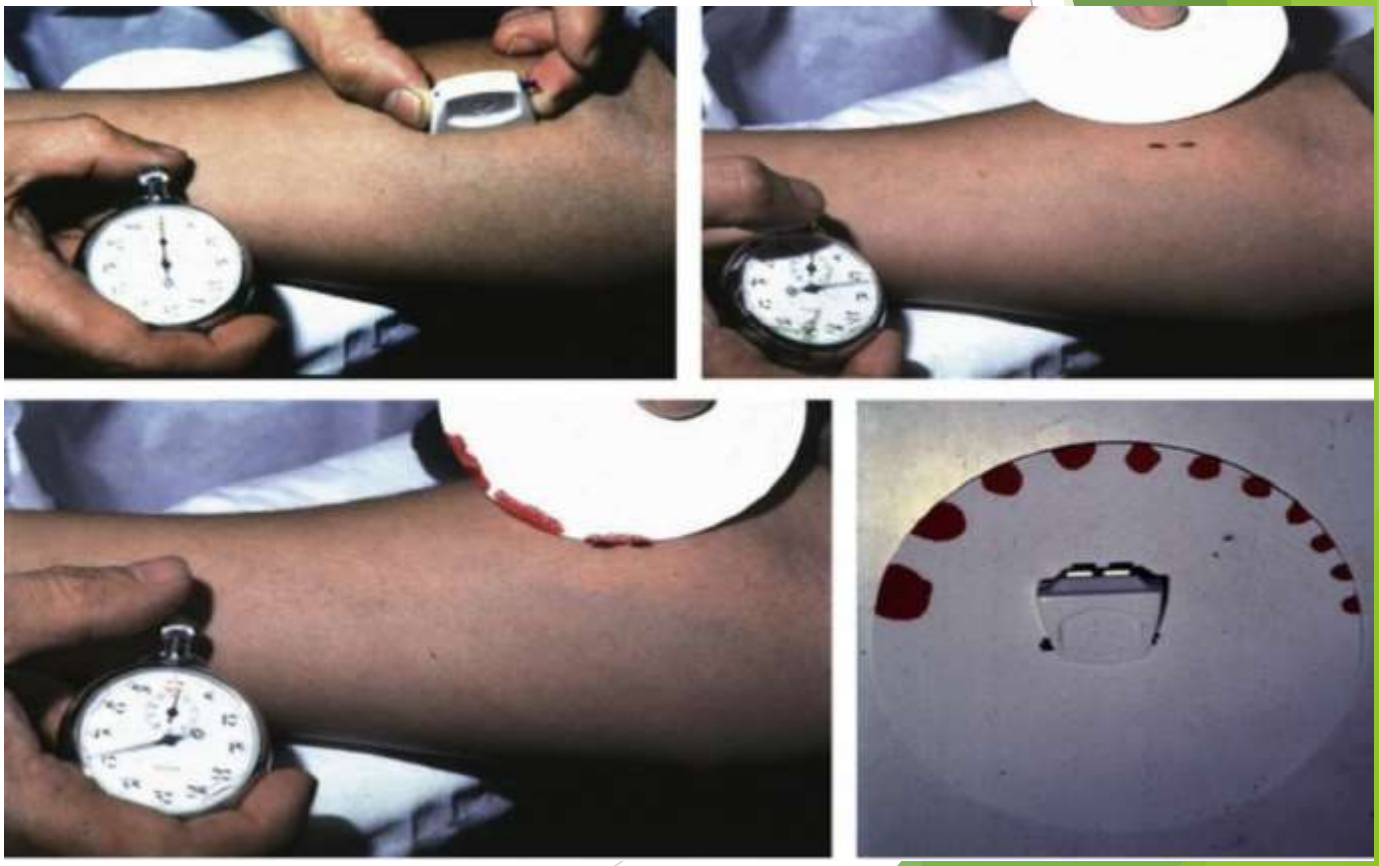
Tests of secondary hemostasis	Assessment
Clotting time	Crude test of coagulation phase
Prothrombin time	Extrinsic and common pathways
Activated partial thromboplastin time	Intrinsic and common pathways
Thrombin time	Clot formation time

Bleeding time

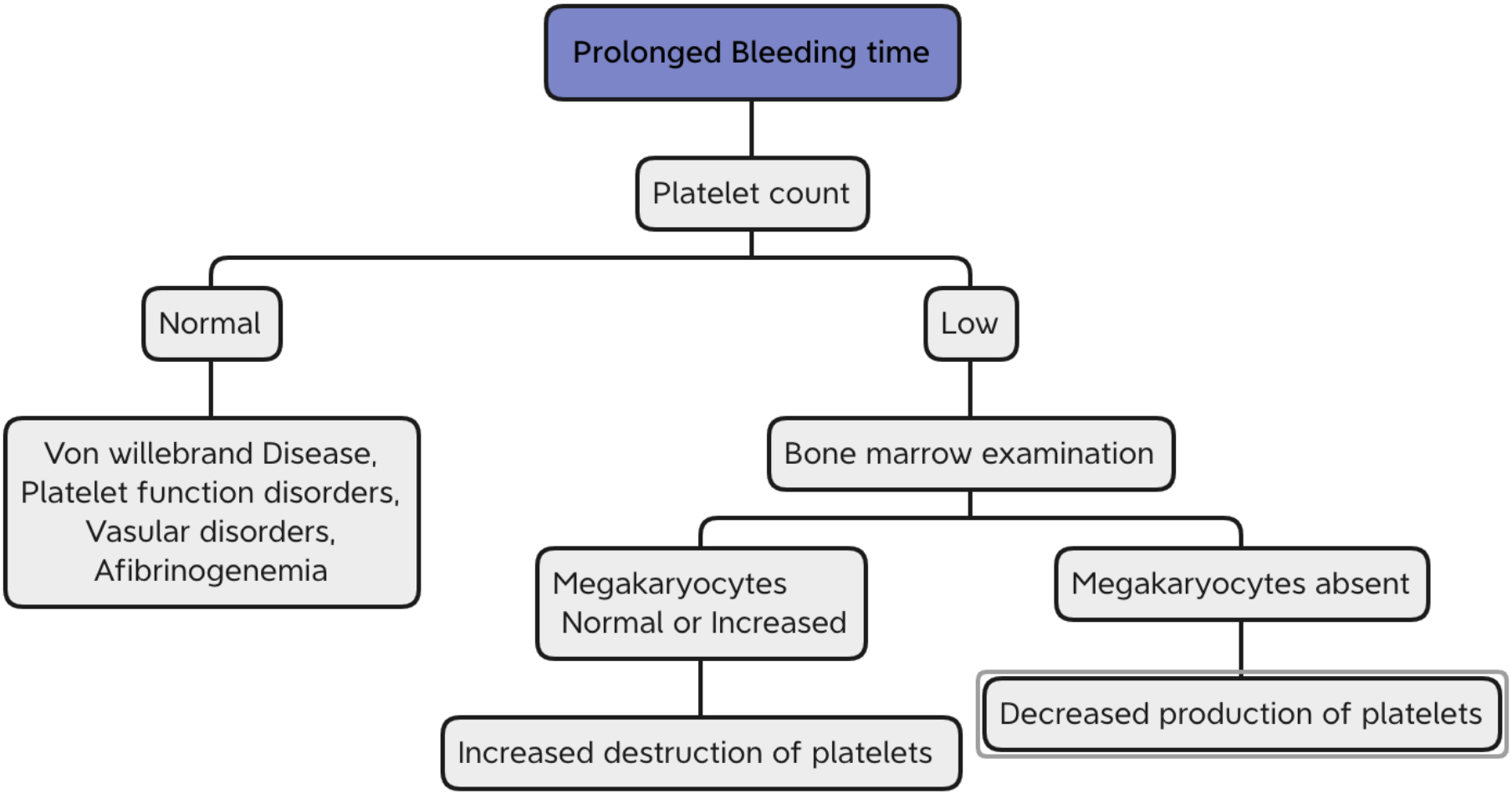
- It is an in vivo measure of primary hemostasis (vascular and platelet components).
- It assess the formation of primary hemostatic plug which is dependent on adequate functioning of platelets.
- When a small slit is made in the skin, the hemostatic mechanisms necessary for coagulation are activated. Without the aid of external pressure, bleeding usually stops within 7 to 9 minutes.

