Board Review- Part 3: Coagulation

1/9/2022

Outline of review

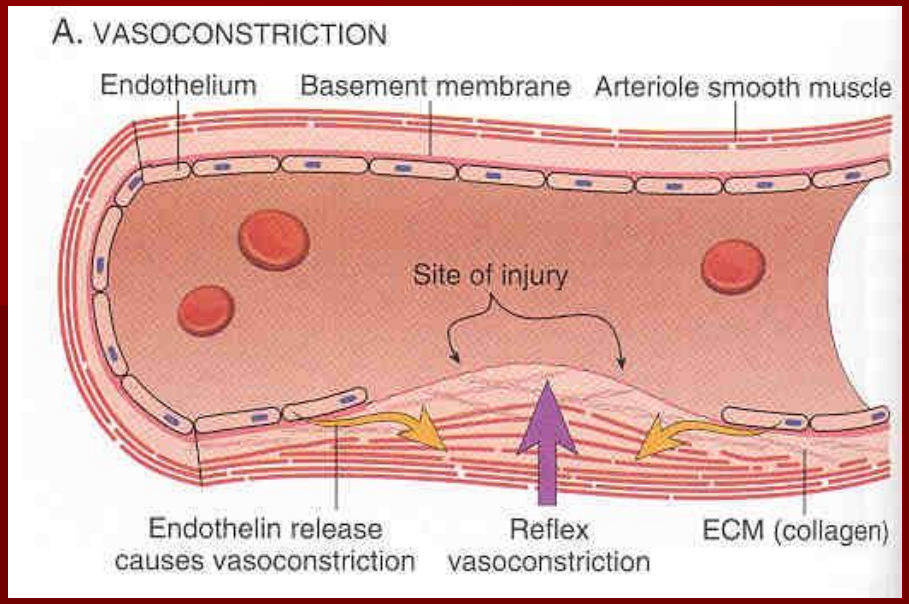
- General hemostasis / coagulopathy
- Selected coagulopathy topics (8)

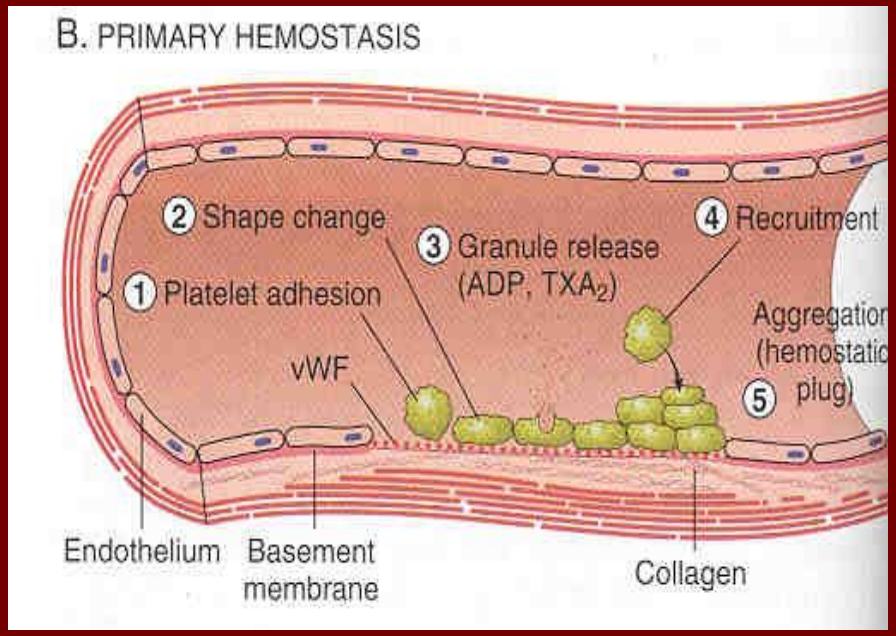
Normal hemostasis

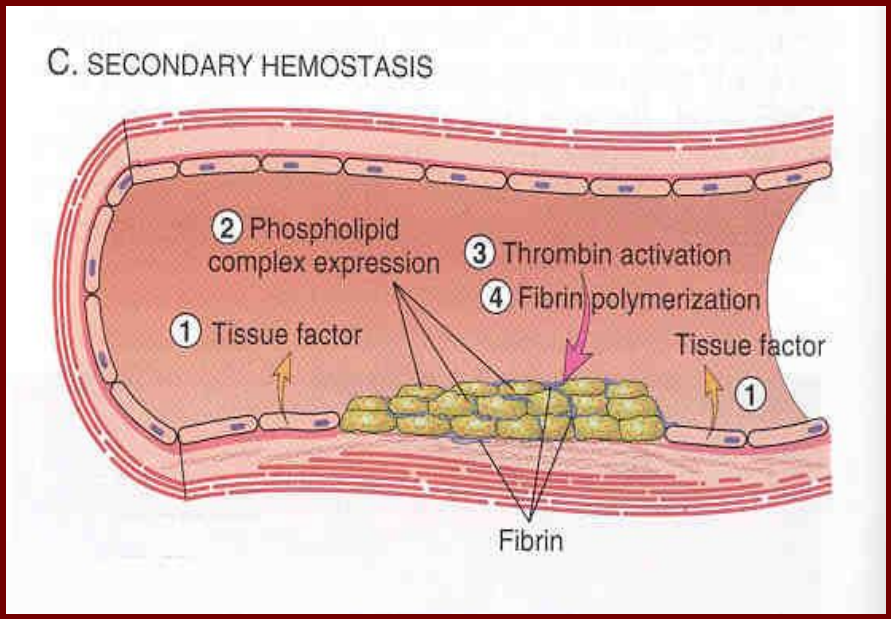
- Vasoconstriction:

  Reflex neurogenic mechanisms

  Augmented by endothelin
- Platelet plug (primary hemostasis)
- Activation of the coagulation cascade (secondary hemostasis)


A. VASOCONSTRICTION
Endothelium
Basement membrane
Arteriole smooth muscle
Site of injury
Endothelin release causes vasoconstriction
Reflex vasoconstriction
ECM (collagen)

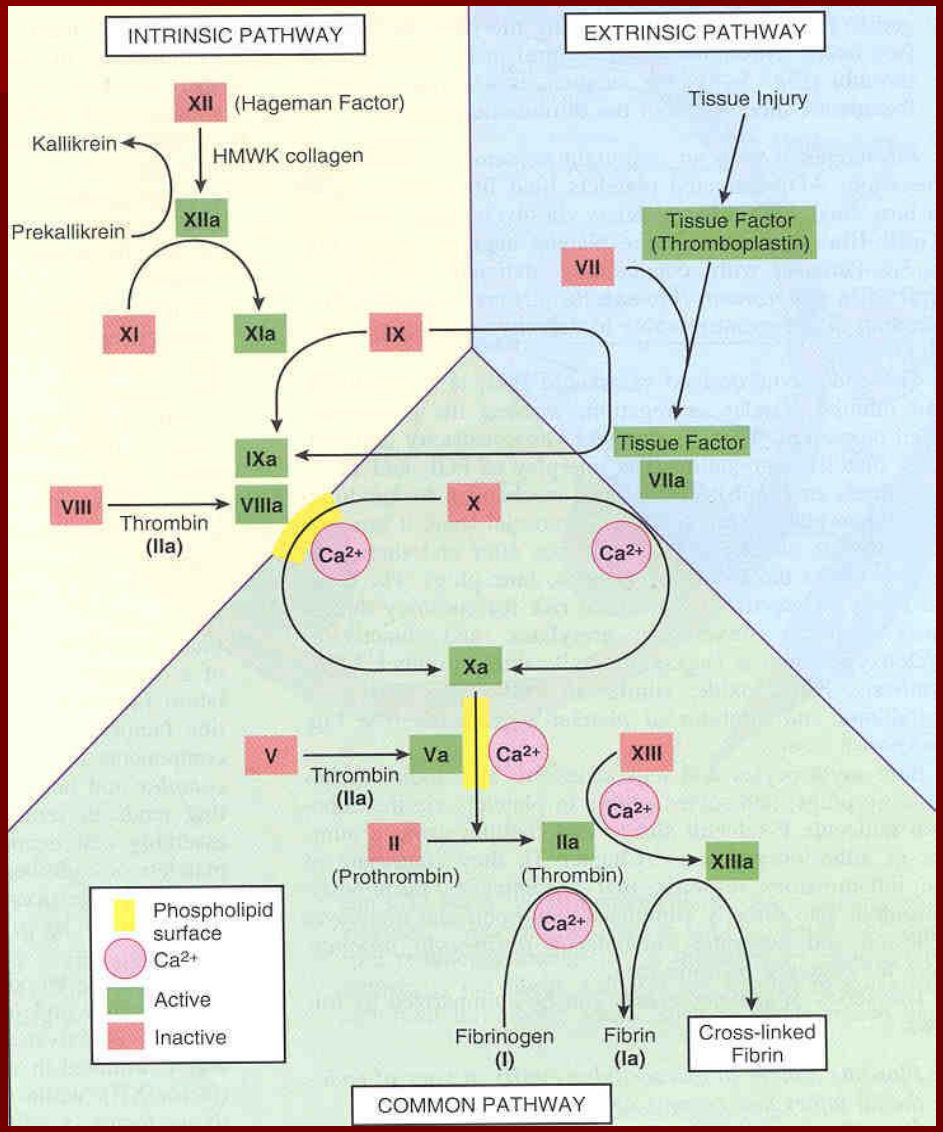

B. PRIMARY HEMOSTASIS
2 Shape change
3 Granule release (ADP, TXA2)
4 Recruitment
1 Platelet adhesion
vWF
Aggregation (hemostatic plug)
5
Endothelium
Basement membrane
Collagen


C. SECONDARY HEMOSTASIS
2 Phospholipid complex expression
3 Thrombin activation
4 Fibrin polymerization
1 Tissue factor
Tissue factor
1
Fibrin

Clinical manifestations in a patient with bleeding disorder

Findings	Coagulation Disorders	Platelet or Vessel Disorders
Petechiae	Rare	Characteristic
Deep hematomas	Characteristic	Rare
Hemarthroses	Characteristic	Rare
Delayed bleeding	Common	Rare
Bleeding from superficial cuts	Minimal	Persistent
Patient gender	Most inherited disorders in men	Most inherited disorders in women
Mucosal bleeding	Minimal	Typical

Secondary hemostasis- cascade model



PT

- Patient's platelet-poor plasma, tissue thromboplastin, and calcium are mixed; and clotting time is determined.
- Assessment of extrinsic pathway and common pathway
- Reported as INR (international normalized ratio)
- Thromboplastin used may vary from lab to lab and country to country; giving variable PT
- INR is used for standardization
- $INR=[\text{Patient PT}/\text{Mean of normal PT range}]^{ISI}$

(ISI: international sensitivity index)

Prolonged PT

- Coumadin
- Vit K def
- Failure of absorption of Vit K (cholestasis, short-bowel syndrome, etc.)
- Liver disease
- Factor def in extrinsic and common pathways

Coumadin

- Vit K epoxide  Vit K

epoxidase reductase

- Coumadin blocks reductase and non-functional epoxide accumulates

Vit K

- The Vit K-dependent factors (II, VII, IX, X) have 9-12 glutamic acid residues near the amino terminal end, which needs to be carboxylated (Vit K-dependent) to bind to phospholipids.
- Vit K dependent proteins:
  II, VII, IX, X, proteins C and S

aPTT

- Patient's platelet-poor plasma, surface activating agent (silica) and platelet substitute (crude phospholipid or partial thromboplastin) are mixed, and clotting time is determined.
- Assessment of intrinsic and common pathways.

An isolated prolonged PTT

- Heparin
- Factor deficiency: VIII, IX, XI, XII
- HMWK (Fitzerald) def
- Pre-kallikrein (Fletcher) def
- Inhibitors: VIII and IX inhibitors, lupus anticoagulant
- vWD

R/O Heparin

- History
- Prolonged Thrombin Time
- Normal reptilase time

Thrombin Time (TT)

- Patient's plasma and thrombin is mixed, and clotting time is determined.
- Heparin produces prolonged TT but normal reptilase time
- Functional fibrinogen (Clauss method) is based on TT, using diluted plasma sample

Prolonged TT

- Heparin
- Hypofibrinogenemia
- Dysfibrinogenemia
- Thrombolytic therapy

Approach to a prolonged PTT: Mixing study

- Patient's plasma is mixed with an equal volume of normal plasma (1:1 mix)
- PTT measured at 0 hour (immediate) and 1-2 hours after incubation.
- Failure of correction of prolonged aPTT means inhibitors
- If results at 0 hour and 1-2 hours are similarly prolonged-> lupus anticoagulant, heparin
- If results show time-dependent prolongation -> coag factor antibody (esp. F VIII inhibitor)

Hereditary clotting factor deficiencies

- Hemophilia A (VIII def), B (IX def), C (XI def)
- I, II, V, VII, X, XIII deficiency
- Dysfibrinogenemia
- Hemophilia A, B are X-linked recessive
- Dysfibrinogenemia : autosomal dominant
- All others: autosomal recessive

Acquired Clotting factor deficiency

- Anticoagulants (coumadin)
- Fibrinolytic therapy
- DIC
- Liver disease
- CP bypass

Factor Inhibitors

- Mixing study: no correction
- Spontaneous inhibitors (typically in autoimmune diseases) can go away
- Acquired inhibitors (in hemophilia A patients with chronic FVIII infusions) are persistent

Inhibitors

- Lupus anticoagulant: dilute Russell Viper Venom Time (DRVVT), confirm with Platelet Neutralization Procedure (PNP)
- Factor VIII or IX inhibitor:

  Mixing study does not show correction

  Factor VIII/IX levels very low (functional activity)

Bleeding with normal PT/PTT

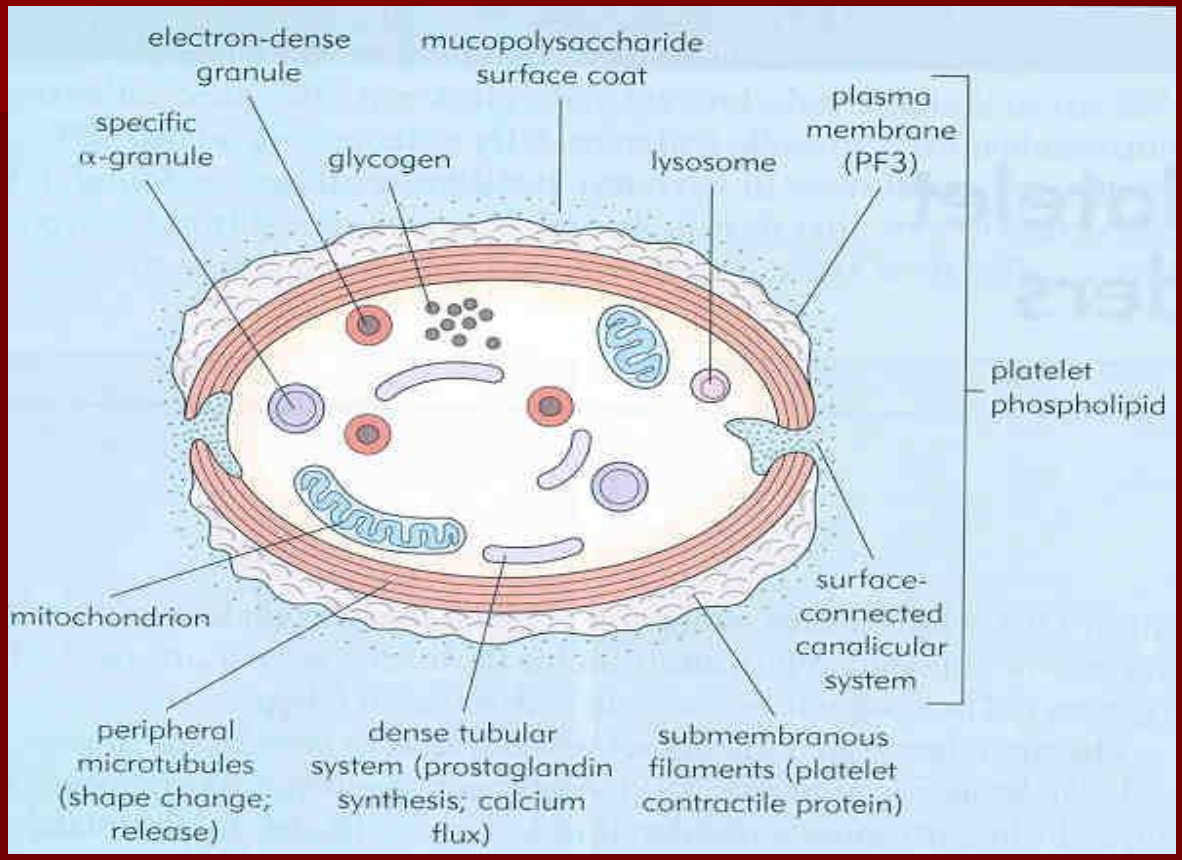
- Factor XIII deficiency (clot is soluble in 5M urea solution in 24 hours); Tx: cryo
- Alpha 2-antiplasmin def, Tx: epsilon amino caproic acid (EACA)

Fibrinogen correction using cryoprecipitates

Number of Units (bags) of Cryo:

- $\frac{\text{Plasma volume ml} \times (\text{desired level} - \text{initial level}) \text{ mg/dl}}{100} = X$
- Number of units of cryo = X/150

Platelets



Circulate for 10 days; 1/3 sequestered in spleen

Platelets

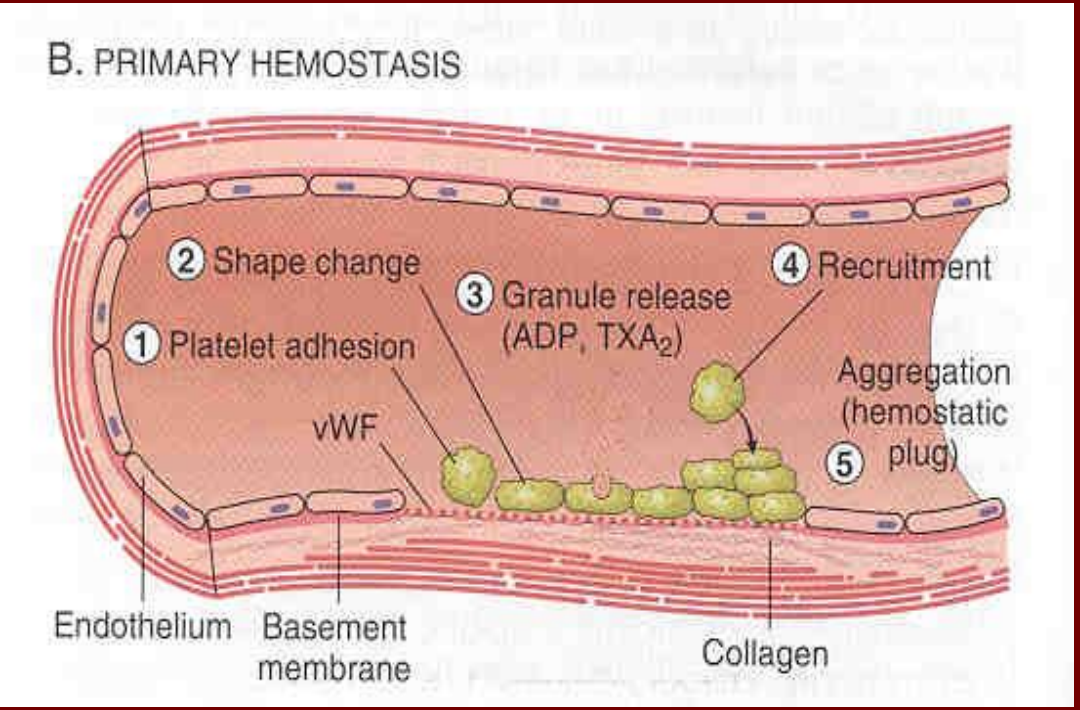
- Alpha granules:
  Fibrinogen, fibronectin
  Factor V, vWF, PF-4, PDGF, Beta Thromboglobulin,
  Thrombospondin
- Dense bodies (delta granules):
  ATP, ADP, ionized calcium, histamine,
  5-HT, epinephrine
- Lysosomes containing acid hydrolases
- Alpha granules: stained by Wright-Giemsa stain
- Delta granules: electron dense due to calcium

Platelet events

- Adhesion and shape change
- Platelet release reaction
- Aggregation

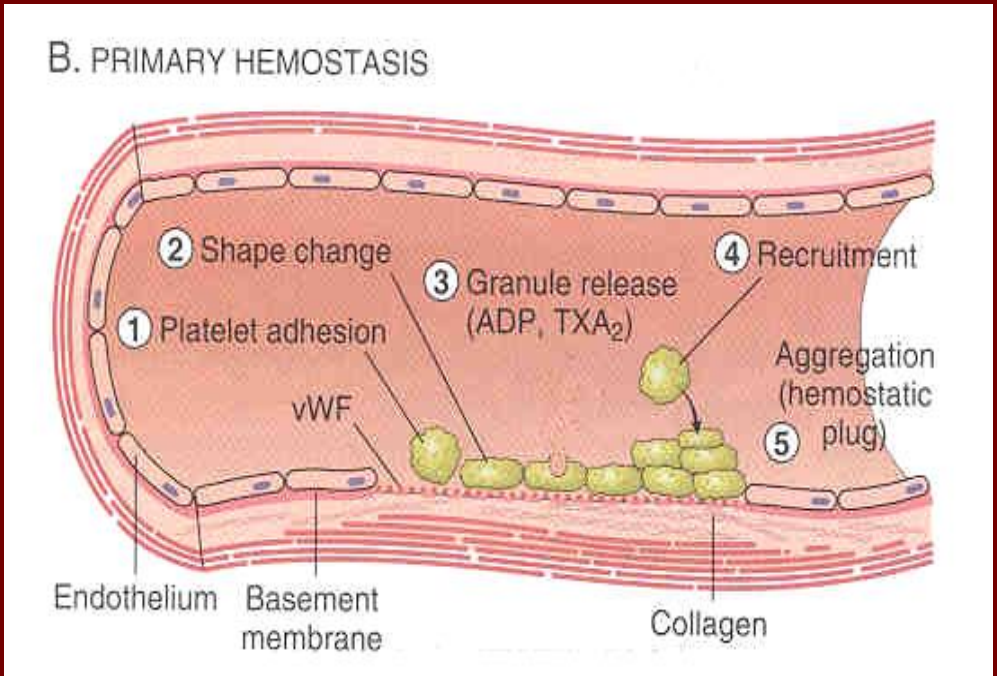
Platelet Adhesion

- Interaction between vWF and GP Ib/IX/V receptors
- Conformational change in HMW multimers of vWF upon exposure to subendothelial collagen



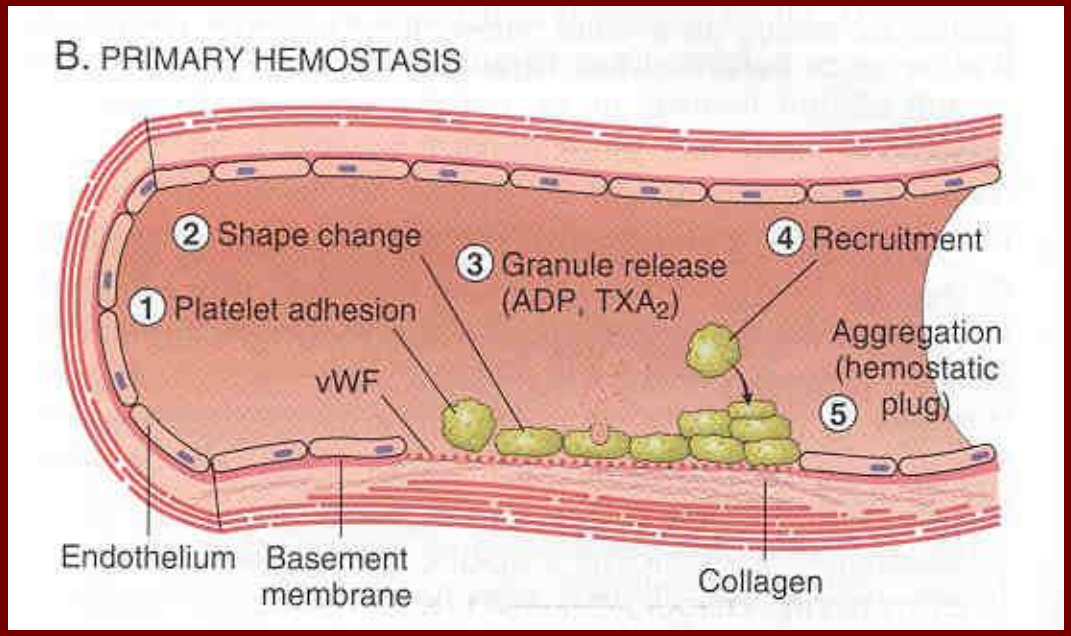
Platelet Activation

- Agonists: ADP, Thrombin, TX A2, Collagen, vWF
- Rapid rise in cytoplasmic calcium
- Shape change; extension of pseudopodia
- Release reaction
- Activation of ligand binding site on GP IIb/IIIa
- Translocation of phosphatidylserine to external surface



Platelet Aggregation

- Fibrinogen mediates binding of activated GP IIb/IIIa receptors on adjacent platelets
- Augmented by Thrombospondin; a component of α-granules



Platelet bleeding disorders

- Thrombocytopenia
- Dysfunctional platelets

Clinical presentation

- Purpura
- Mucosal bleeding
- Prolonged bleeding from superficial cuts and abrasions
- Menorrhagia

Investigations for platelet disorders

- Bleeding time (poor predictive value)
- CBC, peripheral smear
- BM examination
- Platelet aggregation studies

Thrombocytopenia

- Decreased production

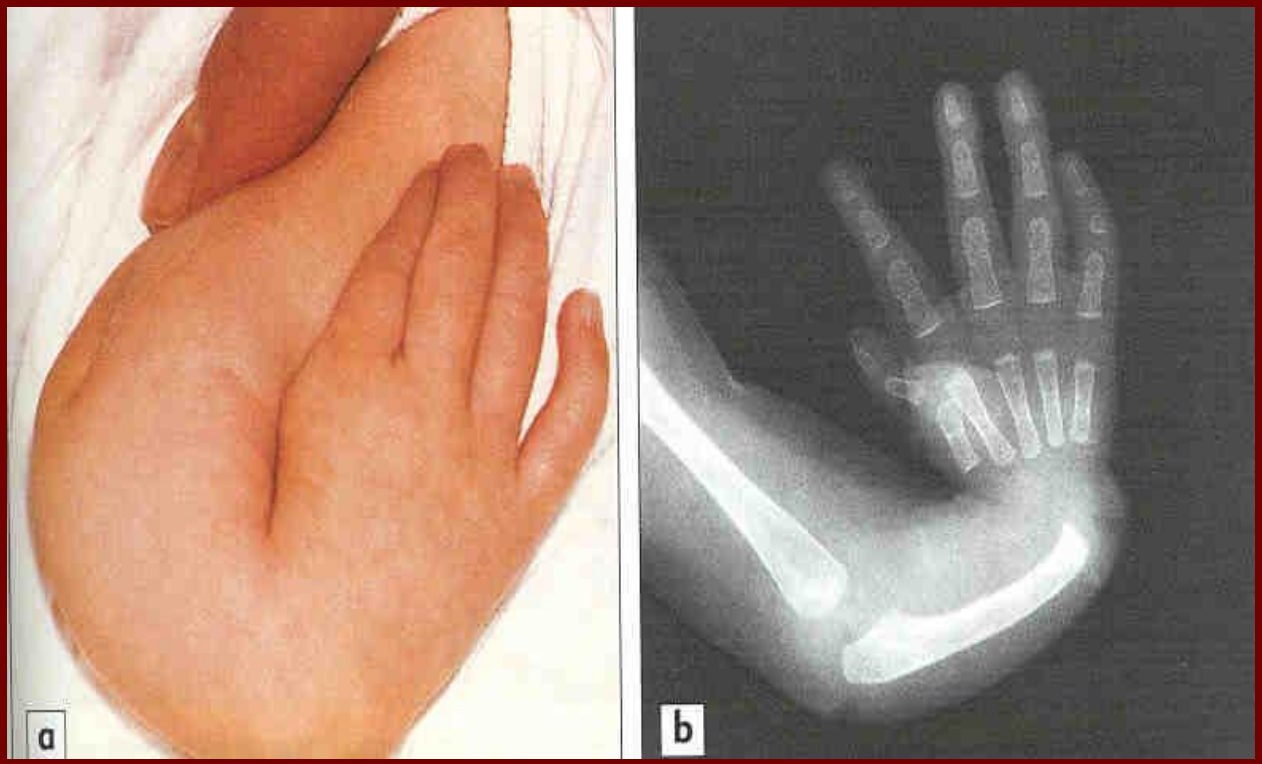
  Generalized BM failure

  Selective megakaryocyte depression
- Increased breakdown: ITP, HIT, Neonatal and post-transfusion purpura
- Increased utilization: DIC, TTP, HUS
- Increased sequestration:
  (a) Kasabach-Merritt syndrome (hemangioma, thrombocytopenia, and coagulopathy)
  (b) Hypersplenism

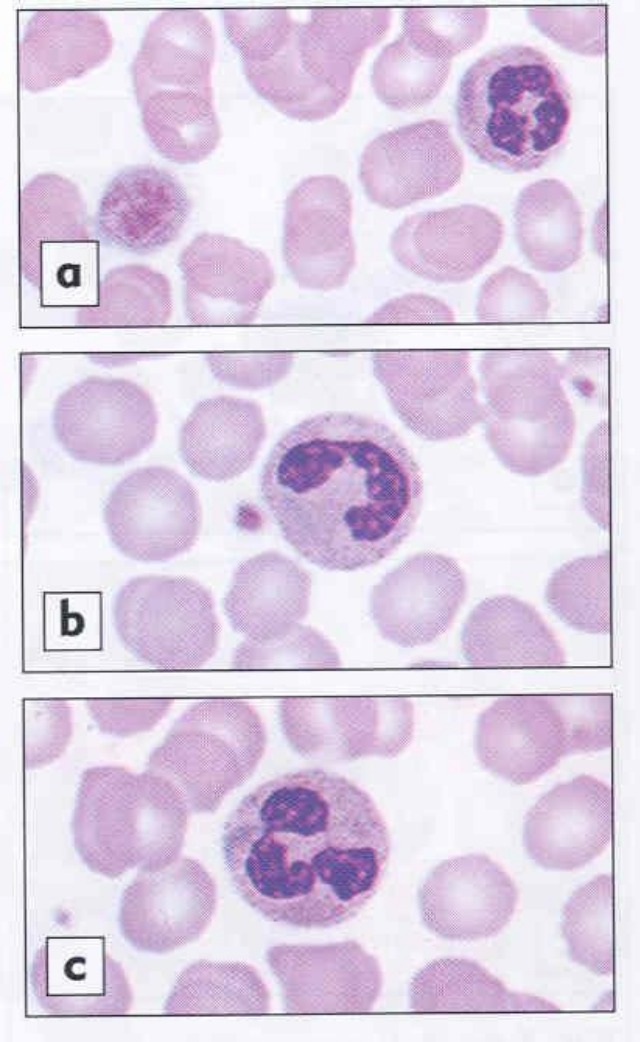
Congenital diseases associated with reduced platelet production

- TAR syndrome (autosomal recessive)
- Fanconi's anemia (autosomal recessive)
- Wiskott-Aldrich syndrome (X-linked recessive): eczema, thrombocytopenia, immune deficiency with decreased IgM
- May-Hegglin anomaly (autosomal dominant): mutations in non-muscle myosin heavy chain IIA (*MYH9*) , Dohle-like bodies

TAR syndrome (thrombocytopenia with absent radius)



May-Hegglin anomaly

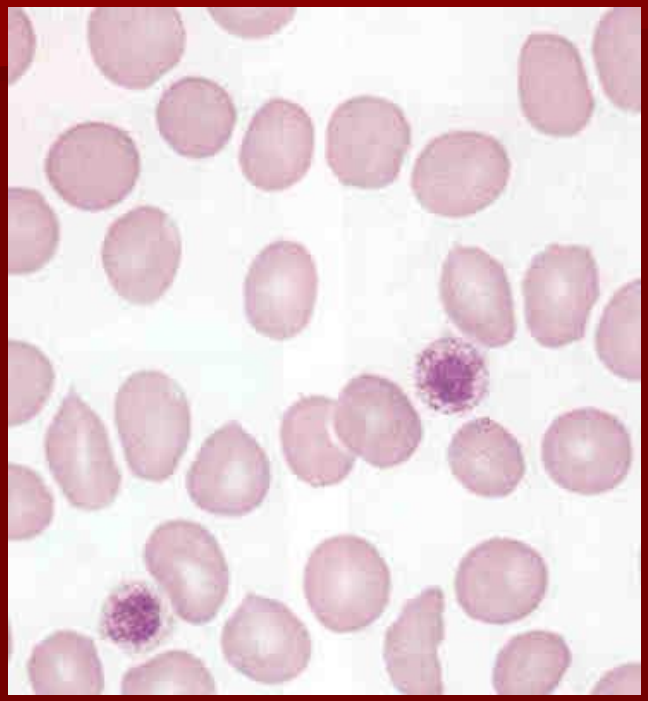


-Thrombocytopenia, giant platelets
-Dohle-like bodies
-Autosomal dominant

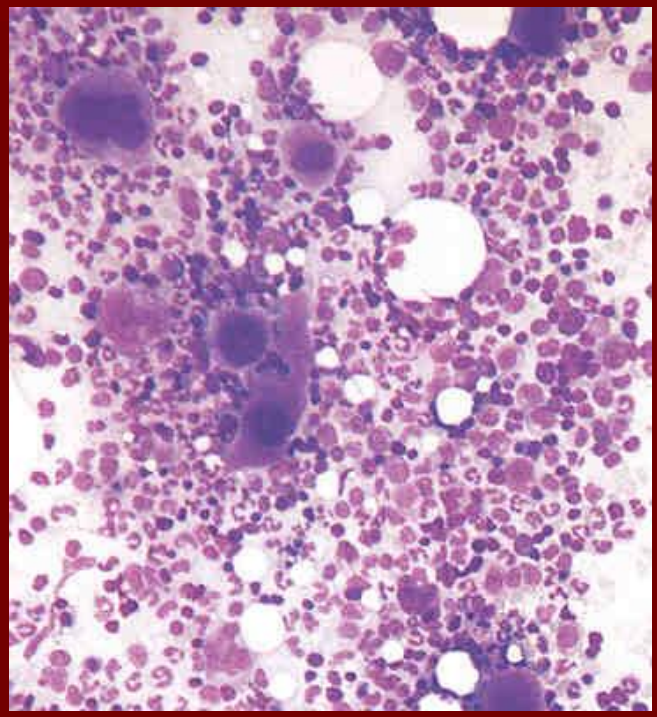
ITP

- Immune destruction of platelets
- Increased megs in BM; large and giant platelets in peripheral smear
- Acute: self limiting
- Chronic: >1 year; 10% with splenomegaly
- Antibody against pathogen which cross reacts with GPIb/IX, GPIIb/IIIa

ITP



Giant platelets



Increased megakaryocytes in BM

ITP

- ITP may present as part of Evan's syndrome (with autoimmune hemolytic anemia)
- ITP may occur in patients with SLE, HIV, CLL and following stem cell transplantation
- Treatment:

  Steroids

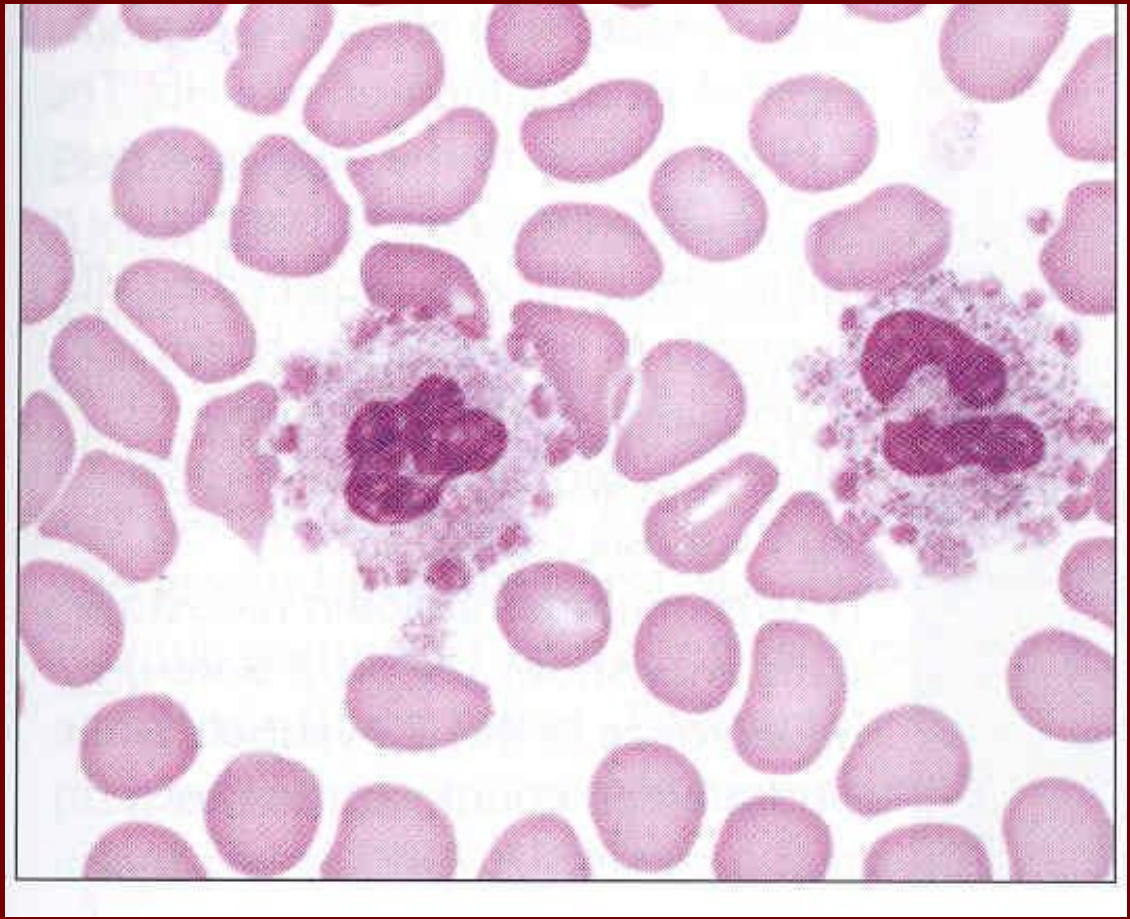
  IVIg

  Rituximab

  Oncovin

  Splenectomy

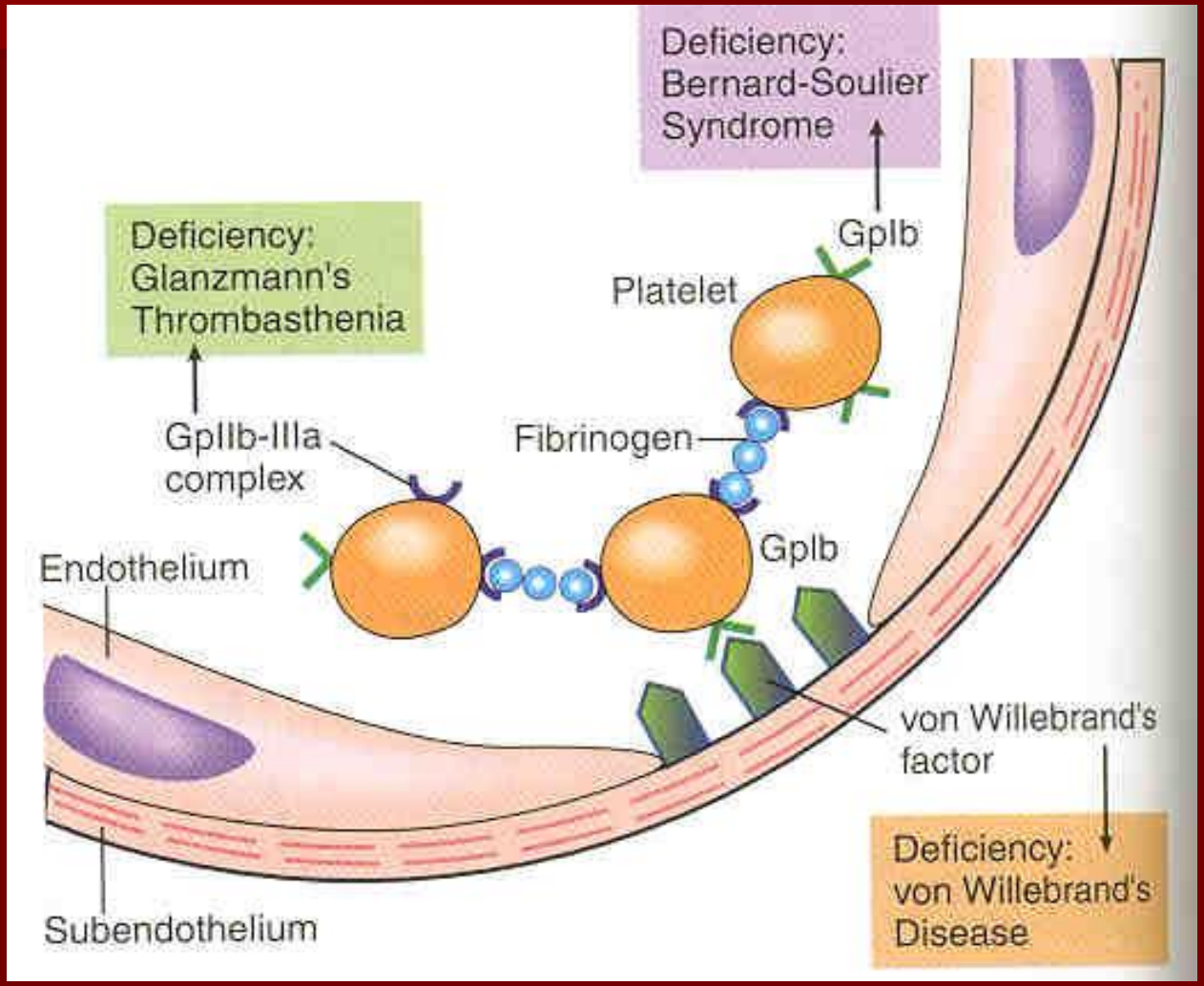
Pseudothrombocytopenia due to EDTA antibody



Congenital Platelet Disorders

- Disorders of platelet adhesion:
  - von Willebrand's disease
  - Bernard-Soulier syndrome
- Disorders of platelet activation
  - Storage-pool disease
- Disorders of platelet aggregation:
  - Glanzmann's syndrome

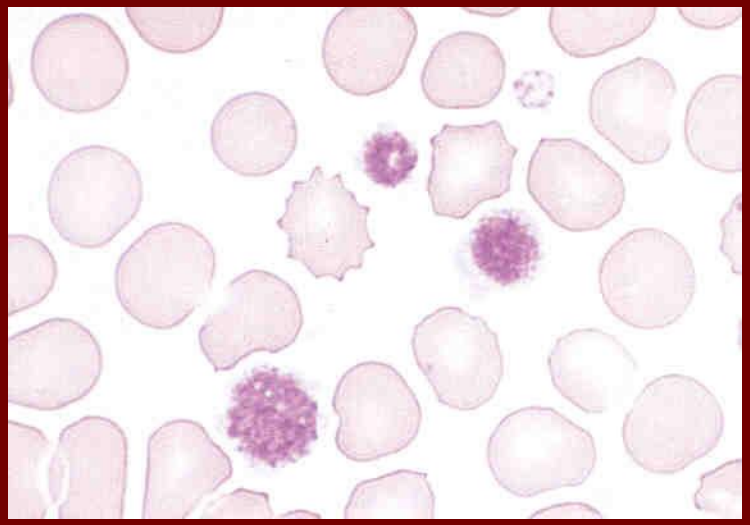
Defects in platelet adhesion and aggregation



Acquired Dysfunctional Platelets

- Drugs: Aspirin, other NSAIDs, other meds
- Uremia
- Acquired VWD
- Myeloproliferative diseases