- Three methods:

1. Ivy’s method
2. Duke’s method
3. Template method



Evaluation of prolonged BT



Clotting time

Capillary tube method:

1-Prick a finger with a sterile lancet, fill 2 non heparinized capillary tube with blood (capillary tube should touch the bleeding point), wipe any excess blood from the outside of the tube.

2- Wait for 4 minutes, then cut the tube after each 30 seconds.

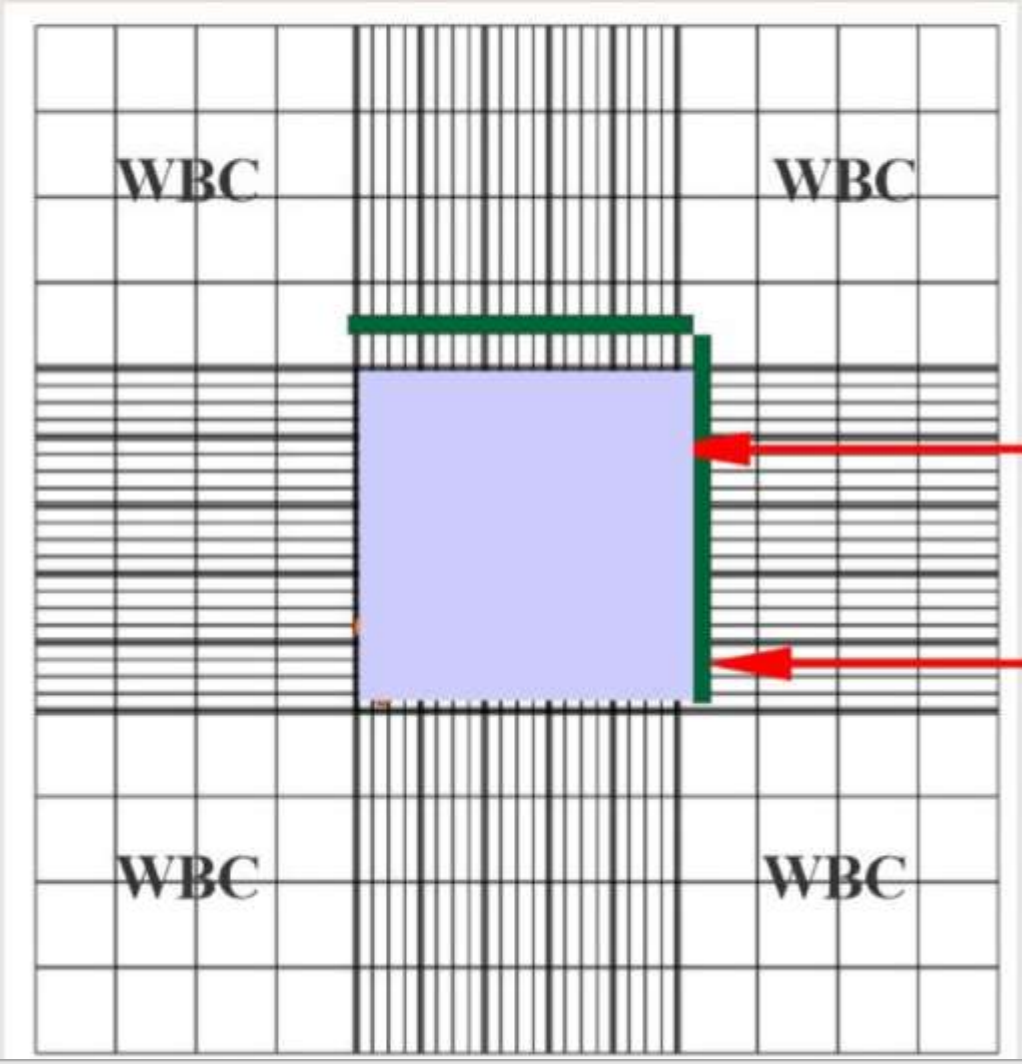
3- Note the time if the fibrin thread is formed between the 2 broken capillary tube.

The test is obsolete and not asked for .

Platelet count

- Can be done manually under microscope or by means of automated hematology cell analyzer.
- Normal range – **1.5-4.5 lakhs/cu mm**.
- In manual method, blood is mixed with 1% ammonium chloride, number of platelets counted in a counting chamber, and result is reported as number of platelets per cubic mm.