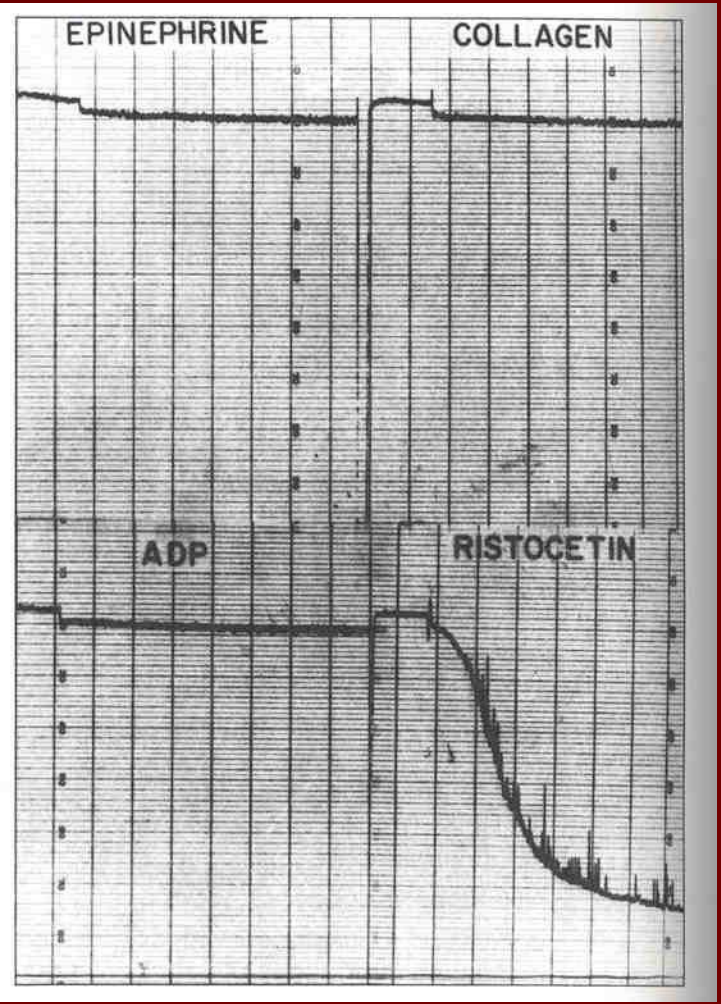
Bernard-Soulier disease Autosomal recessive



Thrombocytopenia with giant platelets

Decreased expression of GP Ib/IX or decreased affinity of GP Ib/IX for VWF

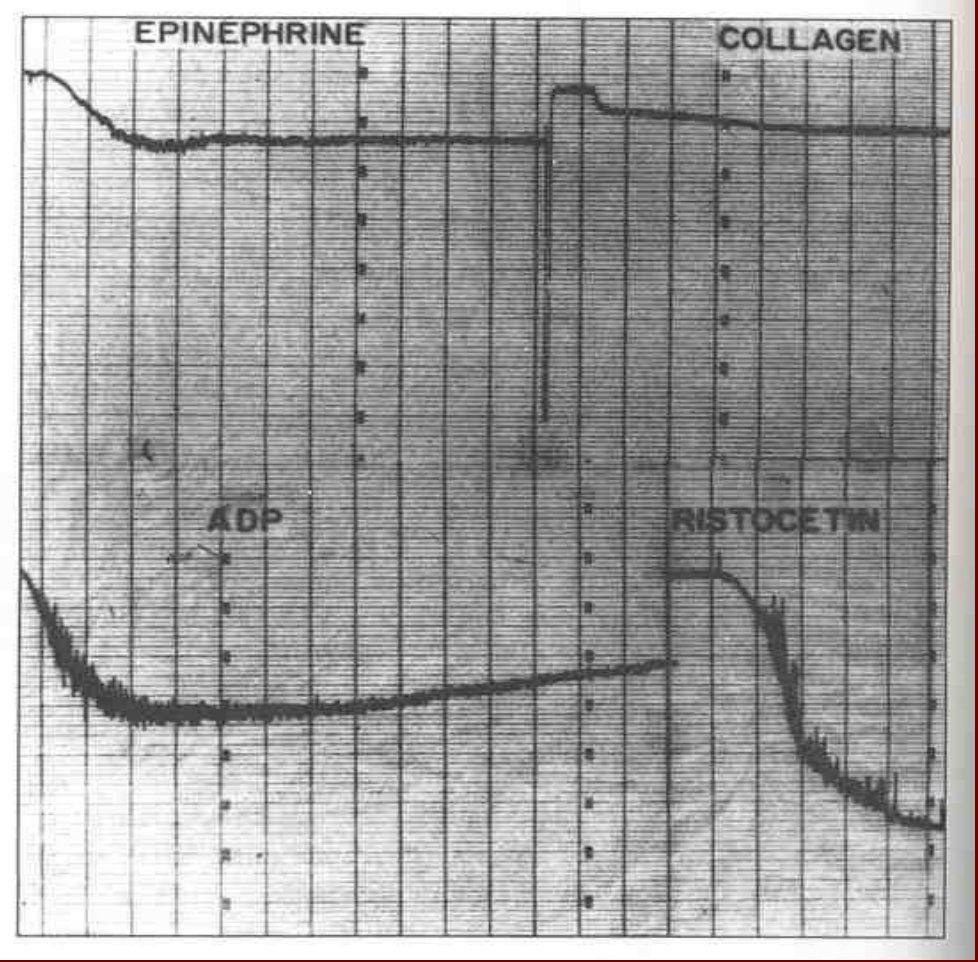
Abnormal platelet aggregation studies: Glanzmann's thrombasthenia



Primary wave defect for all reagents except Ristocetin

Autosomal recessive

Abnormal platelet aggregation studies: Storage pool disease or defective release of storage pool contents (aspirin-like defect)



Secondary waves to ADP and epinephrine absent

Selected Coagulopathy Topics

Topic 1

- vonWillebrand Disease

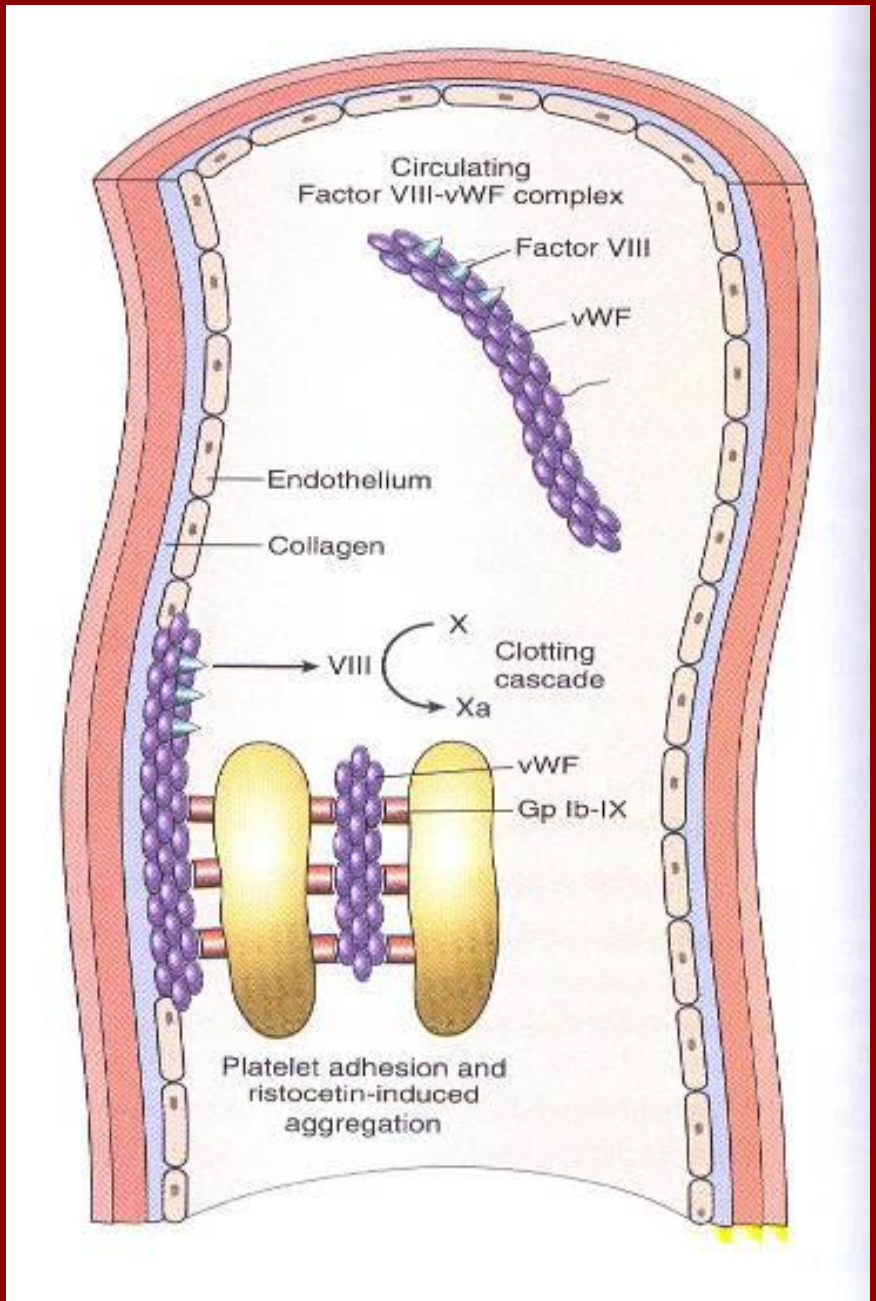
Introduction

- Disorder of primary hemostasis first described in 1926 by Professor Erik von Willebrand : severe mucocutaneous bleeding in a 5 yr old Finnish girl from the Åland Islands; 4 sisters with hemorrhagic deaths before the age of 4; patient died at 13 with her 4th menstrual period
- 1971: the deficient protein was discovered and termed factor VIII-related antigen because it co-purified with factor VIII (FVIII)
- 1976: Zimmerman recognized FVIII R:Ag to be a distinct molecular entity and renamed it vWF protein
- Most common inherited disorder of bleeding in humans with an estimated prevalence of 1-3%

vonWillebrand Disease

- vWD: associated with quantitative and/or qualitative defects of the vWF protein primarily and by deficiency of factor VIII coagulant activity secondarily
- Most types are inherited in a autosomal dominant fashion – males and females, all ethnic groups equally affected
- More than 100 mutations (chromosome 12) in many subtypes of VWD have been described

von Willebrand Factor

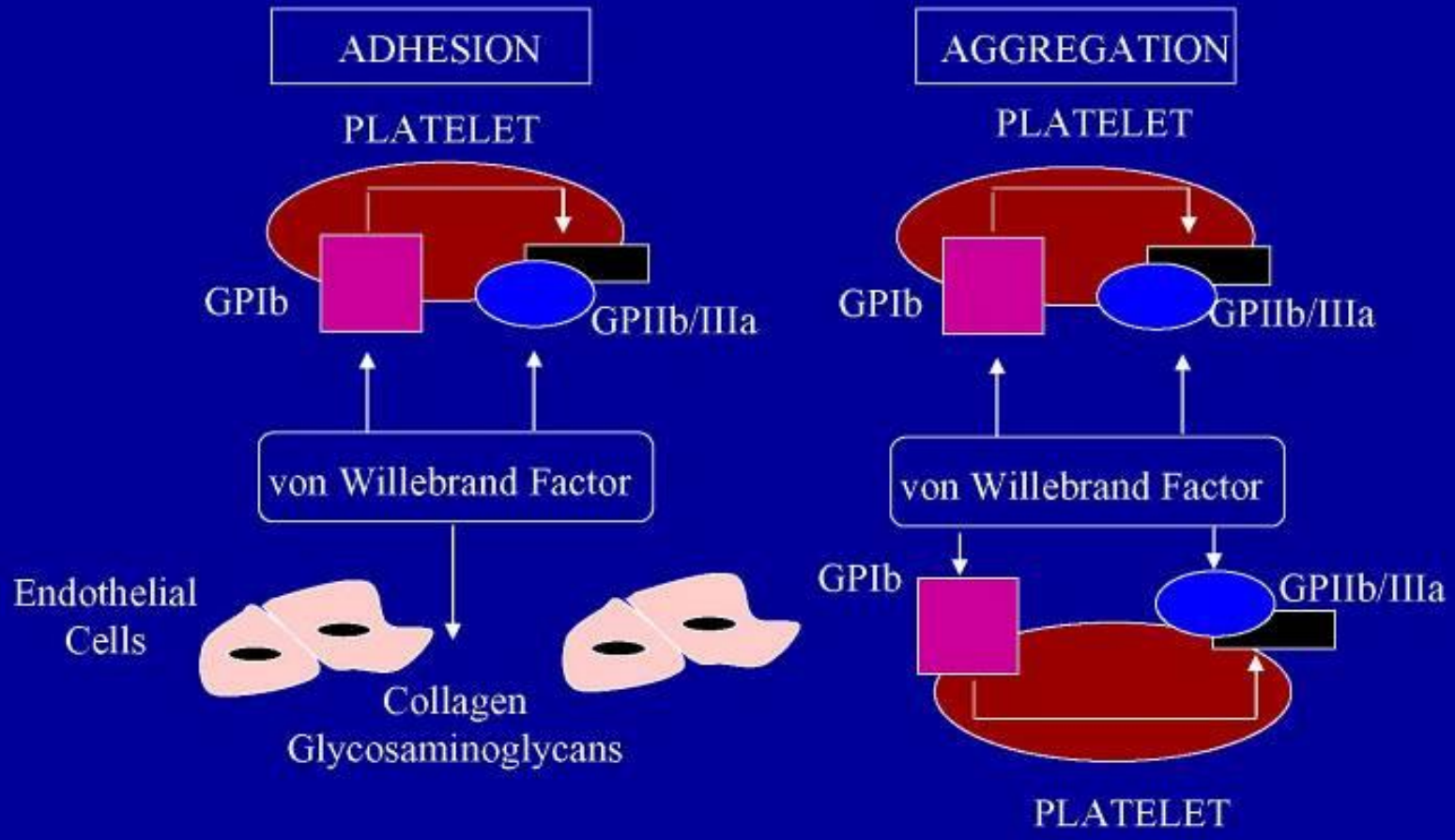


-Synthesized in endothelial cells and megakaryocytes

-vWF stored in Weibel-Palade bodies of endothelial cells & alpha granules of megs/platelets

-vWF levels is higher in:
(a) African Americans, about 15% higher
(b) Chronic inflammation, acute infection, acute trauma
(c) Pregnancy, oral estrogen replacement, or oral contraceptive use
(d) Age, diabetes
(e) Malignancy, stress
(f) Surgery, exercise

vWF Function



Ruggeri ZM, Zimmerman TS. *Blood.* 1987;70:895-904; Meyer D, Girma JP. *Thromb Haemost.* 1993;70:99-104; Furlan M. *Ann Hematol.* 1996;72:341-348.

Symptoms

- Spectrum of clinical severity- many are subclinical
- Recurrent mucocutaneous bleeding, often spontaneous (menorrhagia, epistaxis, gingival bleeding, gastrointestinal/genitourinary bleeding)
- Excessive bleeding from wounds, bleeding following minor trauma, excessive bruising
- Do not have intramuscular or deep subcutaneous bleeding or hemarthroses
- All in the setting of normal platelet count

Type I

- Most common, 75-80% are Type I
- Autosomal-dominant with variable expressivity
- Clinical symptoms usually mild to moderate, sometimes asymptomatic
- Factor VIII activity (VIII:C), vWF antigen (vWF:Ag), and the ristocetin cofactor activity (vWF:RCoF) decreased proprotionately
- Normal spectrum of multimers
- Mild cases respond to DDAVP (1-desamino-8-D-arginine vasopressin)

Type II

- Autosomal dominant
- Much less common with several variants
- Characterized by normal (or slightly-decreased) levels of dysfunctional protein
- Abnormal synthesis (IIa) or consumption (IIb) causing lack of larger multimers in plasma while retaining smaller multimers

Type IIA

- 10-12% of vWD patients
- Autosomal dominant
- Absence of **large** and **medium-sized** multimers in plasma
- Small multimers do not bind effectively to the GPIb receptor on platelets in the presence of ristocetin
- VIII:C N/↓ , vWF:Ag N/↓ , vWF:RCoF ↓↓
- Clinical symptoms usually moderate to severe
- DDAVP ineffective

Type IIB

- 3-5% of vWD patients, much less common than Type IIA
- Autosomal dominant
- Absence of **large** multimers in plasma due to abnormally high affinity for platelet adhesion (via GP Ib)– creates secondary thrombocytopenia
- VIII:C N/↓ , vWF:Ag N/↓ , vWF:RCoF ↓↓
- Increased platelet aggregation with low conc of Ristocetin
- Adverse response to DDAVP due to release of abnormal large multimers

vWD: Types 2N (Rare)

- Also called vWD-Normandy and "autosomal hemophilia"
- 1%-2% of all vWD patients
- Results when a genetic defect prevents vWF from binding to FVIII, causing low level of F VIII
- vWF alleles:
  2N/2N (pt with normal vWF level), or
  2N/vWD-type 1 (pt with low vWF level)
- Often misdiagnosed as mild hemophilia A

vWD: Types 2M (Rare)

- Characterized by decreased binding by vWF to platelet GPIb
- 1%-2% of all vWD patients
- Autosomal dominant
- Normal multimeric pattern

Type III

- Rare (1-3% of vWD patients), very severe, autosomal recessive form
- Usually offspring of two parents, both with mild type I disease
- No detectable vWF or F VIII activity
- Severe & spontaneous mucosal bleeding, sometimes with hemarthroses (similar to hemophilia)
- No response to DDAVP

Platelet Type (Pseudo) vWD

- Mutation in the GPIb gene that produces increased affinity of platelets to vWF
- Lack of large multimers secondary to clearance by platelet binding
- Clinical presentation and laboratory results are similar to thase of Type IIB

Acquired vWD

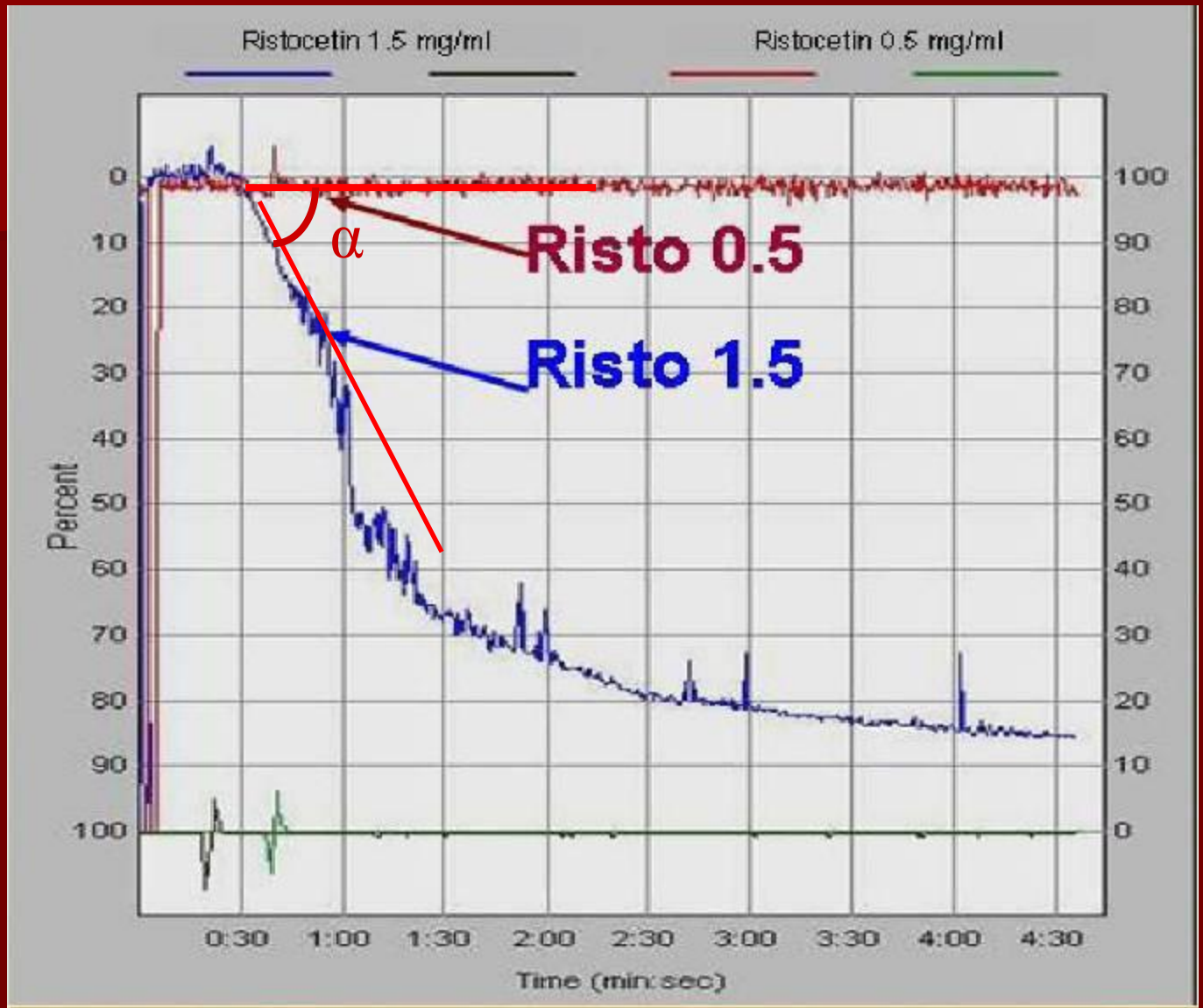
- Extremely rare , fewer than 100 well-documented cases
- IgG autoantibodies to the VIII:vWF complex or absorption by malignant cells (e.g. essential thrombocythemia)
- Associated with immunologic disorders (lymphoma, SLE, MM, myeloproliferative neoplasm, benign monoclonal gammopathy), medications (valproate, ciprofloxacin),
- Inhibitors to vWF may develop following replacement therapy
- Treat the underlying disorder (i.e. corticosteroid therapy or chemotherapy/radiation), as well as acute symptoms of bleeding

Laboratory results for vWD

Parameter	Type 1	Type 2A	Type 2B	Type 3
Bleeding time	↑ or N	↑	↑	↑
Platelet count	N	N	↓ or N	N
vWF:Ag	↓	↓ or N	↓ or N	↓↓
vWF:RCo	↓	↓	↓	↓↓
Multimers	N (decreased amount)	Abn	Abn	Not detectable
VIII	↓	↓ or N	↓ or N	↓↓
RIPA	↓	↓	↑	↓↓