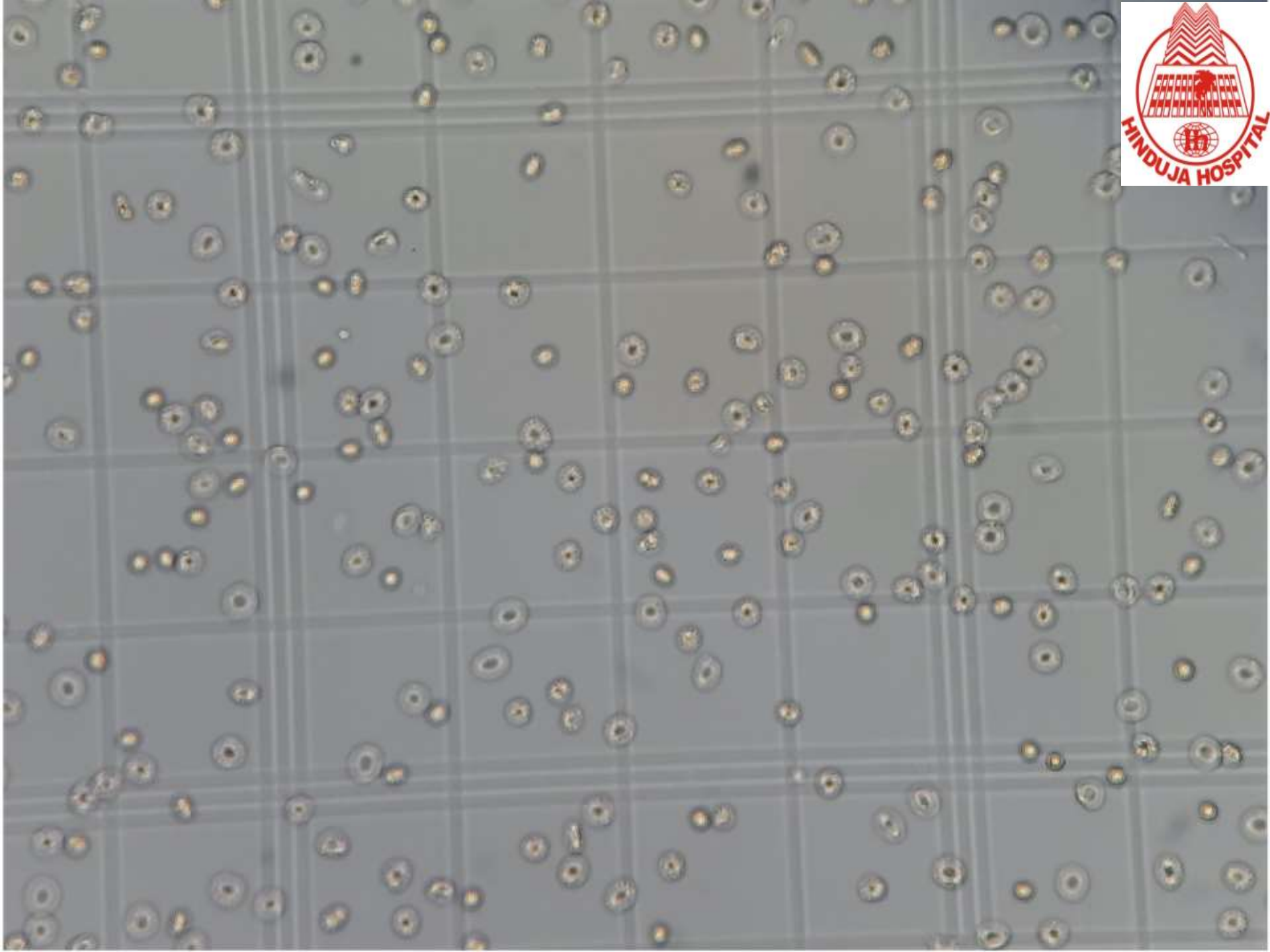
Platelets count



Count the central square

Don't count the platelets along the Green lines (left and upper line)

$$\text{Platelets count} = \frac{\text{average number of platlets} \times 100 \times 1\ \text{mm}^2}{0.1\ \text{mm}} \times 10^6 = \text{Platelets} \times 10^9/\text{L}$$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- They appear as small, spherical, refractile particles.

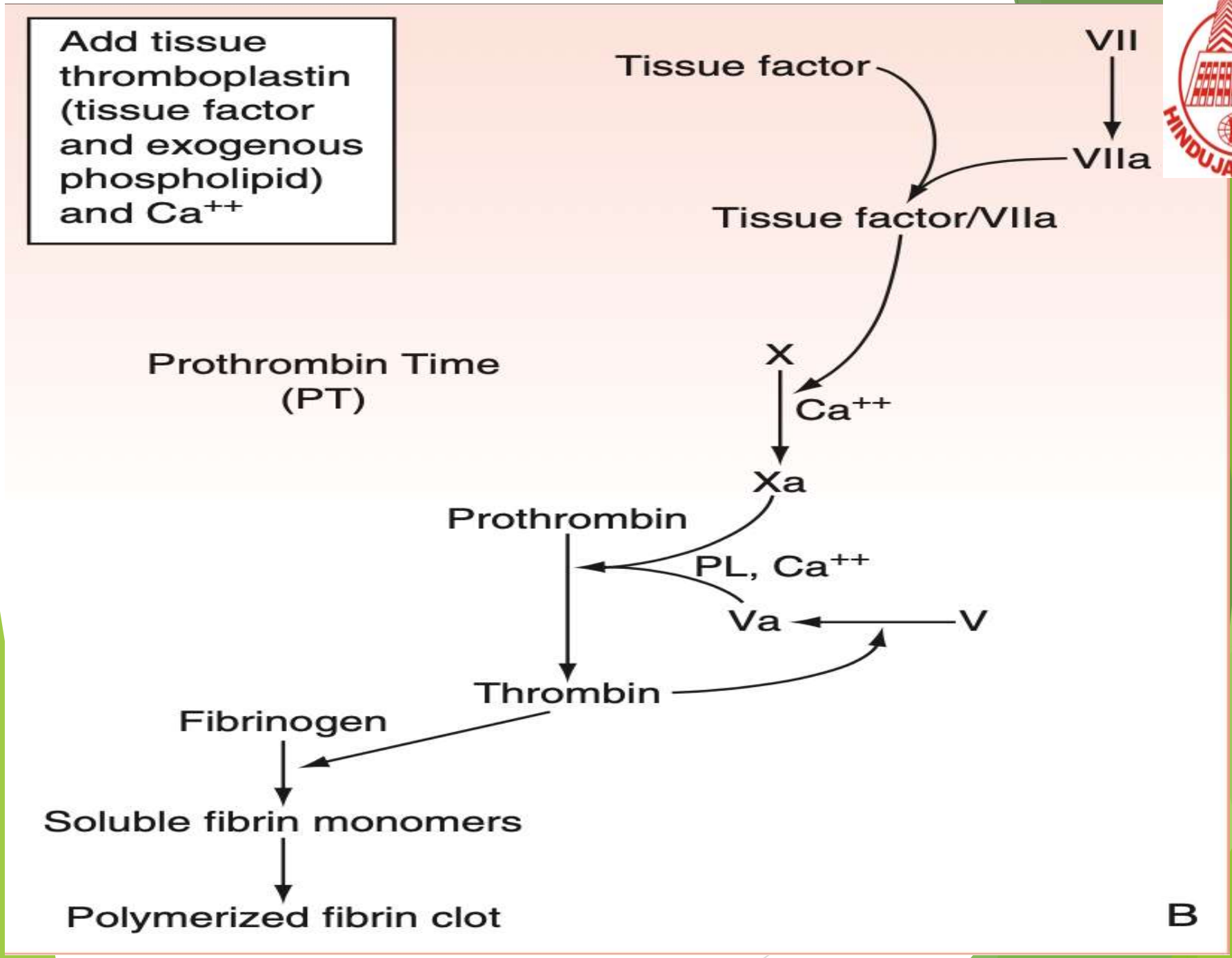
- Normally there are 8-20 platelets/ oil immersion field