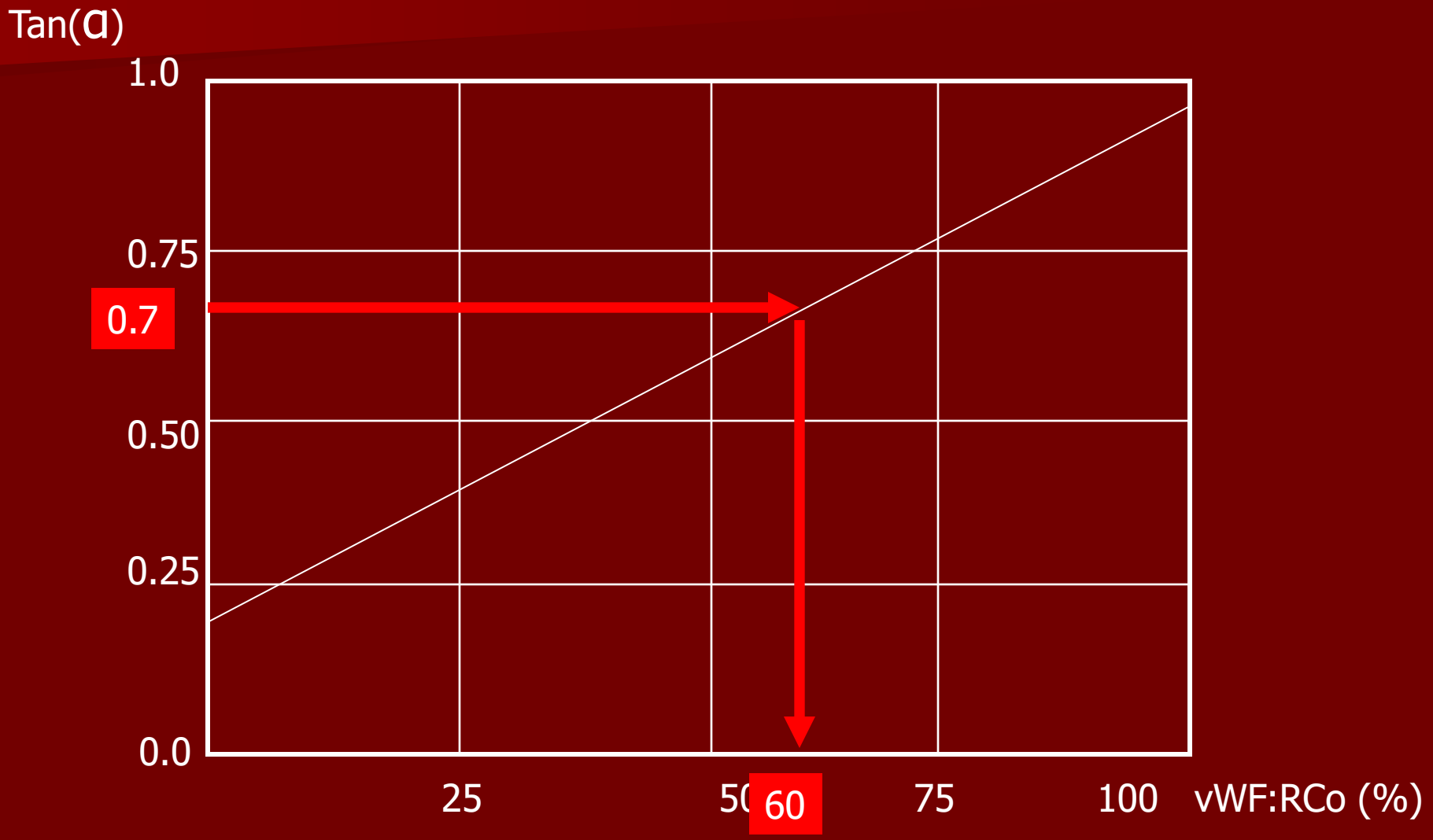
Ristocetin Cofactor (VWF:RCo)

- Ristocetin (ristomycin) is an antibiotic from the vancomycin group, which is active against gram positive bacteria and mycobacteria. It was introduced for clinical use in 1956-7 and removed from use in 1960.
- VWF:RCo : quantitative assay that determines VWF function (activity) in patient's plasma
- A log-log relationship exists between degree of ristocetin-induced platelet aggregation of formalin-fixed platelets and concentration of VWF in patient's plasma

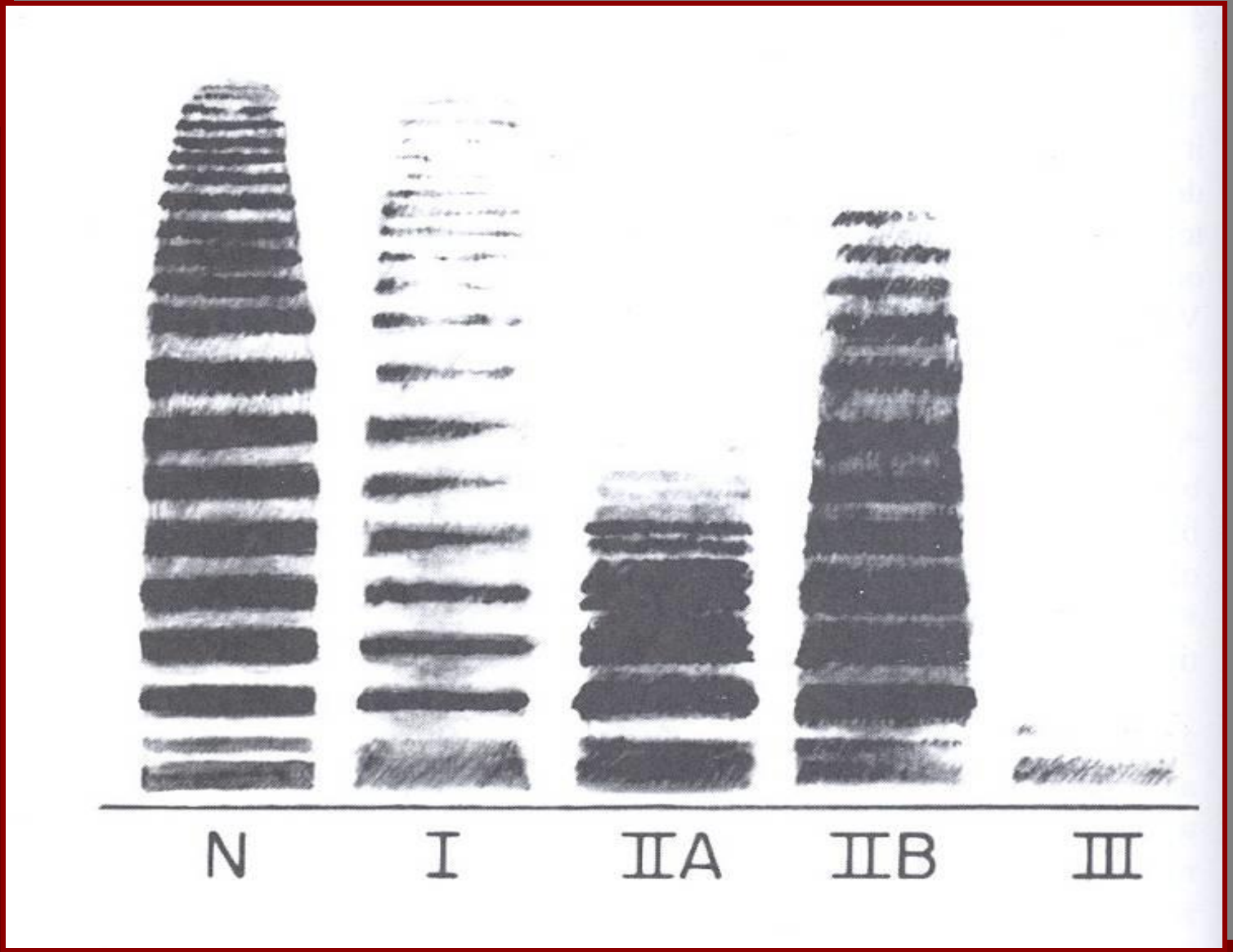


Normal tracing using platelet rich plasma (PRP)

vWF:RCo Standard Curve



Multimer analysis



Special testing for vWD-N and pseudo-vWD

- vWD-N: FVIII-vWF binding assay
- Pseudo-vWD: patients' platelets will aggregate with cryoprecipitate (containing normal vWF)

Treatment

- Type I – DDAVP (1-desamino-8-D-arginine vasopressin), Stimate™- given intranasally or intravenously , 0.3 ug/kg BW (over 30 min) usually sufficient for transient control of bleeding for minor surgical procedures. For severe cases, use vWF/FVIII Concentrates (Humate-P, half-life 12 hrs)
- Type 2A – vWF/FVIII Concentrates
- Type 2B – vWF/FVIII Concentrates , DDAVP contraindicated
- Type 2N- vWF/FVIII Concentrates
- Type 3 – vWF/FVIII Concentrates

- Pseudo vWD-platelet concentrates for thrombocytopenia

FVIII/vWF dosing

- Loading dose of 50-75 IU/kg body weight
- Then, 40-60 IU/kg body weight every 8-12 hours for 3 days
- Then, 40-60 IU/kg body weight daily for 7 days

Targeted levels for bleeding patient

- FVIII: 50%-100%
- vWF:RCo: 50%-100%

- Note that cryoprecipitate should not be used (infection risk) if vWF/FVIII concentrates are available

Topic 2

- Disseminated Intravascular Coagulation

DIC

- Concurrent activation of the coagulation (thrombin) and secondary fibrinolysis (plasmin) with consumption of factors, inhibitors, platelets, and RBCs (microangiopathic hemolysis)
- The major triggering mechanism: exposure of the blood to tissue factor that initiates intense coagulation, overwhelming antithrombin and activated protein C
- Secondary to sepsis, malignancy (pancreatic cancer, APL, others), obstetrical complications (placental abruption, fetal demise, amniotic fluid embolism), tissue injury (esp. brain in head injury), etc.

Clinical presentation

- Most frequent: bleeding due to low levels of clotting factors and platelets
- Less often: vascular thrombosis (if fibrinolytic system or protein C is impaired)

Typical laboratry results in DIC

- ↑ PT & PTT, thrombocytopenia, ↓ fibrinogen, ↑ Thrombin Time, ↑ FSP/D-dimer
- Schistocytes in peripheral blood smear, ↑ LDH, ↓ haptoglobin
- Laboratory results in DIC vary greatly depending on the severity

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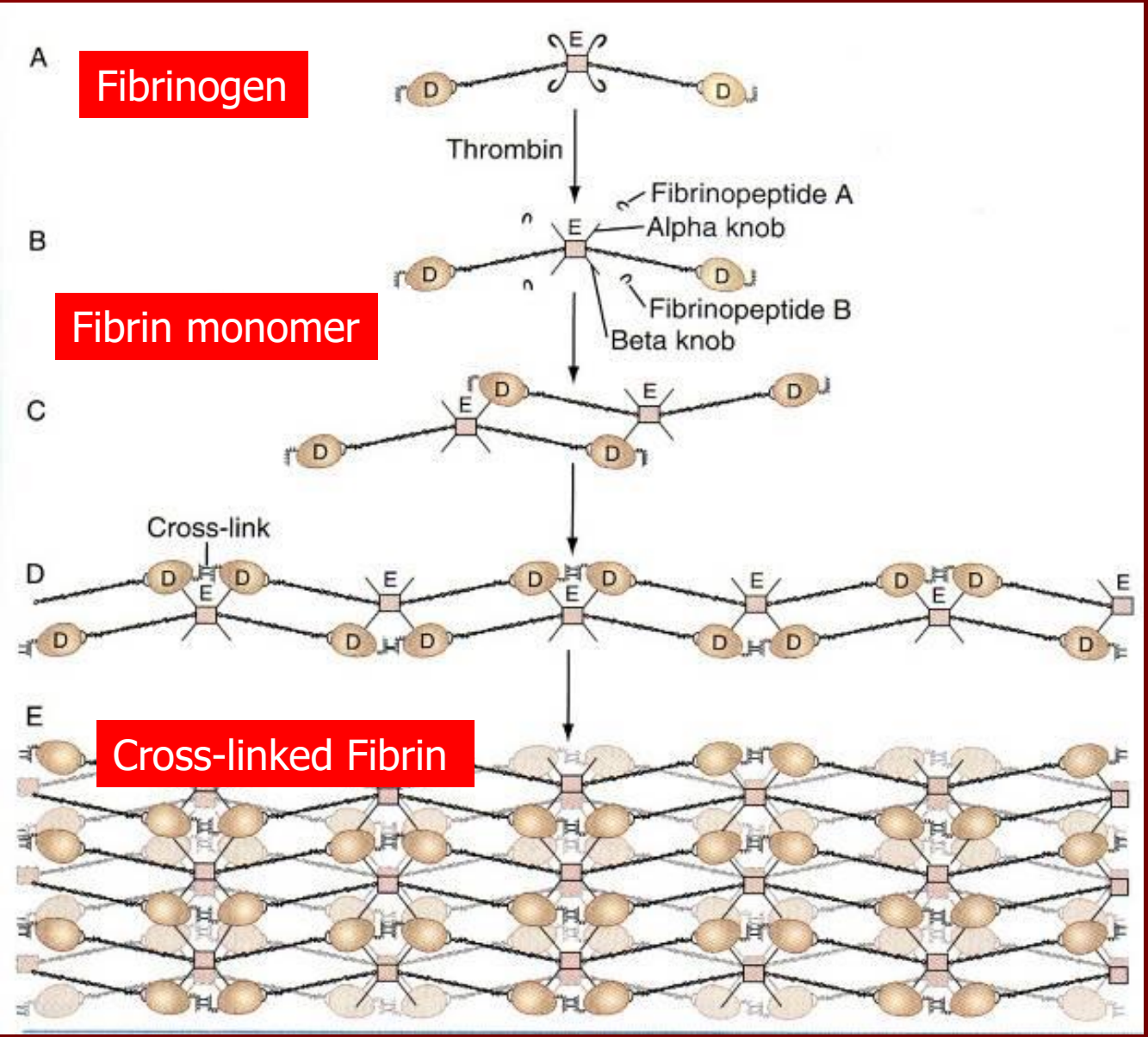
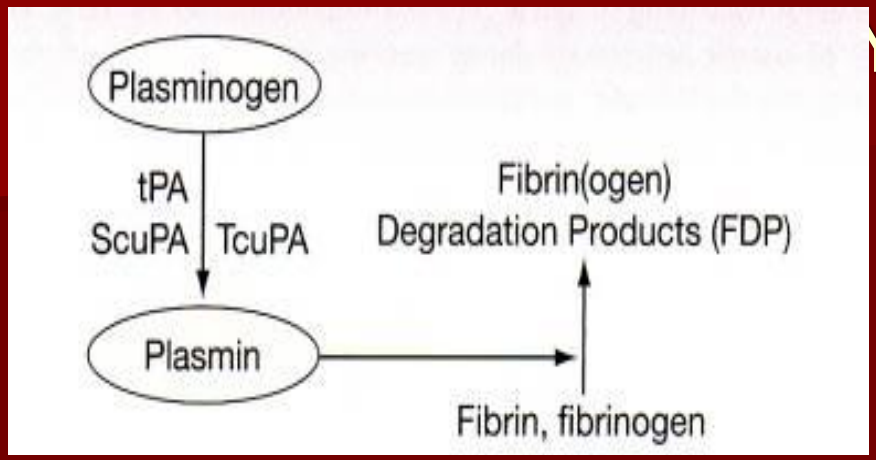
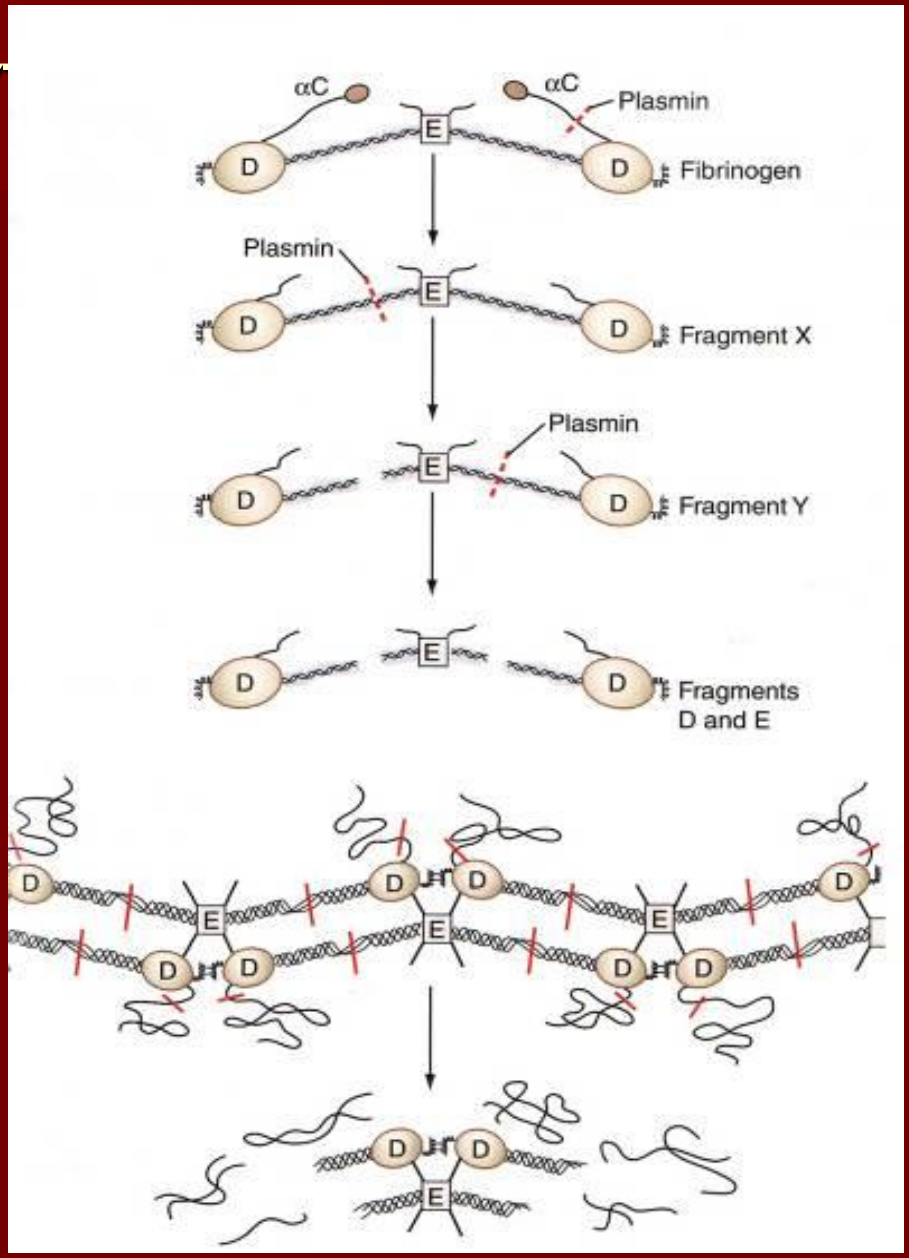




- Activators:
  - Tissue plasminogen activator (tPA)
  - Urokinase plasminogen activator (uPA)
- Inhibitors:
  - Plasminogen activator inhibitor-1 (PAI-1)
  - α2-antiplamin

D-dimer



FDP vs. D-DIMER

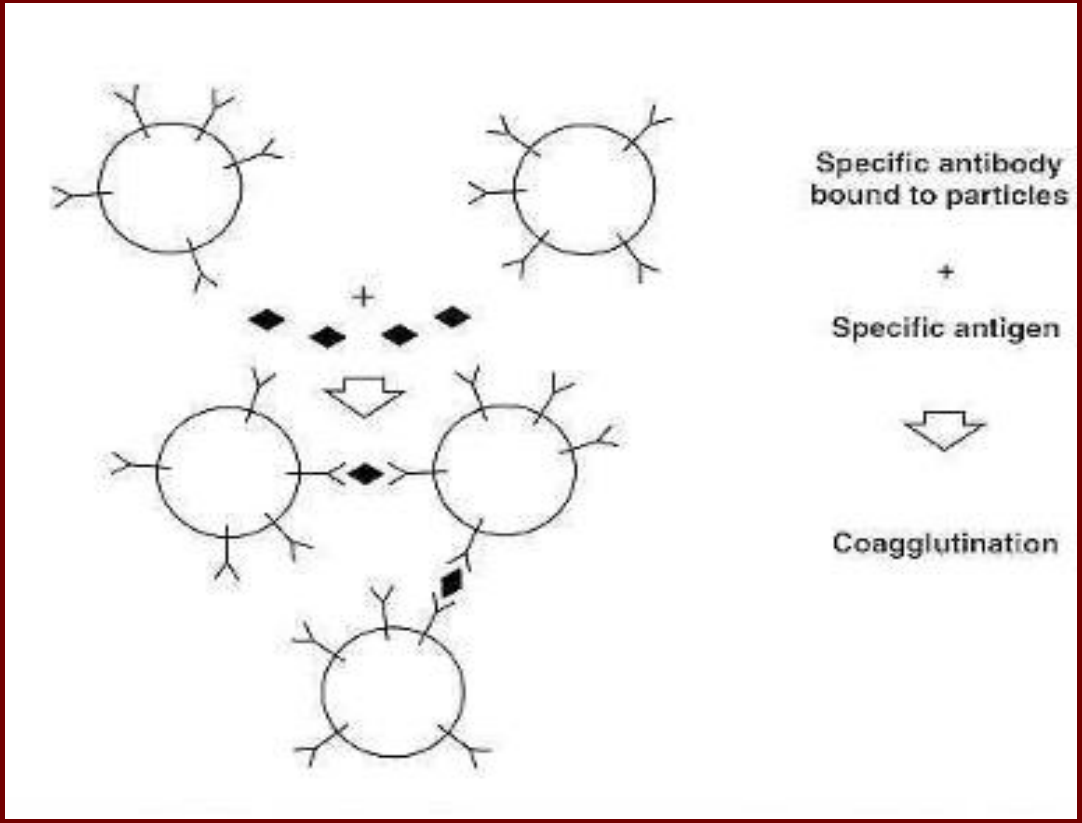
- Fibrin is formed as the end result of coagulation cascade activation
- Fibrinolysis causes cleavage of fibrinogen, fibrin, and fibrin clot, yields FSP (FDP)
- Only cleavage of fibrin clot (crosslinked fibrin) yields D-dimer -> D-dimer is more specific for DIC

Testing: FSP and D-dimer

- Semi-quantitative FSP
- Qualitative D-dimer
- Semi-quantitative D-dimer
- Quantitative D-dimer