OR

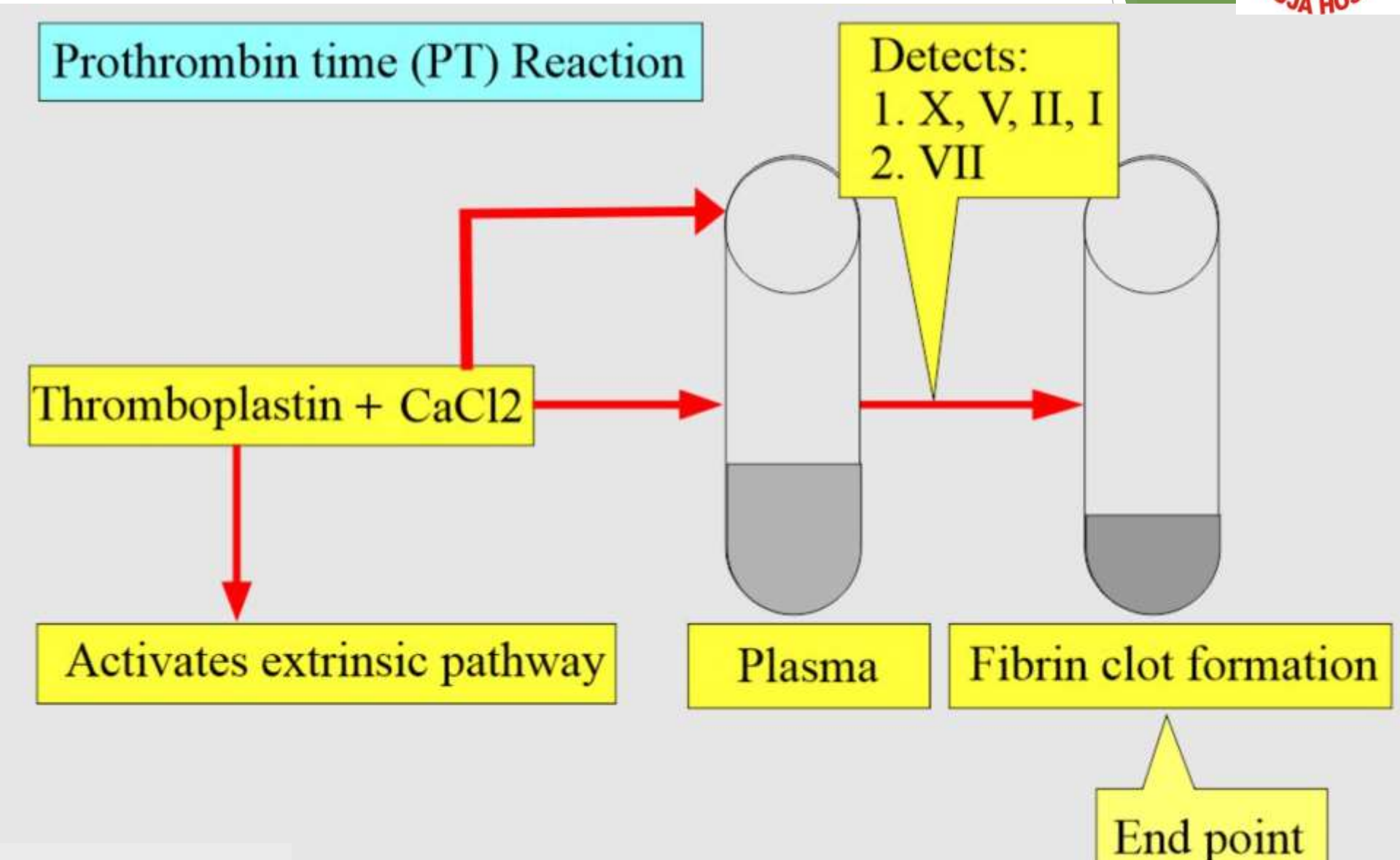
15-30 platelets/high power field

Prothrombin time (PT)

- The PT measures the time necessary to generate fibrin after activation of factor VII.
- It measures the integrity of the "extrinsic" and "common" pathways.
- **Principle**: Calcium ion binds vitamin K dependent factors (F II, VII, IX, X) to thromboplastin(tissue factor +Phospholipids).
- **Normal range**: **11-16 seconds**.


Add tissue thromboplastin (tissue factor and exogenous phospholipid) and Ca++
Tissue factor
VII
VIIa
Tissue factor/VIIa
Prothrombin Time (PT)
X
Ca++
Xa
Prothrombin
PL, Ca++
Va
V
Thrombin
Fibrinogen
Soluble fibrin monomers
Polymerized fibrin clot
B

PROTHROMBIN TIME



Abnormality	Interpretation
Isolated prolonged PT	Factor VII deficiency
Prolonged PT along with other coagulation abnormalities	Vitamin K deficiency Vitamin K antagonists e.g. Warfarin, phenindione Liver disease, Malabsorption High concentration of unfractionated Heparin Direct Thrombin inhibitors e.g. Lepirudin, argatroban Afibrinogenaemia and dysfibrinogenemia Dilutional coagulopathy e.g. massive blood transfusion Multiple clotting factor deficiencies e.g. FV and FVIII deficiency Abnormalities of the vitamin K cycle e.g. mutations within the VKORC1 gene

Mean normal prothrombin time (MNPT)

- It is geometric mean of PT of reference sample group.
- It is determined for every new lot of PT reagent used.
- MNPT is the denominator used for calculation of INR.

How to get MNPT:

- Collect citrated plasma of 20 apparently healthy individual (HCK samples in age group of 18-50 years).
- Spin sample at 2500 g for 15 minutes. Separate platelet poor plasma of each tube.

- Check platelet count of platelet poor plasma (It should be less than 10,000).
- Perform PT on each plasma samples and record the results. Take a geometric mean and ±2 SD. This gives the target value (MNPT) and reference range .
- This MNPT is fed in the instrument along with the ISI for the given Reagent lot. The instrument uses this MNPT and ISI to calculate the INR for the patient's sample using following formula:
- $INR = (Test/MNPT)^{ISI}$

International normalized ratio(INR)

- **Standardises PT**
- Takes into account the ISI of the reagent
- Ratio of patient PT to lab mean normal PT to the power of ISI.

- $INR = \left(\frac{\text{PT of the patient}}{\text{(PT of the normal range mean)}}\right)^{ISI}$

- ISI(international Senstivity Index)- sensitivity of thromboplastin in use as compared to WHO reference reagent
- PT reagents insert has the ISI value
- Closer the ISI is to 1, higher the sensitivity.(Normal range 1-1.4)

The INR test result is given as a number.

Normal range value =0.8 to 1.1

INR is used in monitoring oral anticoagulant therapy

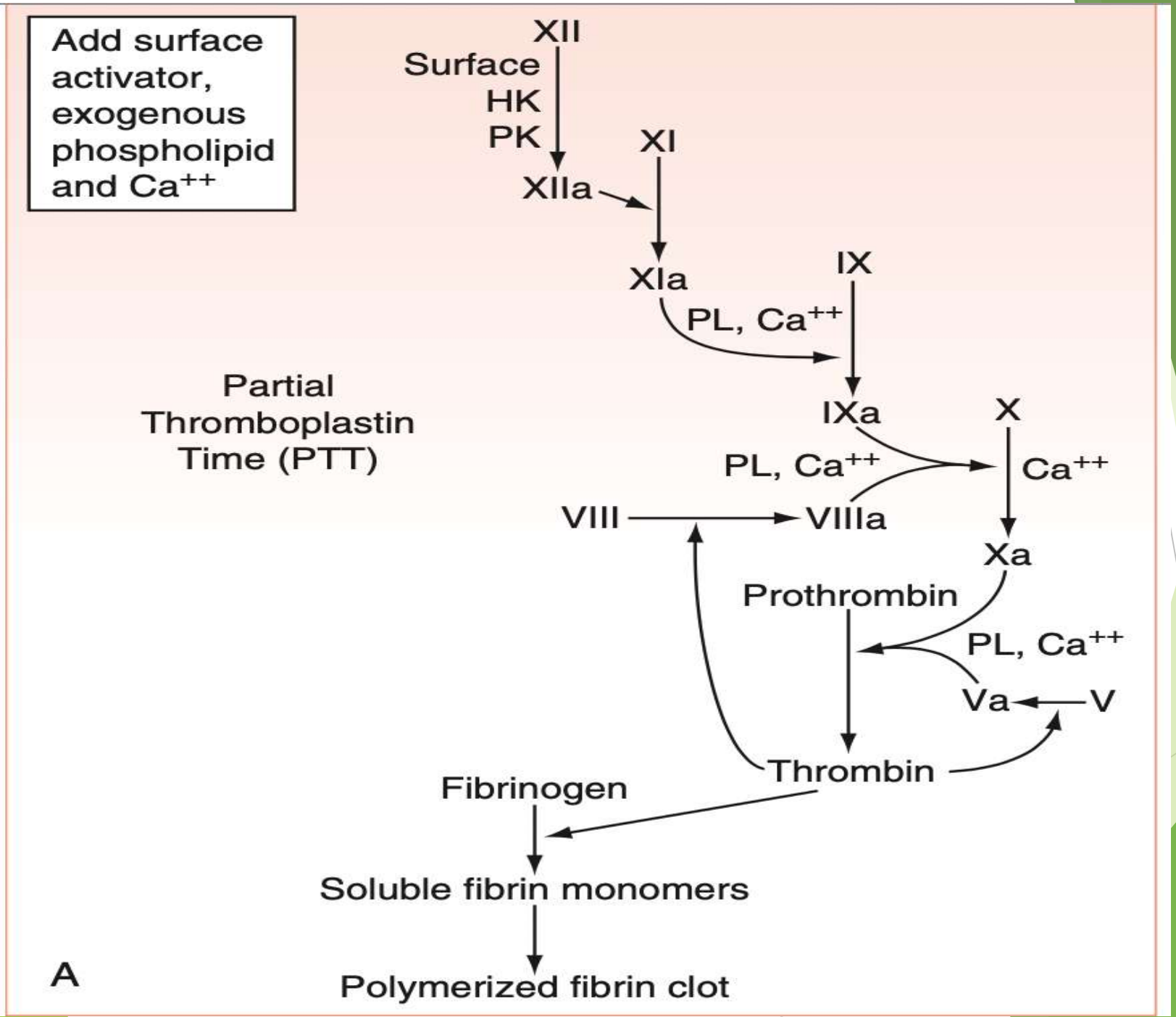
1. For prophylaxis = 1.3
2. All other indications except cardiac = 2.0 to 3.0.
3. In cardiac diseases = 2.5 to 3.5.