SEMI-QUANTITATIVE FSP

- The first test developed (in the early 70's)
- Latex agglutination, FSP antibodies are bound on latex beads, if sample contains FSP, agglutination can be detected



SEMI-QUANTITATIVE FSP

- Semi-quantitation:
  - Serial dilution of sample (1: 20 through 1:640)
  - A positive result at 1:20 corresponds to 20 µg/mL of fibrinogen equivalent units (FEU)
- False-positive result with rheumatoid factor
- Clinical application: DIC, hyperfibrinolysis

QUALITATIVE D-DIMER

- Monoclonal antibodies directed against D-dimer domain
  - More specific for in-vivo fibrin clot formation
- Manual latex agglutination technique (as for FSP), plasma or serum sample:
  - Cut-off value: 0.5 µg/mL FEU
  - Semi-quantitative format: dilutions 1:2 through 1:16
- Abnormal result in DIC
- Normal result in primary fibrinolysis
- False-positive result by rheumatoid factor

QUANTITATIVE D-DIMER

- Automated ELISA, immuno-turbidimetry
- Increased in DIC (>0.66 µg/mL)

- Quantitative D-dimer also has high negative predictive value for venous thromboembolism (VTE including DVT, PE):
  - <0.4 µg/mL, VTE can be ruled out
  - Very sensitive but not specific: high Negative Predictive Value / low Positive Predictive Value

DIC: Treatment:

- Treat underlying conditions
- Blood components (RBC, platelet concentrate, cryo, FFP)

Topic 3

- Factor VIII Deficiency (Hemophilia A)

Early Observations

- 1828: The word "hemophilia" first appeared in a description of the condition written by German Physician Frederick Hopff at the University of Zurich
- 1840: First recorded case of hemophilia treatment by transfusion
- 1893: First documentation of abnormal prolongation of coagulation in capillary tube in hemophilia
- 1920-1930: Hemophilia treatments published; plasma for transfusions introduced
- 1937: IV administration of redissolved plasma precipitate (cryoprecipitate) shown to shorten blood clotting time

New Discoveries

- 1952: Evolution of the definition of hemophilia: a blood clotting disorder affecting males with two possible major protein deficiencies: FVIII -Hemophilia A, FIX - Hemophilia B
- 1966: commercial availability of FVIII concentrates (plasma-derived)
- 1969: FIX concentrate licensed

Hemophilia A

- X linked recessive
- 30% cases result from spontaneous mutation
- Affects all races and ethnic groups equally
- Moderate & mild deficiencies are frequently under-diagnosed
- Affected males -> no sons are affected ; all daughters are carriers
- Female carriers-> affected sons, carrier daughter; normal sons/daughters

Bleeding Pattern correlated with F VIII level

- Severe: <1% , spontaneous hemorrhage, ~1 per week
- Moderate: 1-5% , hemorrhage with incidental injury , ~ 4 –6 / year
- Mild: 5-30% , hemorrhage with injury or surgery, bleeding uncommon
- Subclinical: 30-50%, hemorrhage with major injury or surgery , bleeding very uncommon

Main Sites of Bleeding

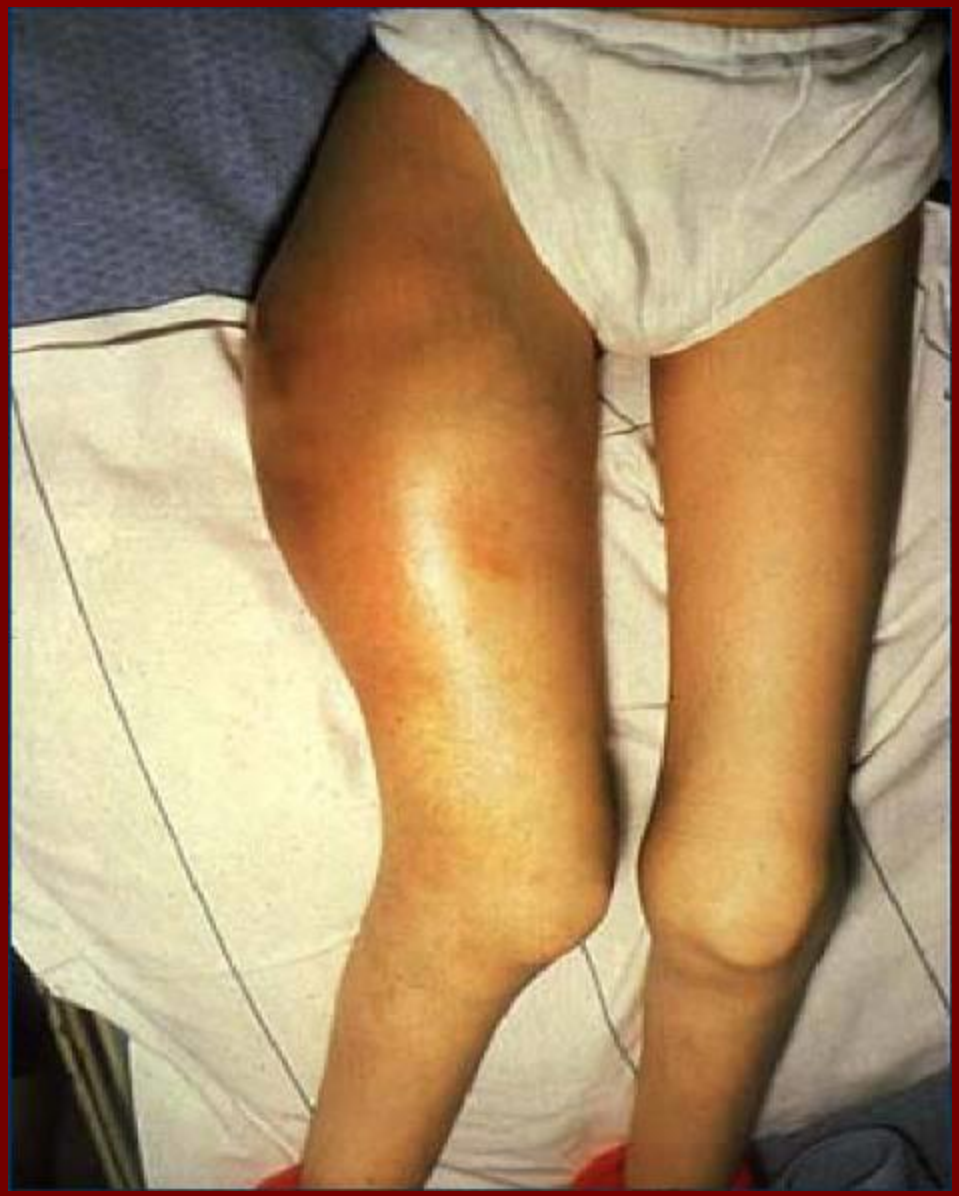
Joint:

- Acute: pain, swelling, interference with normal activities
- Chronic: synovial hypertrophy and synovitis leading to hemophilic arthropathy, disability

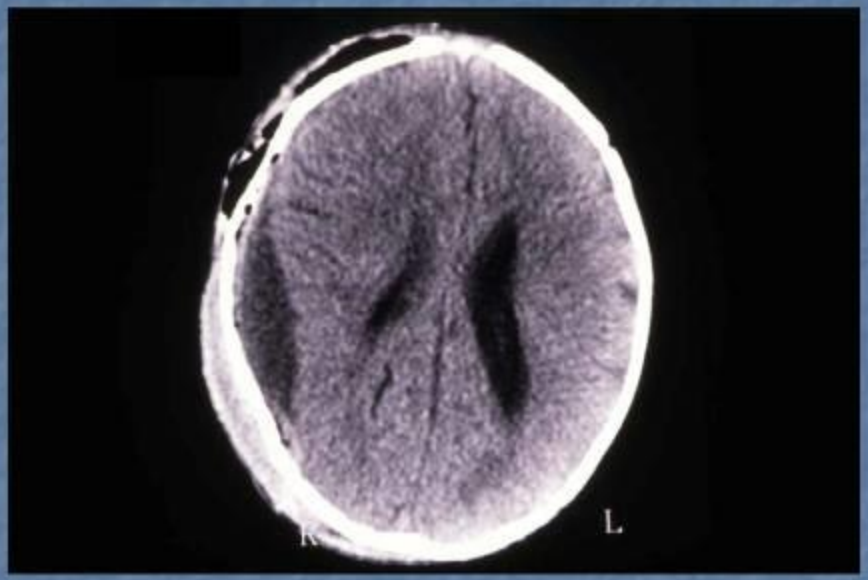
Muscle:

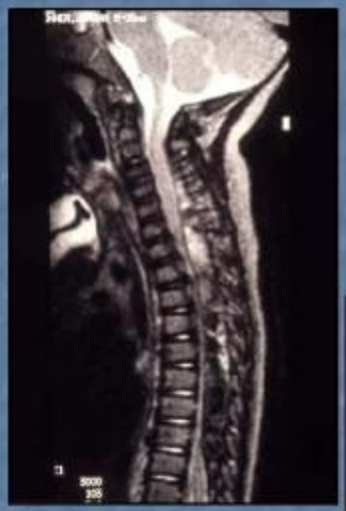
- Limb dysfunction
- Compartment syndrome due to nerve compression

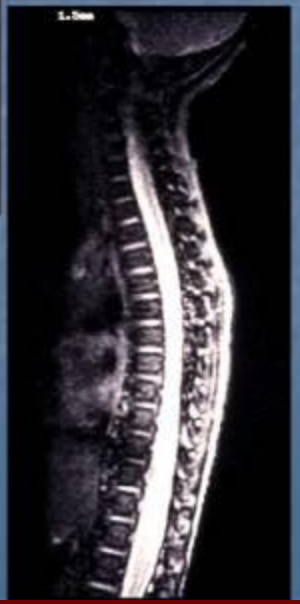
Acute joint swelling due to bleeding



CNS Bleeding







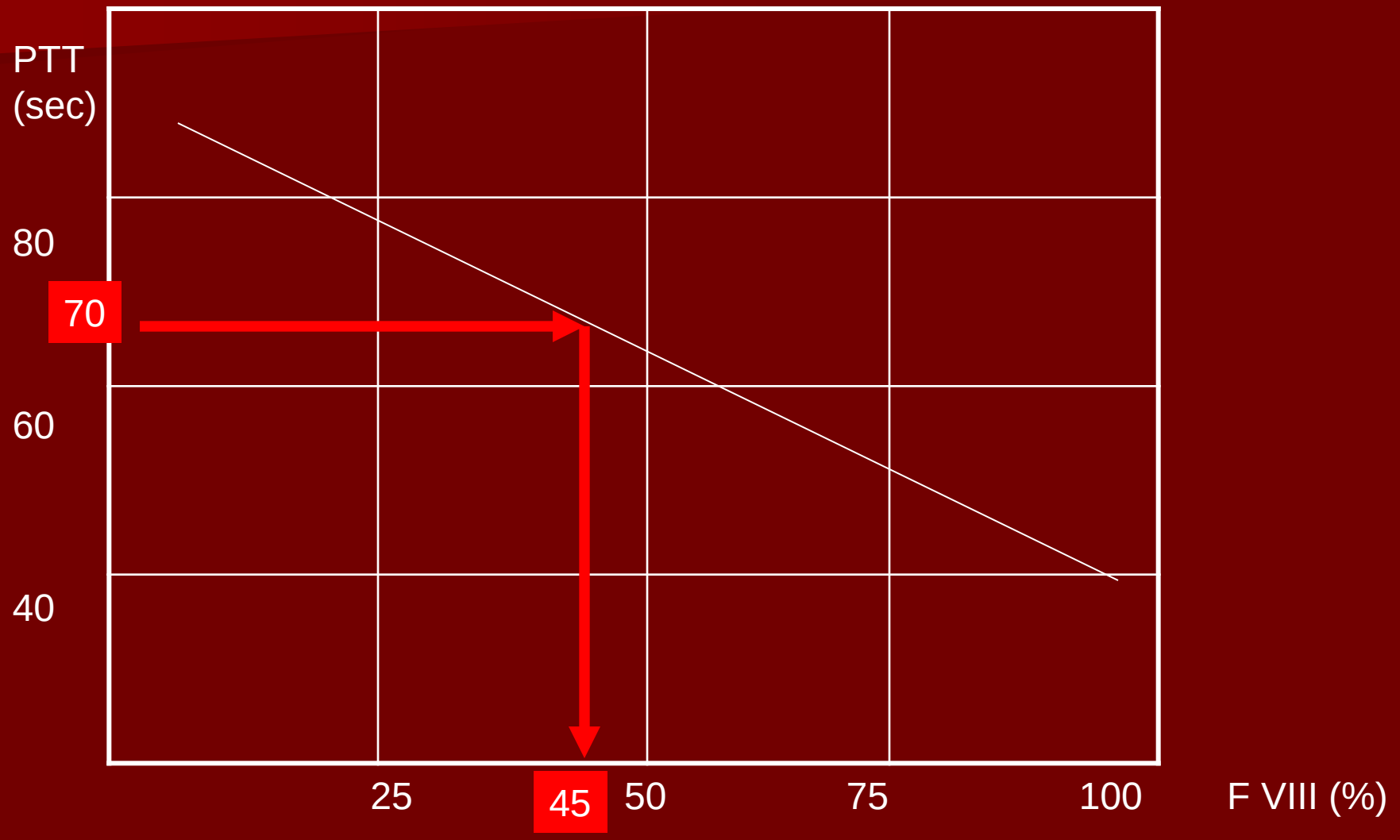
Hemophilia A Carriers

- ~ 1/3 have low factor levels
- May experience bleeding symptoms seen in mild deficient states
- Bleeding after dental extraction, tonsillectomy, other surgery, delivery/post partum
- Treat carriers as potential bleeders

Factor VIII assay

- Factor VIII level is inversely proportional to PTT
- A standard curve (PTT vs F VIII) is first set up using commercial assayed samples
- Mutiple dilutations of patient's sample (using F VIII-deficient substrate) are tested for PTT.
- These PTT's are plotted on the standard curve to intrapolate for F VIII
- Each F VIII is multiplied by the dilutation factor to obtain the actual F VIII before dilution
- F VIII level is the mean of F VIII's from multiple dilutions

Factor VIII Standard Curve



Treatment for Hemophilia A

- DDAVP (1-desamino-8-D-arginine vasopressin) for mild cases (2-10 fold increase in Factor VIII level)
- Factor VIII replacement, 1 unit/kg BW raises FVIII level by 2%, $T^{1/2}$=8hrs
- Factor VIII types: plasma-derived, recombinant, porcine

Example for F VIII Dosage

- 1 IU/kg BW raises FVIII level by 2%
- Patient with 70 kg BW who needs to increase FVIII level from 0% to 100%:
  Dosage = 70 x (100 – 0) / 2 = 3,500 IU

(Note: for hemophilia B: 1 IU/kg BW raises FIX level by 1%)

Target FVIII activity

- Surgery, CNS bleeding, GI and genitourinary bleeds -> 100%
- Bleeding into joints and muscle -> 40-80%

F VIII Inhibitor Development

- Most serious complication of hemophilia A management
- Plasma-dervived FVIII and rFVIII carry similar inhibitor risk
- Inhibitor prevalence:
  - ~ 30% of severe population
  - ~3-13% in moderate deficiency

Factor VIII Inhibitor Assay

- Measured in Bethesda Unit (BU)
- 1 BU = quantity of inhibitor in patient's plasma that results in loss of 50% factor activity in normal plasma sample (1:1 mix) after incubation for 2 hours at 37°C
- Positive for inhibitor: > 0.5 BU
- High responding inhibitor: titer > 5 BU
- Low responding inhibitor. titer < 5 BU despite repeated exposure

Treatment for F VIII inhibitor

- More F VIII concentrates for mild case (double the dosage)
- For more severe cases:
  - Porcine F VIII (potential for development of inhibitor against porcine F VIII)
  - FEIBA (Factor eight inhibitor bypassing activity) consisting of F IIa, VIIa, IXa, Xa
  - Novo Seven (F VIIa)
- High risk of thrombophilia with FEIBA, Novo Seven

Topic 4

- Vitamin K Deficiency

Vitamin K Deficiency

- Vitamin K dependent proteins:
  II, VII, IX, X and protein C and S
- The Vit K-dependent factors (II, VII, IX, X) have 9-12 glutamic acid residues near the amino terminal end, which need to be carboxylated (Vit K dependent) to bind calcium to phospholipid membranes.
- In Vit K deficiency, Vit K-dependent factors cannot bind to phospholipid membranes to maintain the coagulation cascade
- The same effect is seen in Coumadin treatment (Vit K antagonist)

Vitamin K deficiency in adults and children

- Malabsorption of fat-soluble vitamins (bile duct atresia, celiac disease, short-bowel syndrome, etc.)
- Inadequate intake (prolonged fasting)
- Medications (coumadin, antibiotics esp. cephalosporins)

Vitamin K deficiency in Infancy (hemorragic disease of the newborn)

- Premature
- Maternal anticonvulsant medications (phenytoin, phenobarbital, valproic acid, carbamazepine)
- Breast-feeding (human milk is lower in Vit-K compared to cow's)

Typical test results

- Prolonged PT, PTT (PT >> PTT)
- Mixing PT/PTT show correction
- Decreased Vit-K dependent factors (II, VII, IX, X)

Treatment

- For bleeding patients: FFP
- For all patients : Vit-K given subcutaneously
- Dosage:

  -Adults: 10 mg

  -Infants: 1-5 mg

  -Older children: 5-10 mg
- PT is typically corrected in 4-8 hrs

Prophylactic treatment for infants

- For all infants: 1 mg Vit K_1 (IM) at birth (regardless of being premature or not)
- Breastfed infants: 1 mg Vit K_1 (oral) weekly
- Mothers on antibiotics/anticonvulsants: stop medications and take oral Vit-K (10 mg Vit K_1 daily) for 2 weeks before delivery

Topic 5: Hypercoagulation (thrombophilia)

- Hypercoagulation: poorly understood phenomena
- No definite cause is identified in > 40% of cases
- Three major factors in thrombus formation (Rudolf Virchow, 1845): decreased blood flow; changes in the circulating blood (coagulation factors & inhibitors); changes in the vessel wall

Hypercoagulation Disorders

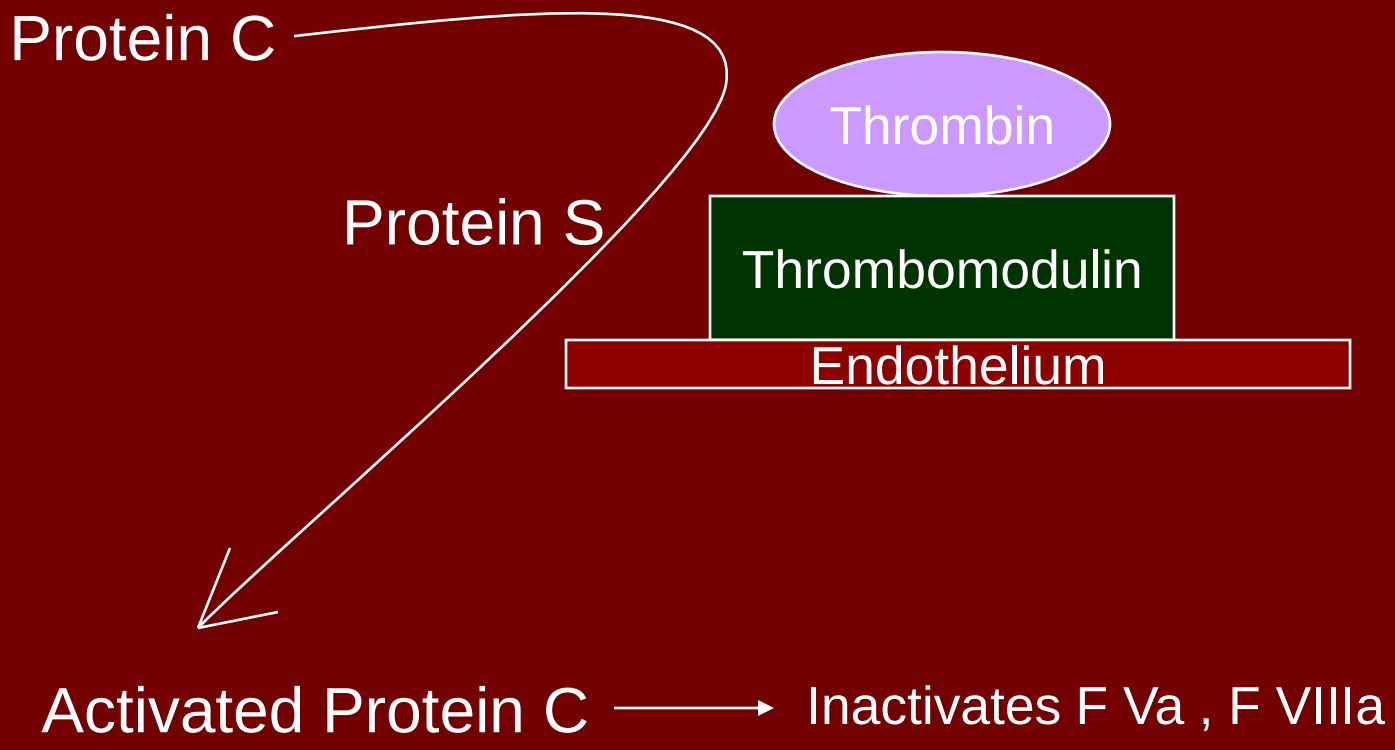
Factor V Leiden (12-40% of hypercoagulation cases), V
Prothrombin gene mutation (6-18%), V
Protein C deficiency (6-10%), V
Protein S deficiency (5-10%), V
Antithrombin III (AT III) deficiency (5-10%), V
Lupus anticoagulant (LA) (10-20%), A+V
Anticardiolipin antibodies (ACA) (5-10%), A+V
Heparin-induced thrombocytopenia, A+V
Hyperhomocysteinemia (10-20%), A+V
Hypercoagulation in COVID19 infection, A

- Legends: A (arterial thrombosis), V (venous thrombosis)