Activated Partial Thromboplastin Time

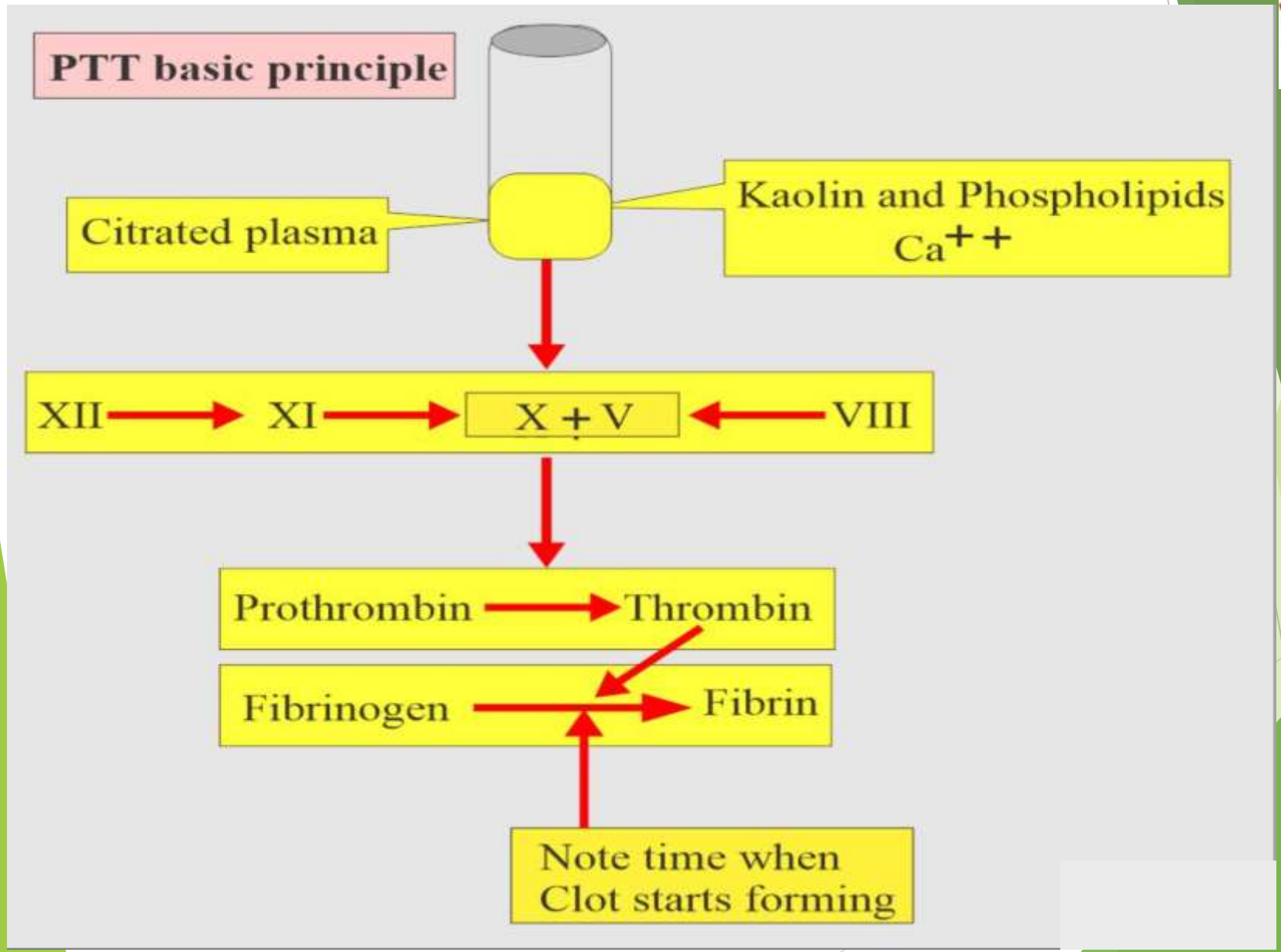
- It is a measure of coagulation factors in intrinsic pathway (F XII, XI, HMWK, Prekallikrien, IX, VIII) and common pathway (F X, V, I, II).

- **Principle**: Plasma is incubated with an activator e.g. kaolin,silica (that initiates intrinsic pathway of coagulation by contact activation).

- Citrated platelet poor plasma, an activating agent, and phospholipid are added together and incubated at 37°C. Calcium is added, and the time necessary for the clumping of kaolin is measured.
- **Normal range**: **<u>26-40 secs</u>**.


Add surface activator, exogenous phospholipid and Ca++
XII
Surface
HK
PK
XIIa
XI
XIa
IX
PL, Ca++
IXa
X
PL, Ca++
Ca++
VIII
VIIIa
Xa
Prothrombin
PL, Ca++
Va
V
Thrombin
Fibrinogen
Soluble fibrin monomers
Polymerized fibrin clot
Partial Thromboplastin Time (PTT)
A

Activated Partial Thromboplastin Time



Abnormality	Interpretation
Isolated prolonged APTT	Deficiencies of either XII, XI, IX & VIII. Acquired clotting factor inhibitors - these are most commonly directed against FVIII. Lupus anticoagulant [LA]
Prolonged APTT +Prolonged PT	Vitamin K deficiency Liver disease Direct thrombin inhibitors including Hirudin, Argatroban and Dabigatran. DIC - due to the consumption of clotting factors Massive blood transfusion leading to a dilutional coagulopathy

- Occasionally the reported value of the aPTT will be lower than normal.

- This "shortened" time may reflect the presence of increased levels of activated factors in context of a “hypercoagulable state.”

- It is seen in some patients in the early stages of DIC but should not be considered diagnostic for that entity.

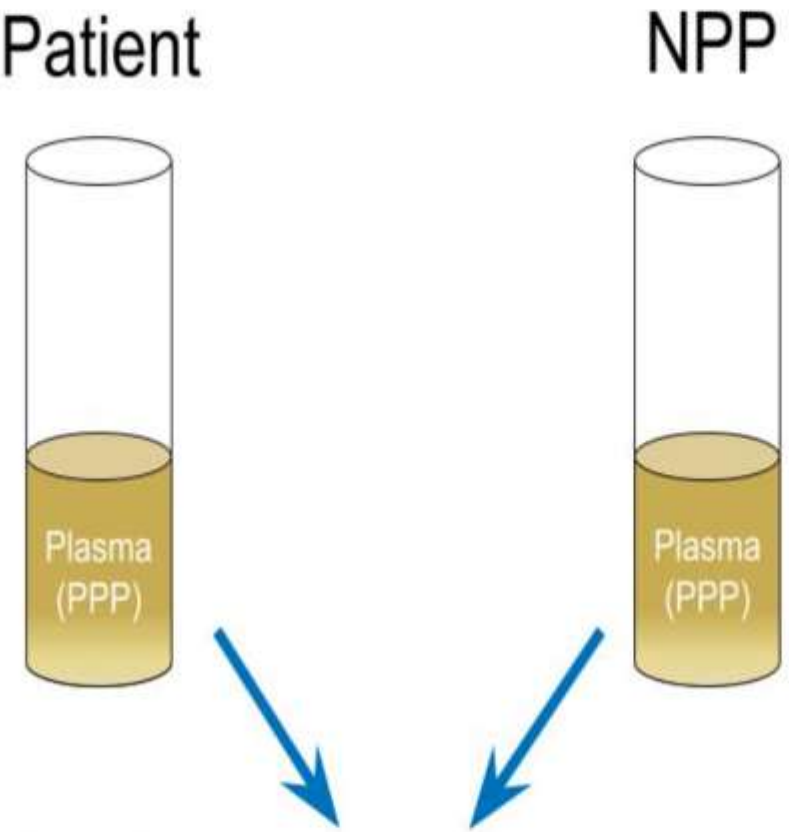
Mixing studies

- Mixing studies are typically used to investigate abnormal clotting time results.

- Mixing studies help distinguish clotting time prolongation due to a coagulation factor deficiency or an inhibitor e.g. lupus anticoagulant (specific or nonspecific).

- If the clotting time corrects, this suggests a factor deficiency; if the clotting time does not correct, this suggests presence of a circulating inhibitor

Mixing Study



1:1 mix = 1 part patient, 1 part NPP

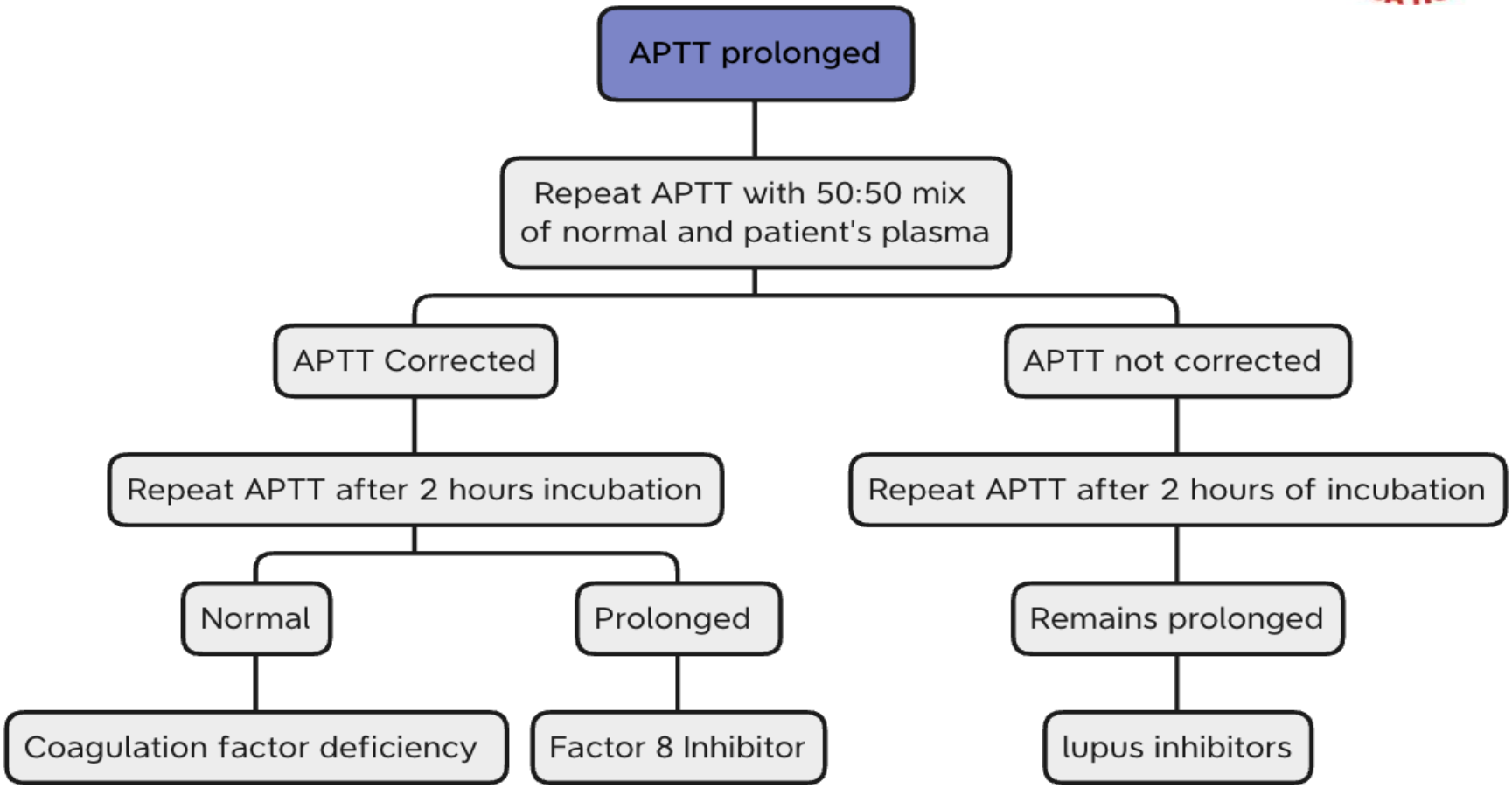
4:1 mix = 4 parts patient, 1 part NPP

Interpretation

Corrects= Factor Deficiency Pattern
Does Not Correct= Inhibitor Pattern

Immediate Mix = measure PT or aPTT immediately

Incubated Mix = incubate samples for 1-2 hours at 37°C, then measure aPTT


APTT prolonged
Repeat APTT with 50:50 mix of normal and patient's plasma
APTT Corrected
APTT not corrected
Repeat APTT after 2 hours incubation
Repeat APTT after 2 hours of incubation
Normal
Prolonged
Remains prolonged
Coagulation factor deficiency
Factor 8 Inhibitor
lupus inhibitors

Thrombin time

- This test measures the time necessary to drive the reaction of fibrinogen to fibrin in the presence of thrombin.
- Thrombin time is affected by the concentration and function of fibrinogen and by the presence of inhibitory substances.
- Citrated plasma is incubated at 37°C and thrombin is added to the solution. Time is measured from the addition of thrombin to the generation of fibrin filaments.
- **Normal range:** 15-19 secs

Factor XIII assay(Clot Solubility Test

- Label 2 glass tubes as test and control
- Add 0.2 ml of test plasma and normal pooled plasma in the respective glass tubes.
- Add 0.2ml of 0.025 mol/L $CaCl_2$ solution to each tube and incubate for 30 minutes at 37^0 C(in waterbath).
- Tap each tube gently. To loosen the clot from the sides of the tube and 3 ml of 5 mol/L urea so that the clot is suspended.
- Leave at room temperature(18-25^0 C) overnight.
- Inspect the clot after 24 hours.