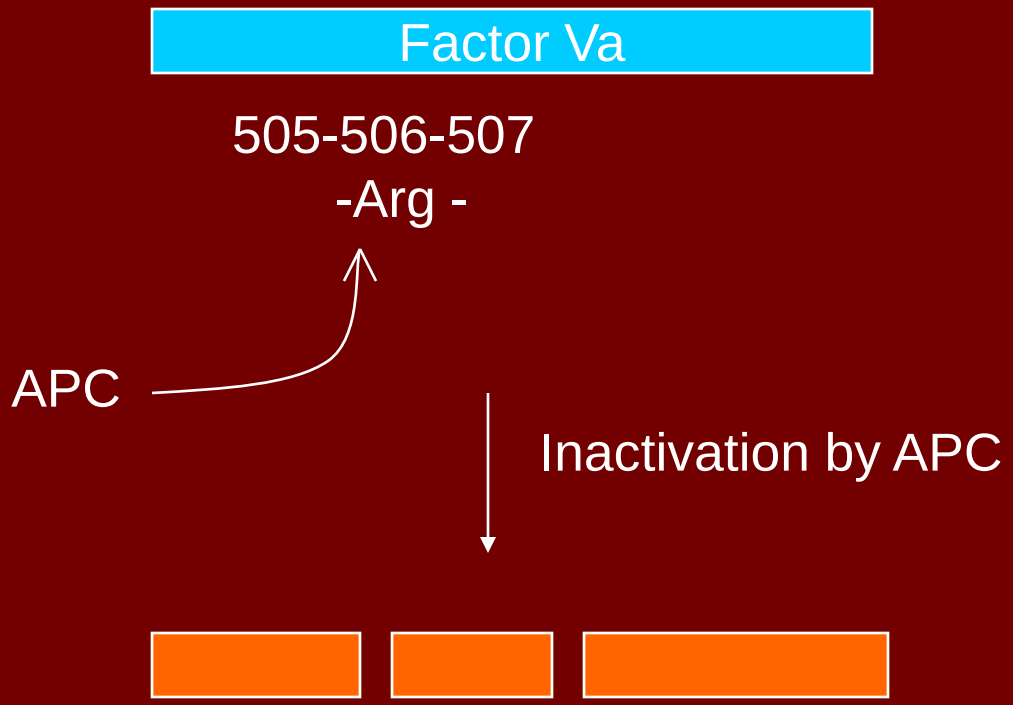
F V Leiden: INTRODUCTION

- Dahlback described an inherited (autosomal dominant) disorder associated with venous hypercoagulation (1993).
- This disorder is due to a mutation in Factor V gene on chromosome 1 (the mutated gene is called Factor V Leiden). Mutation at nucleotide 1691: Guanine-> Adenine, causing substitution at position 506: Arginine-> Glutamine [V506Q]
- Note: FV HR2 haplotype (A4070G, His199Arg) has unknown risk

Protein C Pathway

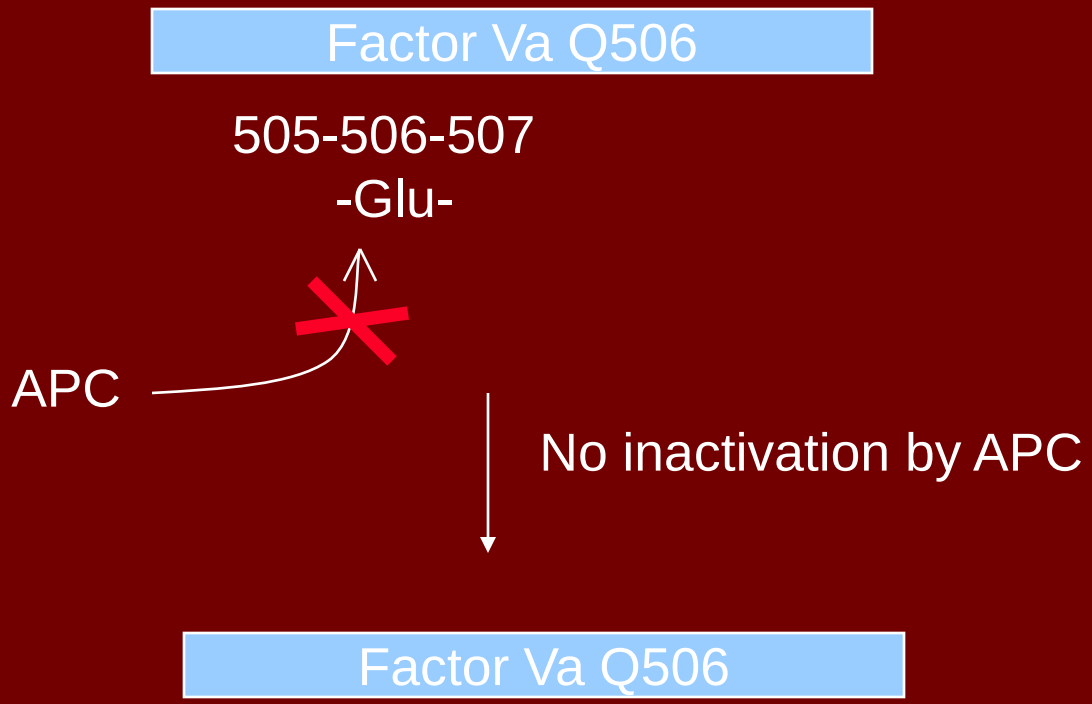


Cleavage Site on Factor V by APC:
Inactivation of Factor V in normal patient



Cleavage Site on Factor V by APC:

No inactivation of Factor V in patient with Factor V Leiden (95% of APC resistance cases)



Two Forms of Factor V Leiden

- Heterozygous: 3-7% of general population, 3-5 fold increase in risk of deep vein thrombosis, 20% have thrombosis by 33 y/o (mean age of first thrombotic episode)
- Homozygous: 0.06-0.25% of general population, 50-100 fold increase in risk of deep vein thrombosis, 40% have thrombosis by 33 y/o

Other Relevant Information on Factor V Leiden

- Some patients do not have thrombosis unless exposed to hemostatic challenge
- Increased risk for hypercoagulation in combination with other risk factors (such as Lupus anticoagulant, Protein C or S deficiency)
- Factor V procoagulant activity is normal
- Treatment: heparin, coumadin

PCR Testing for F V Leiden

- Amplifies the mutated gene fragment. Results: negative, heterozygous, homozygous.
- Results not effected by factor deficiency, lupus anticoagulant, anticoagulant (as in clot-based tests)

Note: PCR testing cannot detect APC resistance that is not due to FV Leiden

PCR Testing (cont'd)

- Genomic DNA from lymphocytes
- DNA sequence flanking the mutation site is amplified by PCR, resultant product is analyzed by restriction enzyme digestion
- Normal (wild type): two normal FV alleles
  Heterozygous: one abnormal allelle
  Homozygous: two abnormal allelles

New PCR Testing: LightCycler (Roche)

- Melting curve analysis method
- “Real time” analysis
- 35 thermal cycles in 25 min, followed by melting curve analysis in 5 min -> results in 30 min
- Batch of 32 samples
- Designed for clinical lab setting
- Optional module for automated DNA extraction (60 min for 32 sample extraction)

MagNA Pure LC and LightCycler

Magnetic Bead Technology for DNA Extraction

LightCycler Schematics

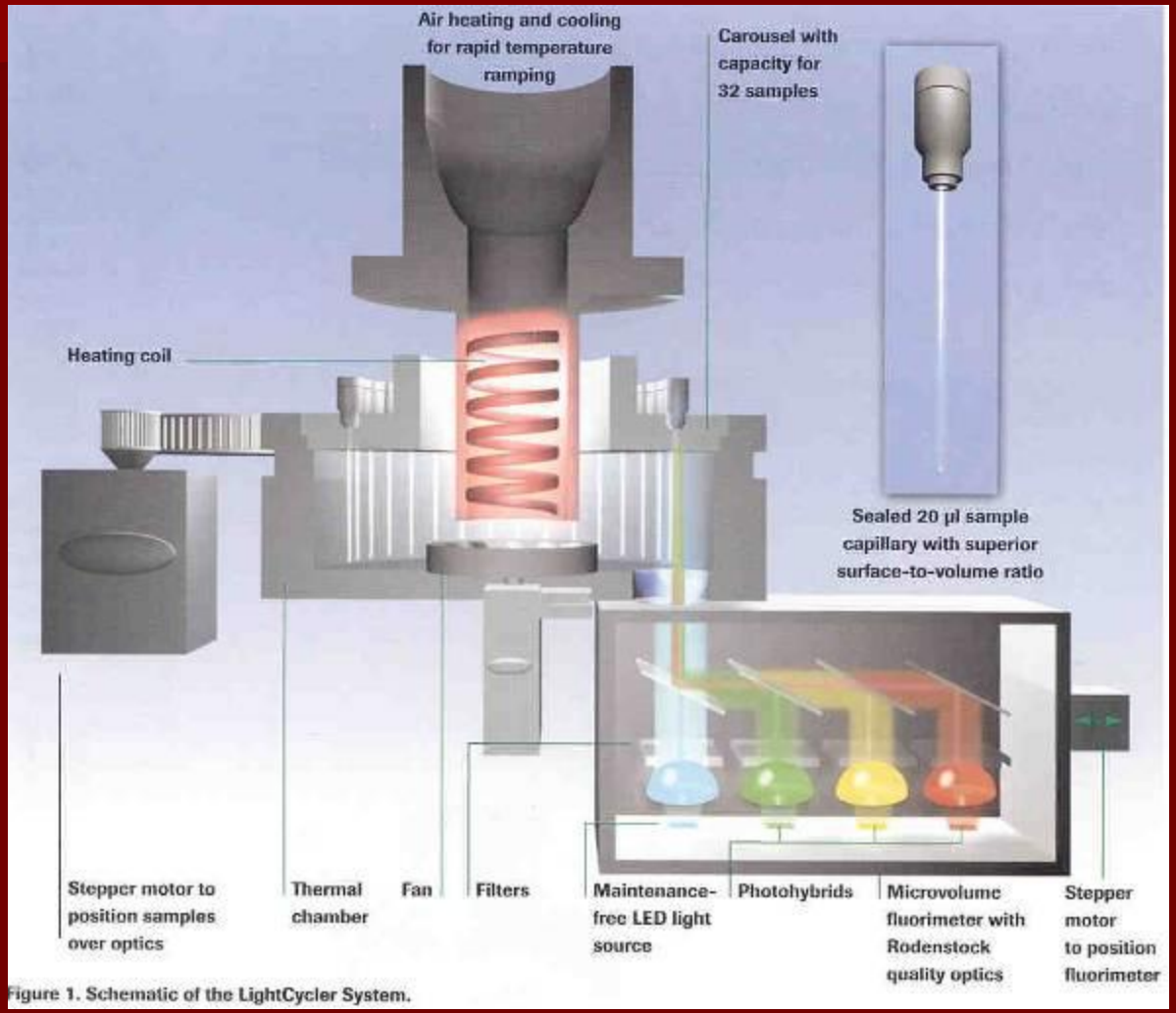
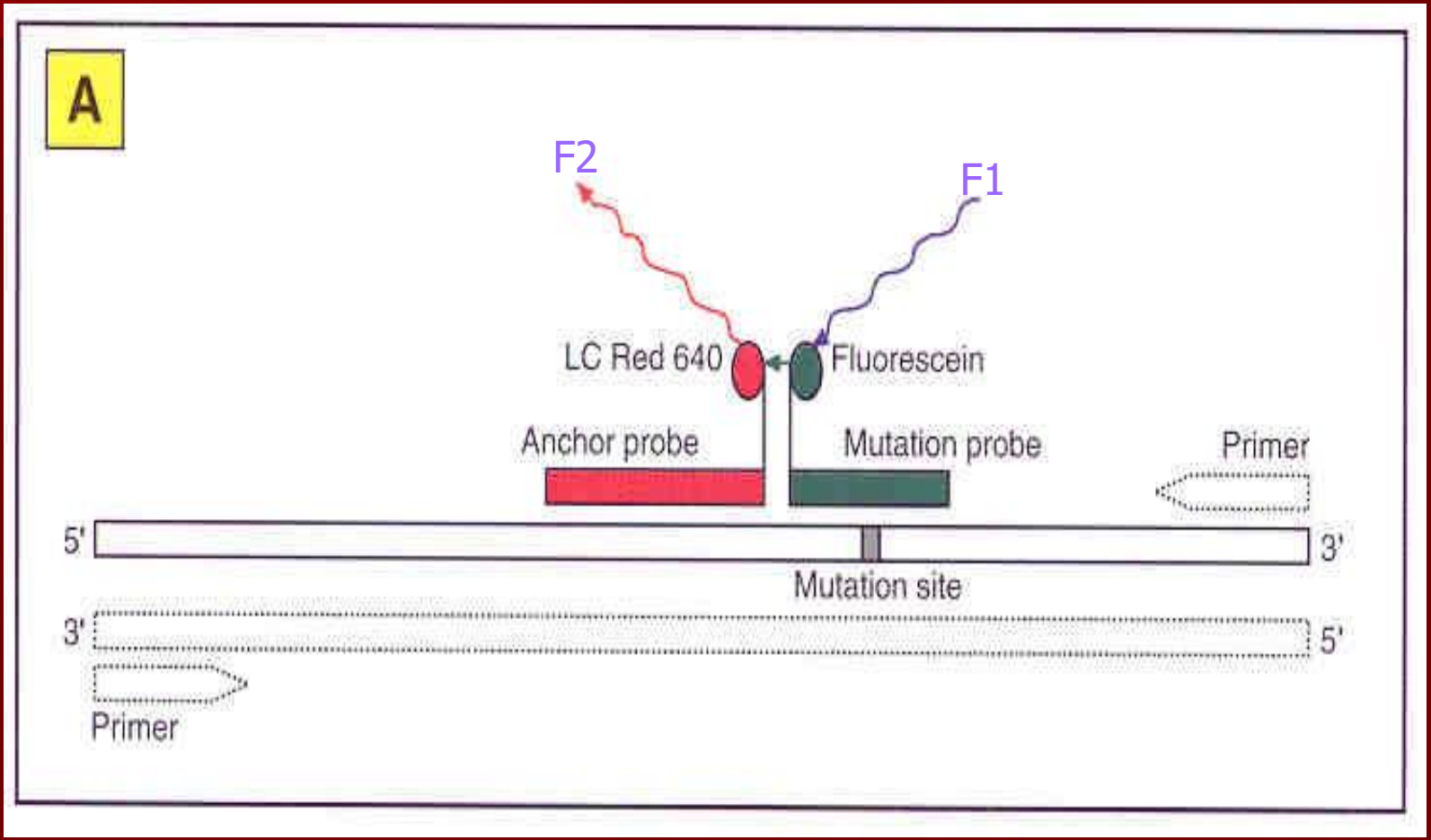
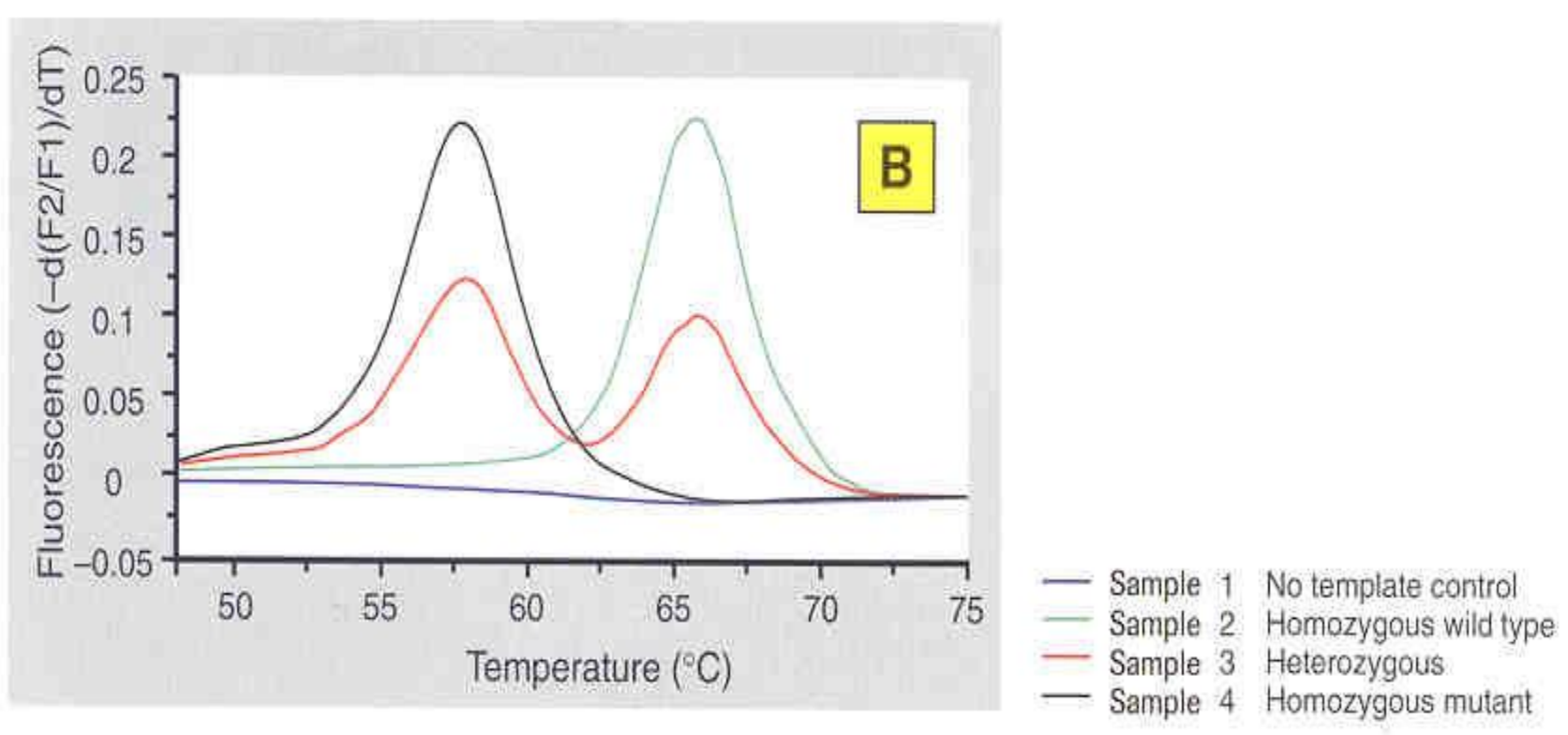


Figure 1. Schematic of the LightCycler System.

FV Leiden Mutation: Hybridization Probe with Fluoresence Resonance Energy Transfer (FRET)



FV Leiden Mutation: Melting Curve Analysis



Prothrombin Gene Mutation

- Single base pair substitution at nucleotide position 20210 in chromosome 11, guanine-> adenine (G20210A) . This results in relatively high prothrombin level with increased risk for venous thrombosis (132% vs 105% of normal)
- Autosomal dominant. 1-3% of Caucasion population; risk increased 3 fold (heterozygote)
- 5-18% of hypercoagulation cases. Tx: heparin, coumadin
- Laboratory: PCR testing for G20210A, Factor II assay (optional)

Protein C Deficiency

- Protein C: a vitamin K-dependent coagulation inhibitor; synthesized in the liver; inactivating F Va and F VIIIa
- Protein C deficiency: autosomal dominant; 0.14-0.5% of population; risk increased 6.5-8 fold; 6-10% of hypercoagulation cases
- Clinical manifestation: recurrent deep vein thrombosis, pulmonary embolism, neonatal purpura fulminans (in homozygote). Tx: heparin, Coumadin
- Laboratory: immunological, functional assays, no mutation testing (>160 mutations)

Protein S Deficiency

- Protein S: a vitamin K-dependent protein; synthesized in the liver and megakaryocytes; cofactor of protein C
- Protein S deficiency: autosomal dominant; 0.7% of population; risk increased 1.6-11.5 fold; 5-10% of hypercoagulation cases
- Clinical manifestation: recurrent deep vein thrombosis, pulmonary embolism, neonatal purpura fulminans. Tx: heparin, coumadin
- Laboratory: immunological assay, functional assay, no mutation testing (>70 mutations)

AT III Deficiency

- AT III: inactivates thrombin and other factors (Xa, IXa, XIa, XIIa, kallikrein); accelerated by heparin
- AT III deficiency: autosomal dominant; 0.17% of population; risk increased 5-8.1 fold; 5-10% of hypercoagulation cases
- Clinical manifestation: recurrent deep vein thrombosis, pulmonary embolism. Tx: AT III, heparin, coumadin
- Laboratory: functional assay (chromogenic), immunologic assay, no mutation testing (>250 mutations)

Lupus Anticoagulant

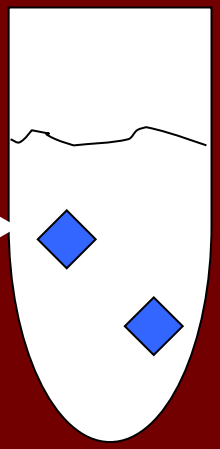
- Immunoglobulins that prolong in-vitro phospholipid-dependent clotting times
- Found in various conditions; 30% of patients have thrombosis ; 10-20% of hypercoagulation cases
- Antiphospholipid antibodies (lupus anticoagulant, anticardiolipin antibody, antiphosphatidyl serine, anti Beta 2 Glycoprotein I, etc): 1-2% of population, 50% of SLE patients
- Clinical manifestation: variety of thrombotic diseases. Tx: Heparin, Coumadin, Aspirin & prednisone (to prevent fetal demise)
- Laboratory: aPTT, dilute Russell Viper venom time (dRVVT), Hexagonal Phospholipid Neutralization, Platelet Neutralization Procedure

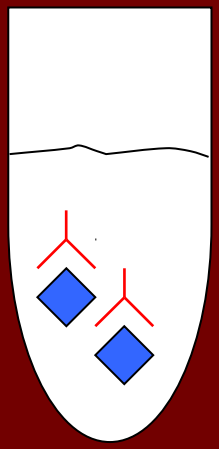
Evaluation of lupus anticoagulant

- Mixing studies
  - Mix equal parts patient and control plasma
  - aPTT will correct if prolongation due to factor deficiencies
  - If LA present will fail to correct aPTT
    - Usually immediate acting (before incubation)

Dilute Russell Viper Venom Time

Dilute Phospholipid





Normal plasma
dRVVT 36-42 sec

Plasma with lupus anticoagulant
dRVVT > 43 sec

Neutralization Procedure

Neutralization study:

Addition of phospholipid will neutralize lupus anticoagulant

Two common neutralization tests:

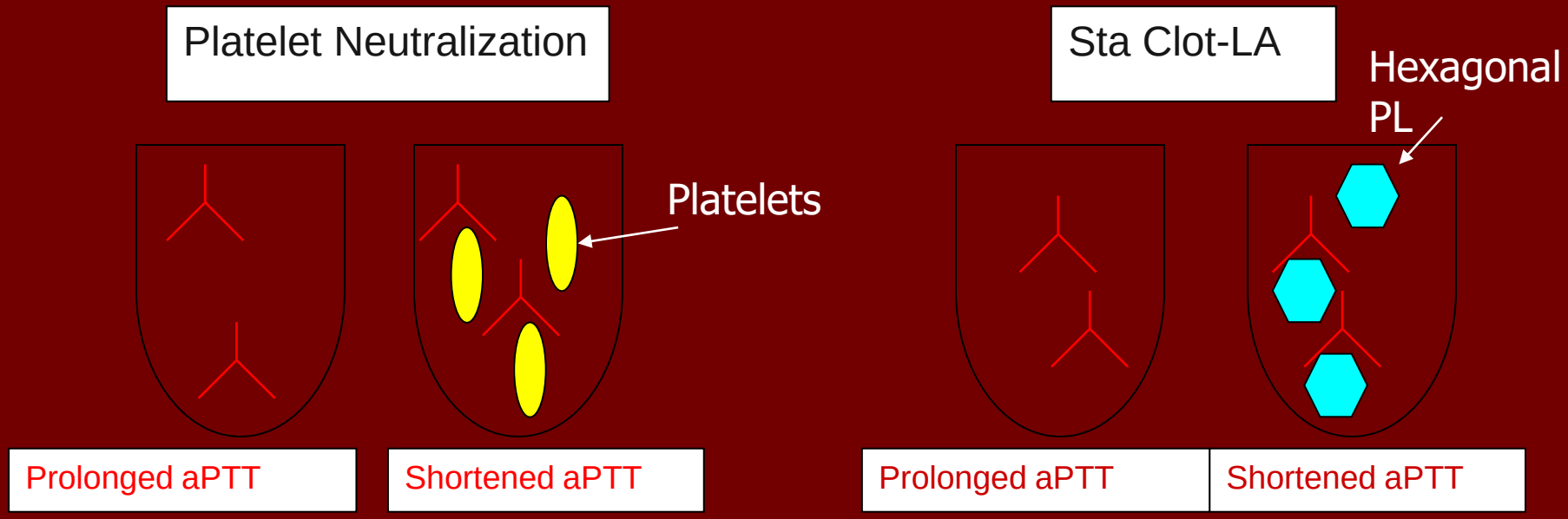
- Platelet neutralization:

  lysates of frozen platelets-> thawed and washed before testing

- Hexagonal phase phospholipid neutralization:

  hexagonal phospholipid with high affinity for lupus anticoagulant

Neutralization Procedure Tests



Clotting time decreases for >10 seconds after addition of PL -> pos for LA

Anticardiolipin Antibodies

- ACA: IgG, IGM, IgA
- Found in various conditions; thrombotic manifestations; in 5-10% of hypercoagulation cases. Tx: not well worked out, including Heparin, coumadin, and steroid
- Laboratory: ACA by ELISA; high levels are associated with high risks of thrombosis

Anticardiolipin Antibodies (cont'd)

	Normal range	Clinically insignificant	Moderate risk	High risk
IgG	< 15 GPL	15-20	20-80	> 80
IgM	< 12.5 MPL	12.5-20	20-80	> 80
IgA	< 15 APL	15-20	20-80	> 80

Genetic and Environmental Factors in Thrombophilia

- Combination of risks: genetic abnormality + environment factor (trauma, surgery, immobility, pregnancy, oral contraceptive, etc.)

- Multiple genetic abnormalities lead to synergistic effect:
Example: Factor V Leiden heterozygote (risk 3-6 fold) + Prothrombin gene mutation heterozygote (risk 3 fold)-> risk 25 fold

- Hypercoagulation is relatively expensive since the panel includes all significant tests

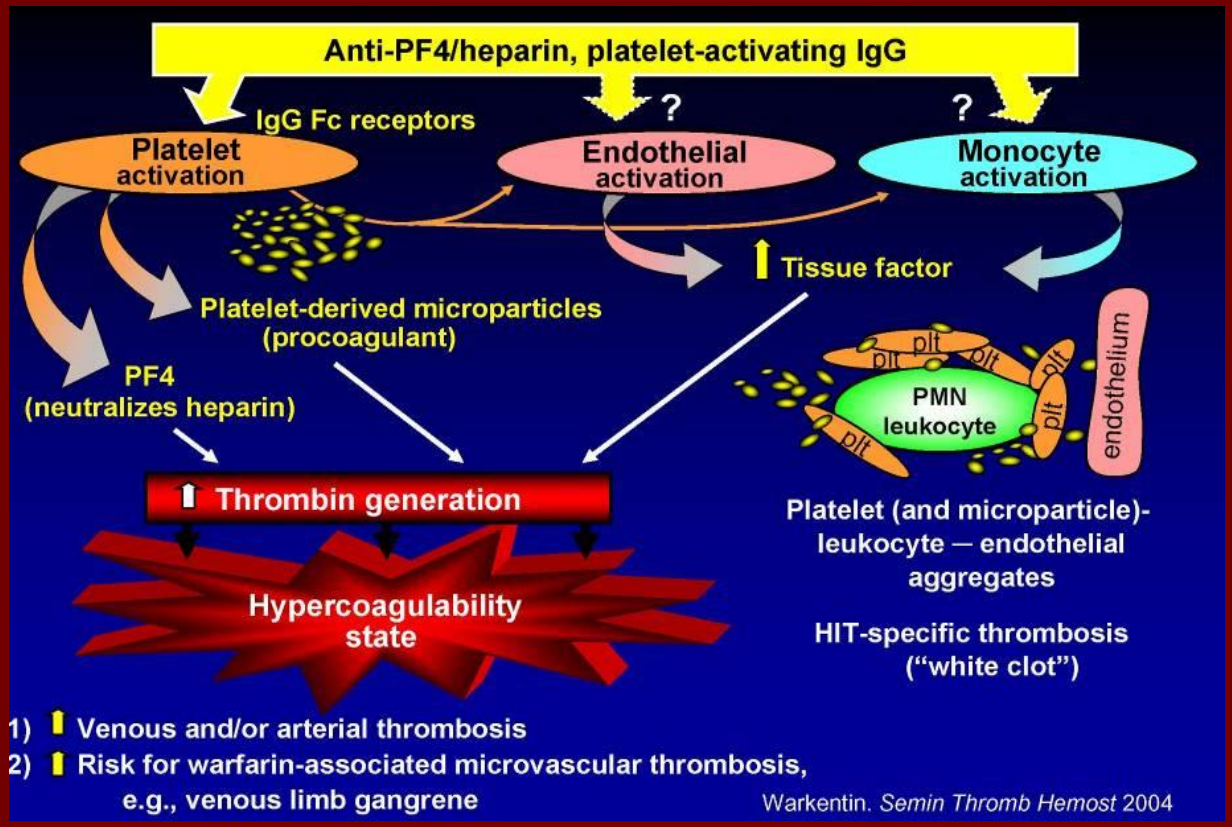
Interference in hypercoagulation tests

- Patients in active DVT/PE may have low levels of: AT, Protein C, protein S (normal levels are still useful)
- Heparin affects: lupus anticoagulant
- Coumadin affects: protein C, protein S

- For baseline testing of the above:
  - Get pre-anticoagulant samples
  - Test patients after discharge (follow-up visit)
  - Patients off heparin for one day
  - Patients off coumadin for 2 weeks

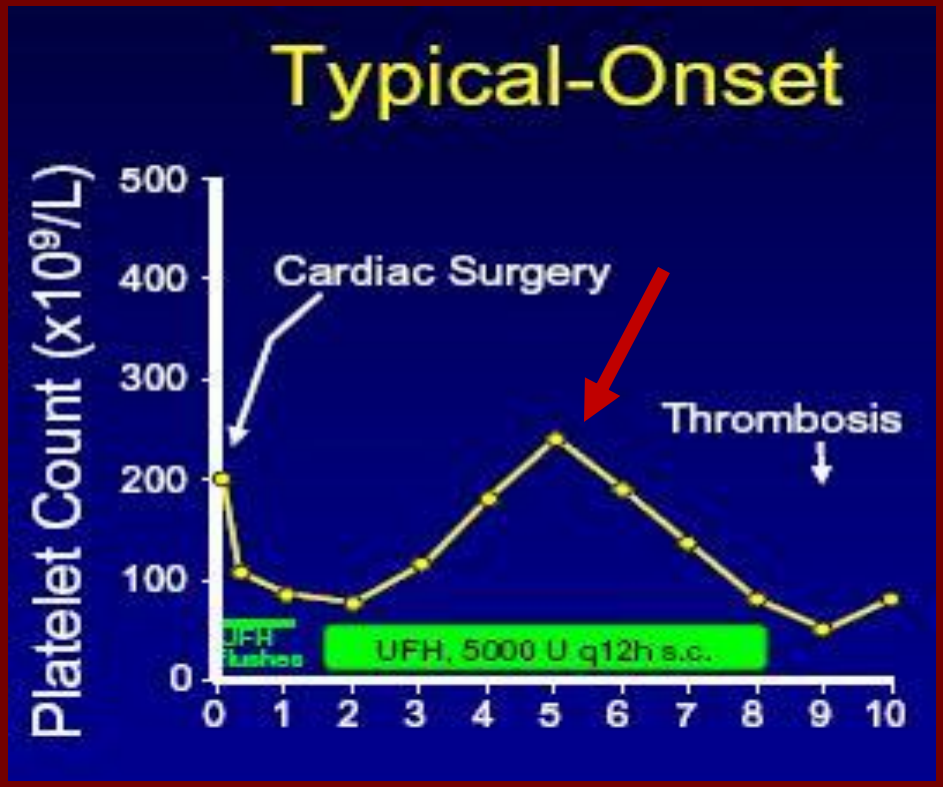
Heparin-induced thrombocytopenia (HIT)

-Upon exposure to heparin, the immune system may form antibody against heparin and platelet factor 4 (PF4)
-Upon re-exposure to heparin, the development of these antibodies takes about 5 days to activate platelets forming thrombosis and subsequent thrombocytopenia



HIT

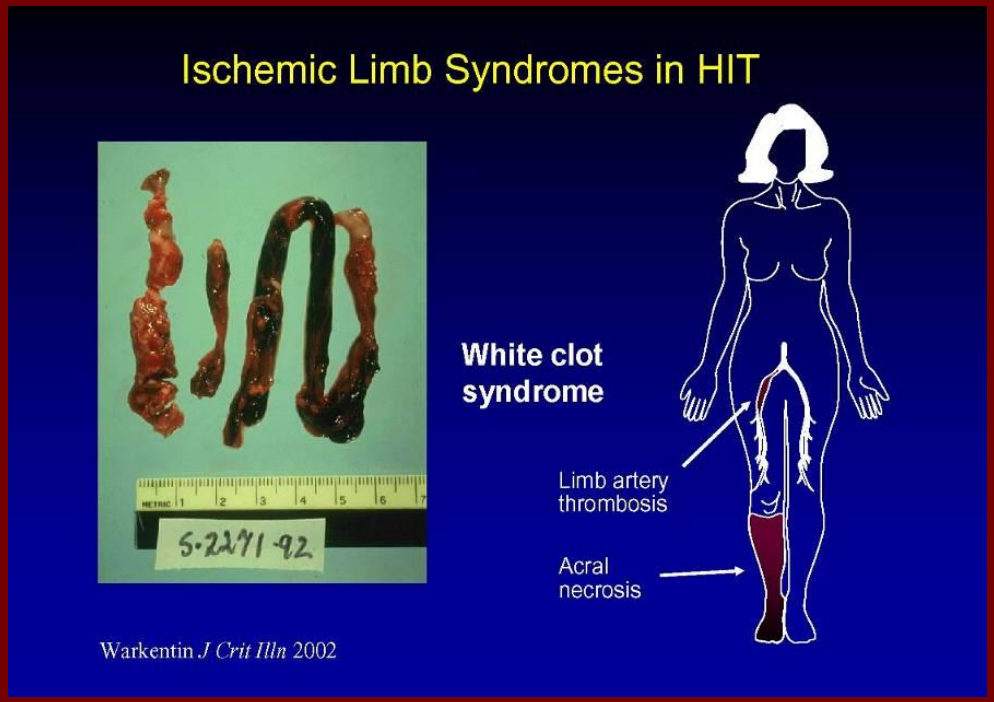
- Risk of HIT is higher with UFH than with LMWH
- Typical onset: 5 days after heparin exposure



Example for cardiopulmonary bypass

Complications of HIT

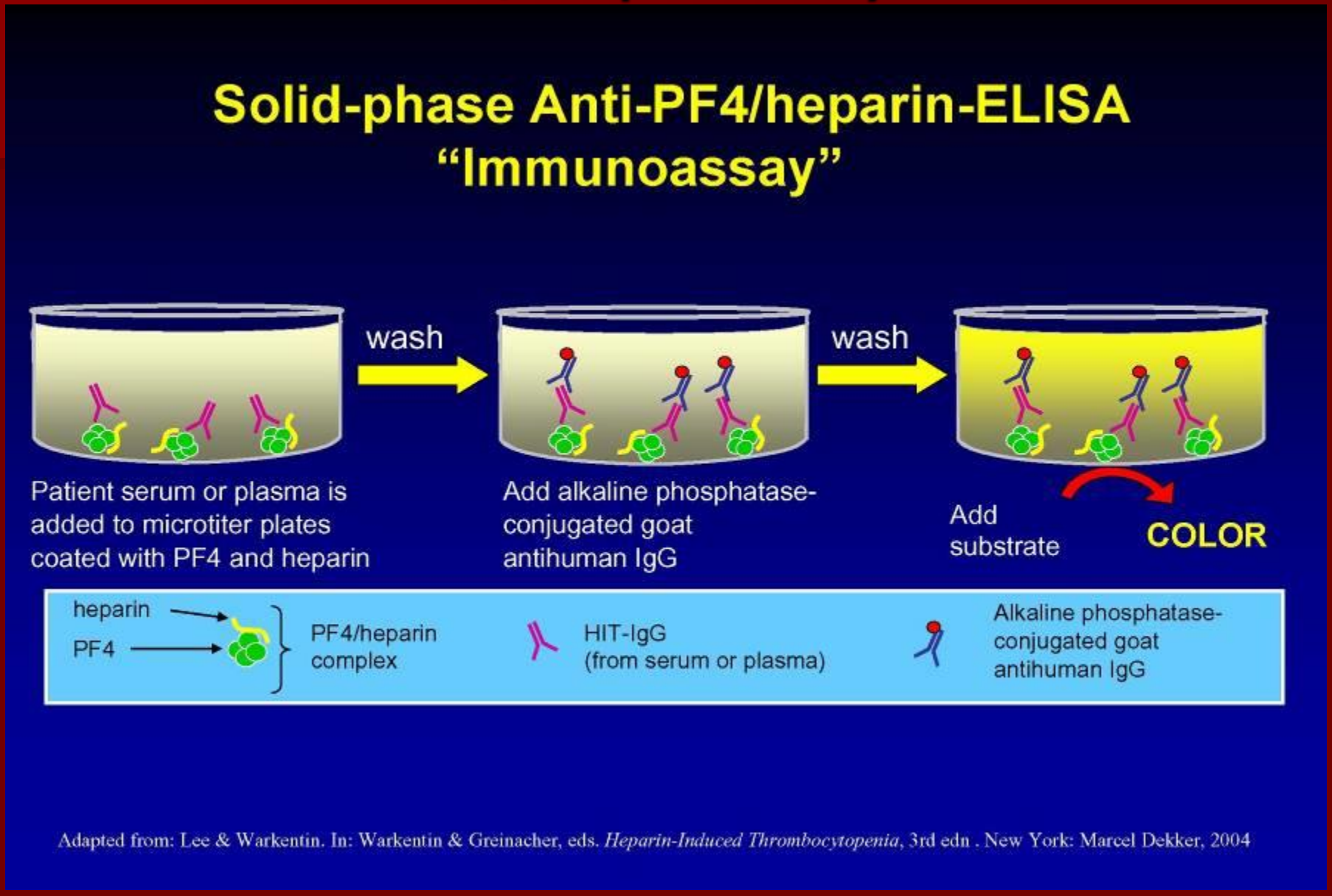
- Venous thrombosis (50%)
- Arterial thrombosis (10-15%)



Testing for Heparin Antibody

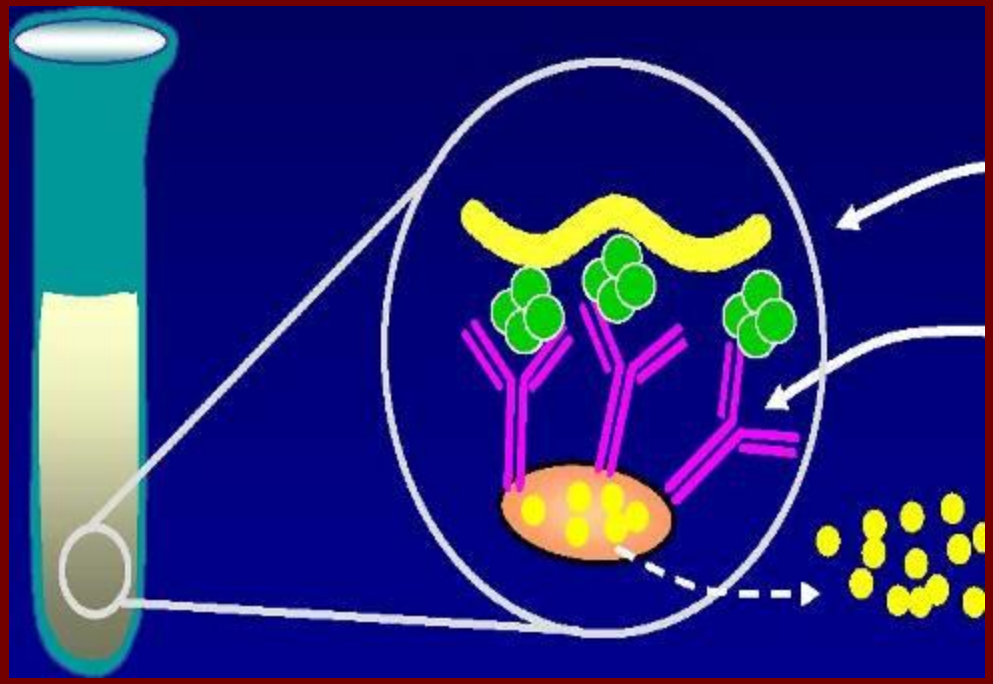
- Heparin-PF4 antibody (ELISA): sensitivity 82%, specificity 70%
- Serotonin release assay: sensitivity 80%, specificity 85%, limited use due to radioisotope (^{14}C)

Anti-PF4/heparin by ELISA



Adapted from: Lee & Warkentin. In: Warkentin & Greinacher, eds. *Heparin-Induced Thrombocytopenia*, 3rd edn . New York: Marcel Dekker, 2004

Anti-PF4/heparin by Serotonin release assay



Heparin/PF4 complex

HIT IgG

Radiolabeled serotonin released from platelets

HIT Treatment Principles

- Stop Heparin
- Switch to alternate anticoagulant (Danaparoid, Lepirudin, Argatroban, Angiomax)
- No warfarin (which would decrease proteins C and S)
- No prophylactic platelets

Hypercoagulation in COVID19 Infection

- J Thrombosis Research (April 2020): 31% of 184 COVID19 patients suffered thrombotic complications
- New England Journal of Medicine (May 2020): young patients in the 30's and 40's with strokes
- The Lancet (April 2020): the virus enters cells via the angiotensin converting enzyme 2 (ACE2) receptors, which are most commonly found in the alveolar epithelial cells, followed by endothelial cells. When the virus binds to these cells, it may damage the blood vessel, especially the microcirculation of the small blood vessels, and thus activates platelet aggregation, leading to hypercoagulation.
- Pulmonary intravascular coagulopathy (Lancet Rheumatol 2020, May 7, 2020): elevated D-Dimer/fibrinogen, normal platelet count, elevated cardiac enzymes with pulmonary hypertension). Lungs filled with microclots helped explain why ventilators work poorly for patients with low blood oxygen. The microclots block circulation and blood is leaving the lungs with less oxygen.