CASE 1

A 4 year old with DNS presented to the OPD for routine pre-operative check up

	Test	Range
PT	12secs	10.5-13.5 secs
APTT	97 secs	24-36 secs
TT	11secs	10-13secs
Fibrinogen	2.7g/L	1.5-4.0g/L

- Repeat Assay
- Mixing studies: The prolonged APTT corrects in a mix with normal plasma
- What factor assays would you request and why?

FVIII, IX , XI assays.

The FIX assay was normal but the FVIII assay was <5 IU/dl.

CASE 2

- 39 year old female was referred for evaluation of coagulopathy and thrombosis.
- She had previous history of spontaneous abortion and now presented with pain in the lower limbs.
- On doppler she was diagnosed with DVT and started on LMWH and warfarin.

CBC revealed platelet count -114000/ul

APTT-68.2sec (NR-21-28sec)

PT-11.2 sec (NR-9.6-12.6 sec)

- Mixing studies done

TIME	APPT-PATIENT (T) Secs	Normal Pooled Plasma (C)secs	T+C(secs)
0 hour	68.2	34.5	55.8
1 hour	75	35.8	58.4
2 hour	85.8	36.6	61

Lupus Anticoagulant was positive

QUALITY CHECK IN COAGULATION

Concept of quality in a coagulation lab

Lab has ethical obligation to provide results that are

- Reliable
- Accurate
- Reproducible

Quality Assurance is intended to ensure reliability of lab results

- According to CLIA (Clinical Laboratory Improvement Amendment) QA stands for **Quality Assessment**
- QA measures quality through all aspects of the testing procedure

1. **Pre analytic**
2. Analytic
3. Post analytic

Issues in Coagulation Testing

Pre-analytic

- Preparation of the patient
- Drugs
- Sample collection
- Transportation of sample

Analytic

- Equipment
- Calibration & Controls
- Reagents

Post-analytic

- Reference range
- Report format
- Interpretation

Factors influencing sample quality before testing

Sample collection: Patient preparation

Patient identification and sample labelling

Phlebotomy technique

Sample volume

Sample collection tube

Sample handling: Storage

Centrifugation

Transport conditions

Delays in transportation

Patient factors: Physiological variables

Pathological states

- **Sample for Blood Coagulation**

- After patient identification collect venous blood in 3.2% ,0.109 M sodium citrate in the ratio of 1 vol of Na Citrate to 9 vol of blood using evacuated tube system or plastic syringe.
- Venipuncture from peripheral vein using evacuated tube system preferred
- Coagulation tube should be the first draw or next after a non-additive collection (not beyond –in vivo changes due to tourniquet)
- Touniquet application should be less than a minute

- Blood should be mixed with anticoagulant < 1 min.
- 3-6 gentle inversions immediately.
- Anticoagulant to blood ratio should be maintained (tubes should not be overfilled/underfilled+/-10% fill volume min)

- **In abnormal Hct with polycythemia or severe anemia adjust blood : citrate ratio**

C= 1.85 /1000 x (100-H) V

C= citrate in ml (anticoagulant)
H=haematocrit
V=volume of blood (sample)

Hct (%)	Citrate (ml)
0.2	0.70
0.25	0.65
0.30	0.61
	0.55
0.55	0.39
0.60	0.36
0.65	0.31
0.70	0.27

Storage

Perform the assay immediately or as soon as possible.

The allowable time interval between collection and analysis depends on the test-

- PT - 24 hrs
- APTT - 4 hrs
- Others - 4 hrs

Cold storage of citrated whole blood, either by placing samples in an ice bath or refrigerated (2–8°C) may lead to

- activation of platelets
- activation of factor VII and significant time dependent loss of both FVIII and VWF.

Sample should be kept at room temperature if it is to be used for PT tests, lupus anticoagulant (LAC) or factor VII assays

If tests are to be performed later, plasma to be separated and frozen.

- -20 deg upto 2 weeks
- -70 deg upto 6-12 months
- Thawed at 37 deg quickly (and not to be refrozen)

Plasma samples should be rapidly thawed at 37^0C while gently mixing and tested immediately

Common Collection Problems

Error	Consequence	Comment
Short draw <2.7 mL	PT/PTT falsely prolonged	Anticoagulant to blood ratio exceeds 1:9
Failure to mix specimen after collection	PT/PTT falsely prolonged	Blood clots form when anticoagulant & blood do not mix
Excess vigorous mixing	PT/PTT falsely shortened	Hemolysis and platelet activation cause start of cascade
Hemolysis	PT/PTT falsely shortened	Reject specimen
Improper storage: wrong temperature or held too long	PT/PTT falsely prolonged	Must follow storage requirements
Chilling in refrigerator or placing on ice	PT falsely shortened	Chilling to 4 ^{o}C activates factor VII.
Inadequate centrifugation	PTT loses sensitivity for lupus anticoagulants and heparin. Factor assays inaccurate	Desire platelet poor plasma
Prolonged tourniquet application	Falsely elevates vWF, factor VIII	Tourniquet causes venous stasis,

Sample rejection

Sample is rejected if

- Collected in wrong anticoagulant
- Clotted
- Shows fibrin strands after centrifugation
- Ratio of blood to anticoagulant not proper
- Hemolysed samples
- Lipemic or icteric samples are not rejected on electromechanical coagulometers but cannot be performed on optical instruments
- Mention condition of sample in the report

Q) Vitamin K is essential for the synthesis of which group of coagulation factors

1)Factors I, II, V and VII

2)Factors II,VII,IX and X

3)Factors V, VIII,IX and X

4)Factors XI,XII, XIII and XIV.

QHemophilia A is a bleeding disorder caused by the deficiency of which coagulation factor?

a) Factor VII

b) Factor VIII

c) Factor IX

d) Factor XI

- Dr Shanaz Khodaiji
- Hematology Laboratory Staff

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Thank You

Q Normal Value of clotting time

1)2-8 mins

2)3-6 mins

3)4-5 mins

4)8-12 mins

Q1)All are components of hemostasis except-

a) Blood Platelets

b) Red blood cells

c) Endothelial Cells

d) Plasma Coagulation Factors

Ans b)Red Blood cells

Q2)Which test evaluates the extrinsic pathway?

a)PT(INR)

b)PTT

c)TT

d)Bleeding Time

Ans a)PT(INR)

Q3)Thromboplastin contains

a)Tissue Factor

b)$CaCl_2$

c)Phospholipids

d)Tissue factor + Phospholipids

Ans d)Tissue factor+ Phospholipids

Q4)Platelet poor plasma means platelet count less than---

a) 50,000/cmm

b) 10,000/cmm

c) 1,00,000/cmm

d) 15,000/cmm

Ans <10,000/cmm