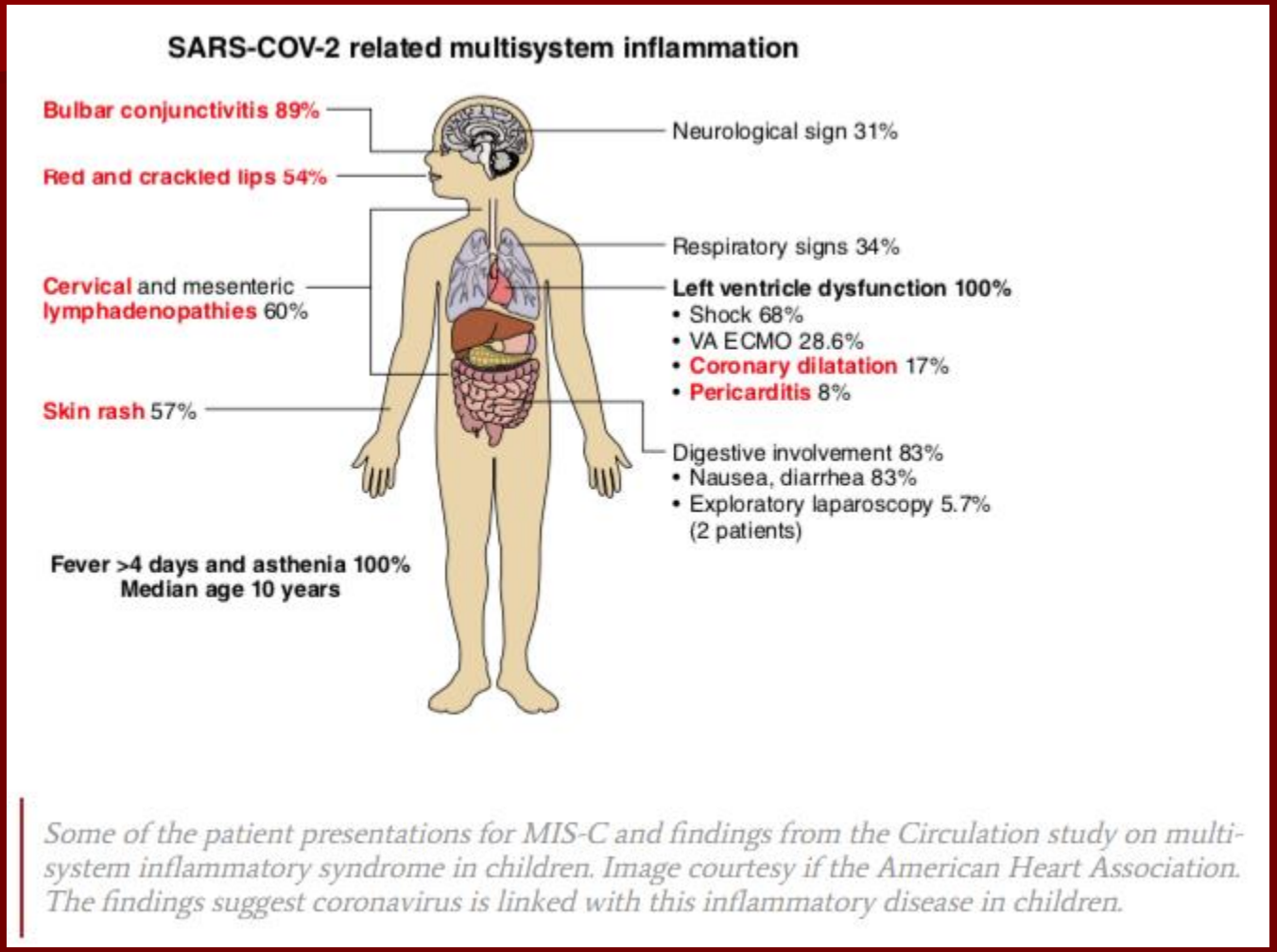
Hypercoagulation in COVID19 Infection

- The International Society on Thrombosis and Haemostasis (ISTH) recommended that all hospitalized COVID-19 patients, should get prophylactic-dose low molecular weight heparin (LMWH), unless they have contraindications (active bleeding and platelet count $<25\times10^3/\mu L$).
- Many institutions choose threshold values upon which to start systemic anticoagulation: D-dimer >1,500 ng/mL or fibrinogen >800 mg/mL

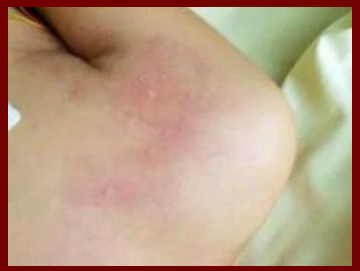
Multisystem Inflammatory Syndrome in Children (MIS-C) Associated with COVID19

- While children are less likely to become severely ill than older adults, there are subpopulations of children with an increased risk for more significant illness (MIS-C).
- On April 26, 2020, reports in the United Kingdom (UK) of 8 previously healthy children presenting with a severe inflammatory syndrome with Kawasaki disease-like features: persistent fever and a constellation of symptoms including hypotension, multiorgan (e.g., cardiac, gastrointestinal, renal, hematologic, dermatologic and neurologic) involvement, and elevated inflammatory markers. As of May 12, 2020, the New York State Department of Health identified 102 patients with similar presentations
- Most patients responded well to intravenous immunoglobulin (IVIG) and high-dose aspirin (ASA) [Veena Jones et al, J Hospital Pediatrics 2020]

Multisystem Inflammatory Syndrome in Children (MIS-C) Associated with COVID19



Some of the patient presentations for MIS-C and findings from the Circulation study on multi-system inflammatory syndrome in children. Image courtesy if the American Heart Association. The findings suggest coronavirus is linked with this inflammatory disease in children.



Skin rash

Topic 7

- Platelet functional disorders

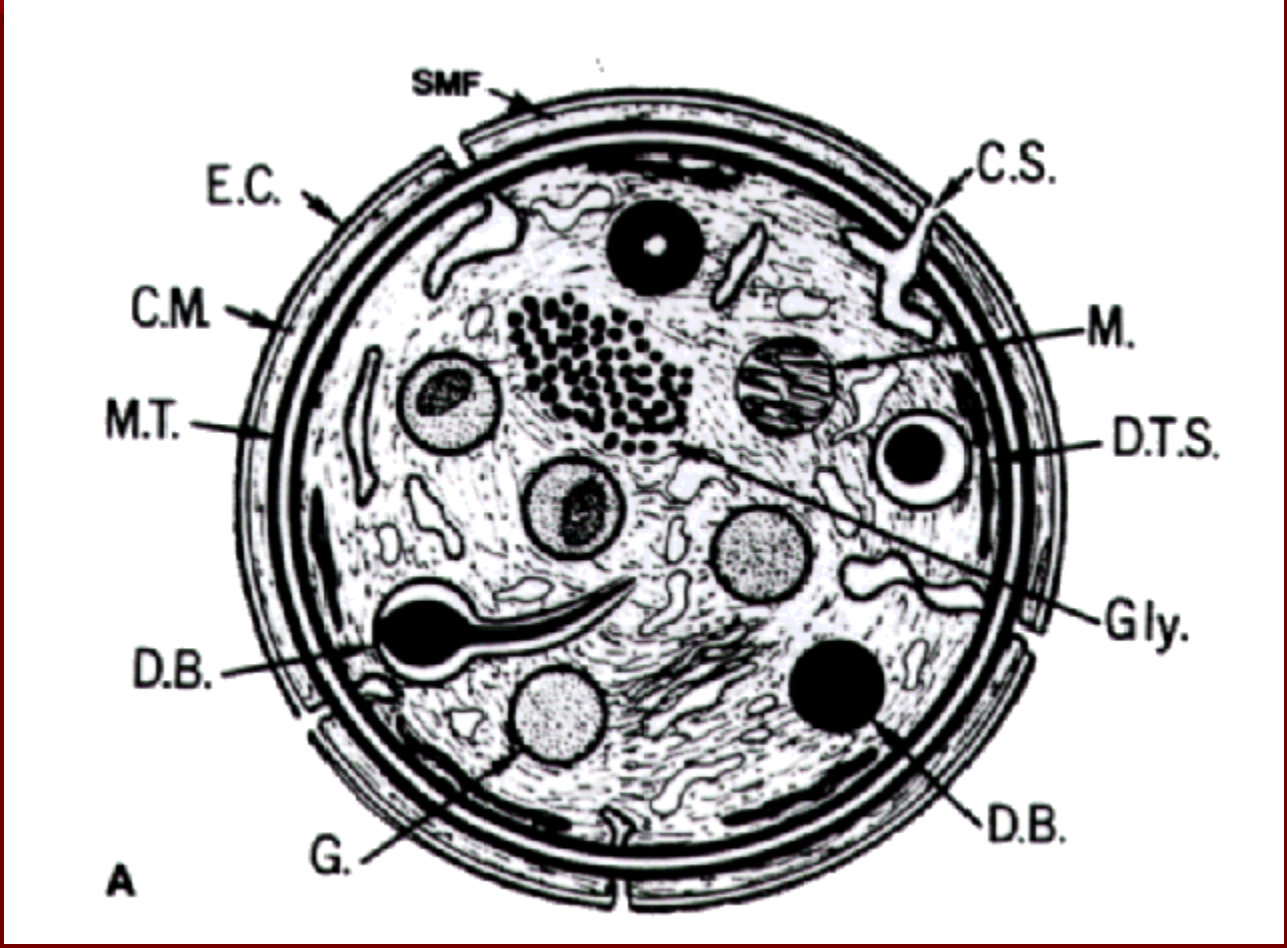
Review of platelet functional anatomy

- Glycocalyx: outer surface, rich in glycoproteins
- Microtubules: sub-membranous band, protein tubulin, provide structural support
- Contractile microfilaments: actin, myosin
- Open canalicular system: direct communication with extracellular environment
- Dense tubular system: derived from smooth endoplasmic reticulum, site for arachidonic acid metabolism

Review of platelet functional anatomy

- Mitochondria
- Glycogen
- Alpha granules: platelet fibrinogen, platelet-derived growth factor, vonWillebrand factor, beta-thromboglobulin, heparin neutralizing factor (PF4)
- Dense granules: adenosine diphosphate, adenosine triphosphate, serotonin, calcium
- Lysosomes

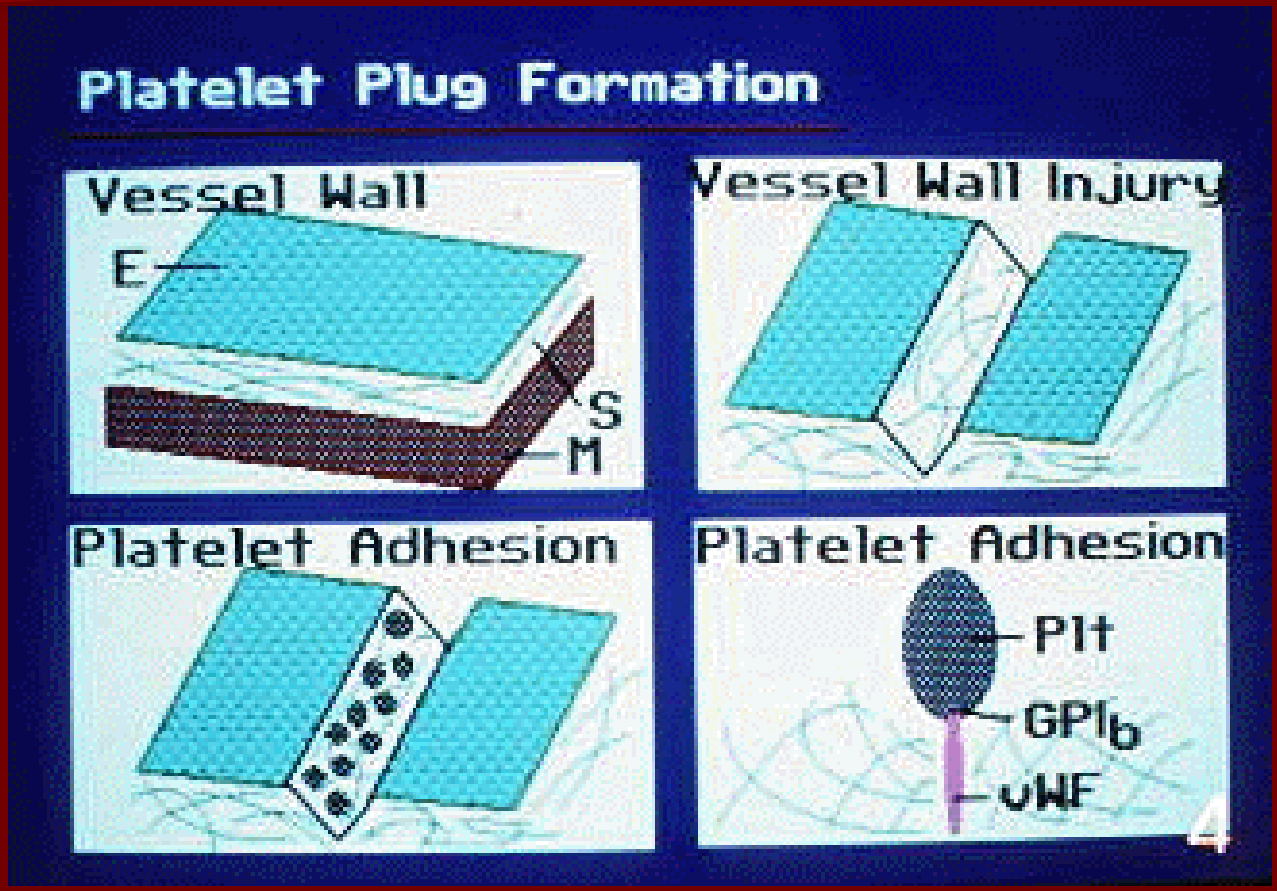
Review of platelet functional anatomy



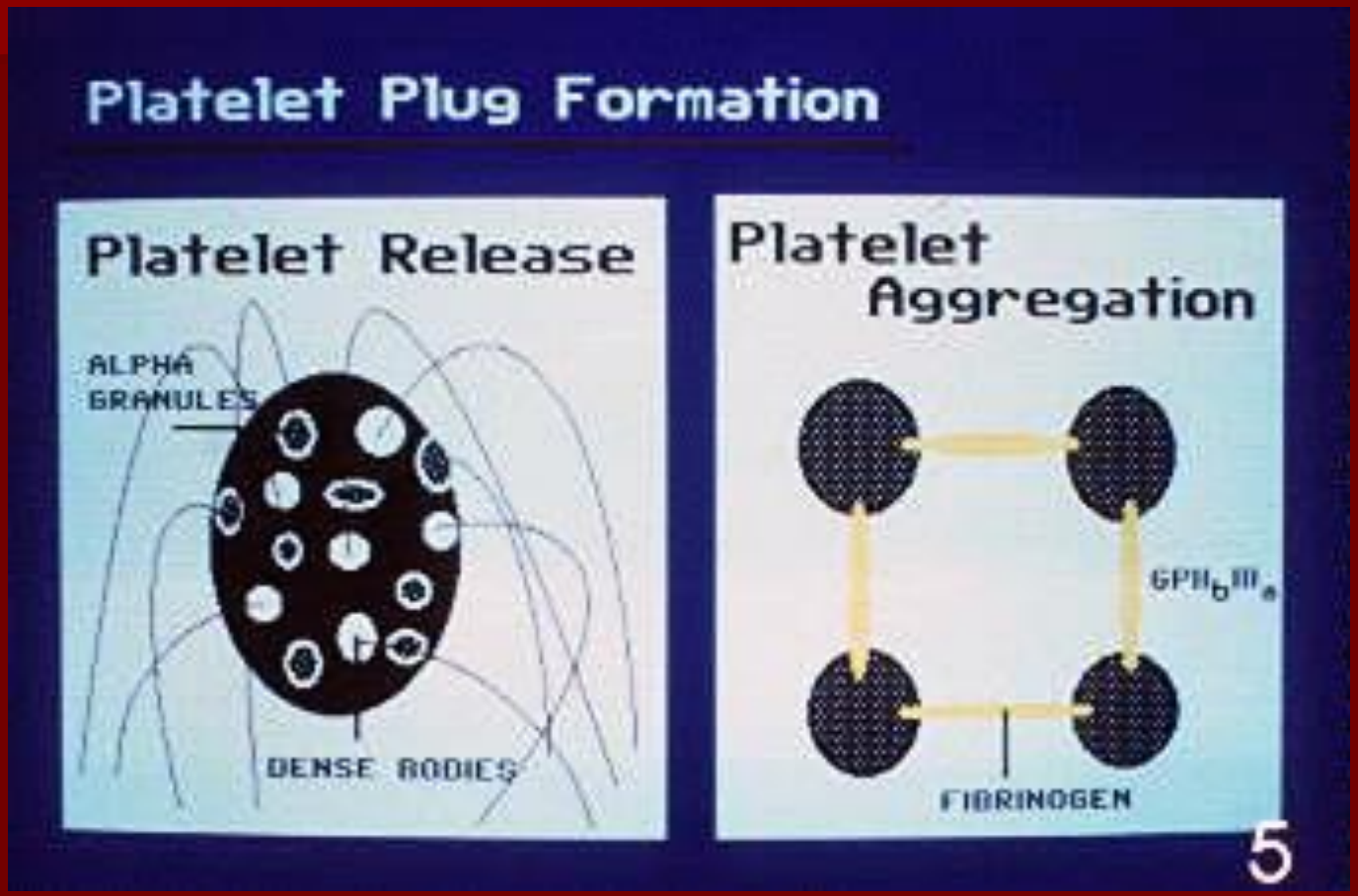
Platelet membrane glycoproteins

- Identified by radio-active labeling of surface glycoproteins, solubilization of the membranes, electrophoresis on polyacrylamide gels
- Clinically important: GP Ib, V, IX, IIb, IIIa

Platelet activities in hemostasis



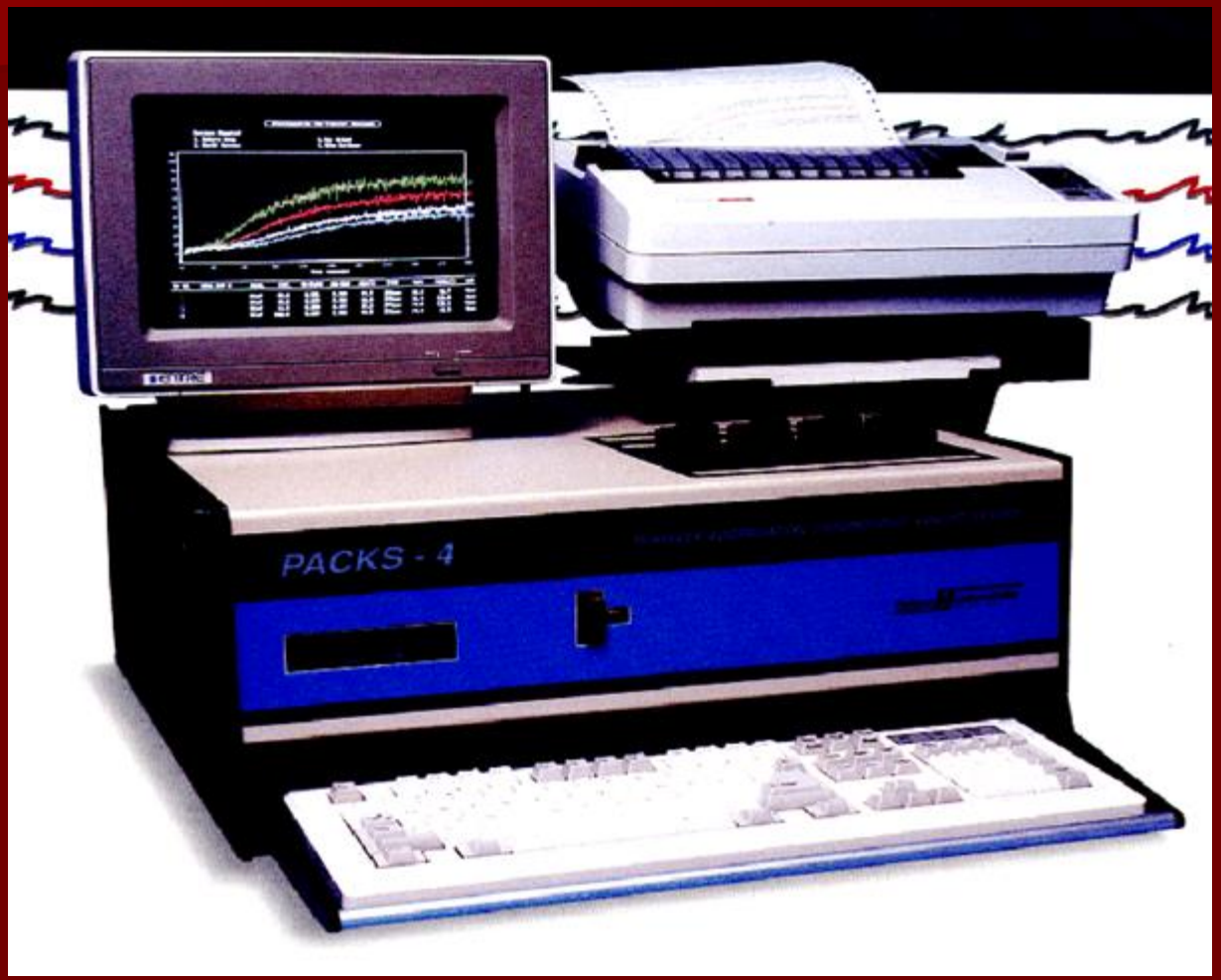
Platelet activities in hemostasis (cont'd)



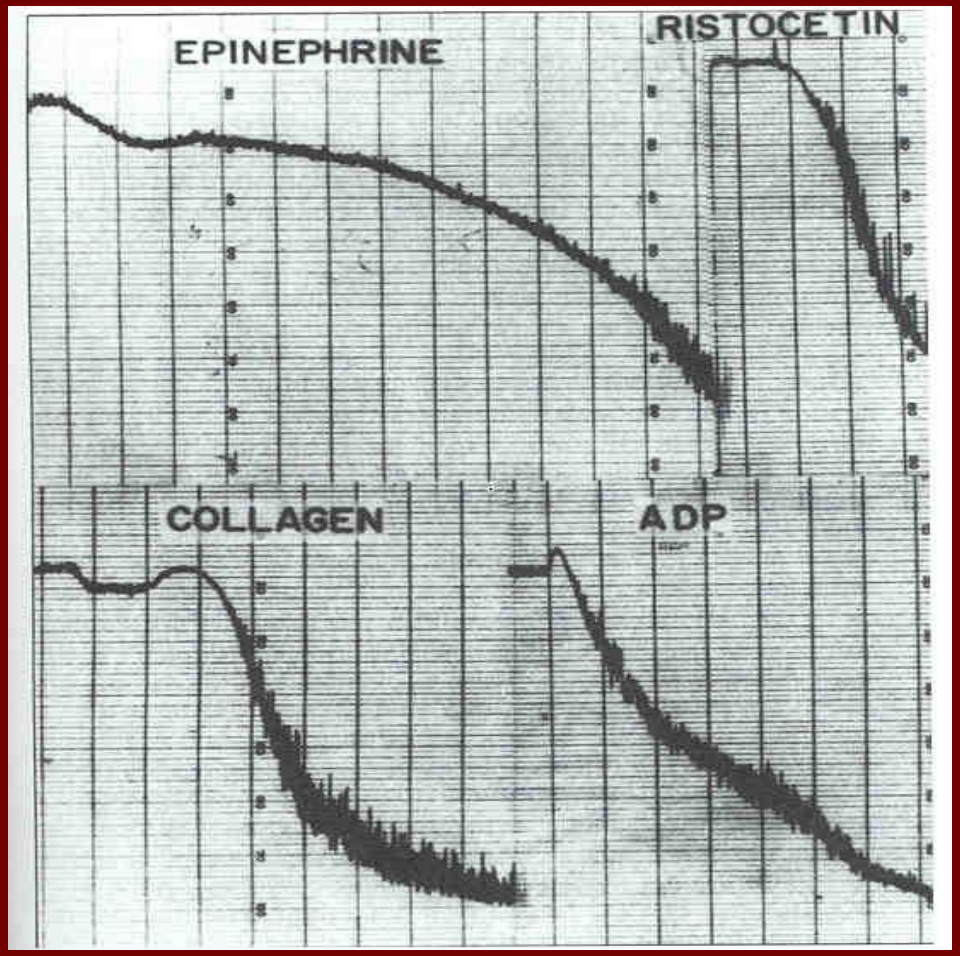
Platelet aggregation study

- Principle: aggregation in response to an added chemical stimulus can be monitored by change in transmittance
- Stimulating agent: arachidonic acid, ADP, collagen, epinephrine, and ristocetin
- Platelet functional disorders have typical aggregation patterns

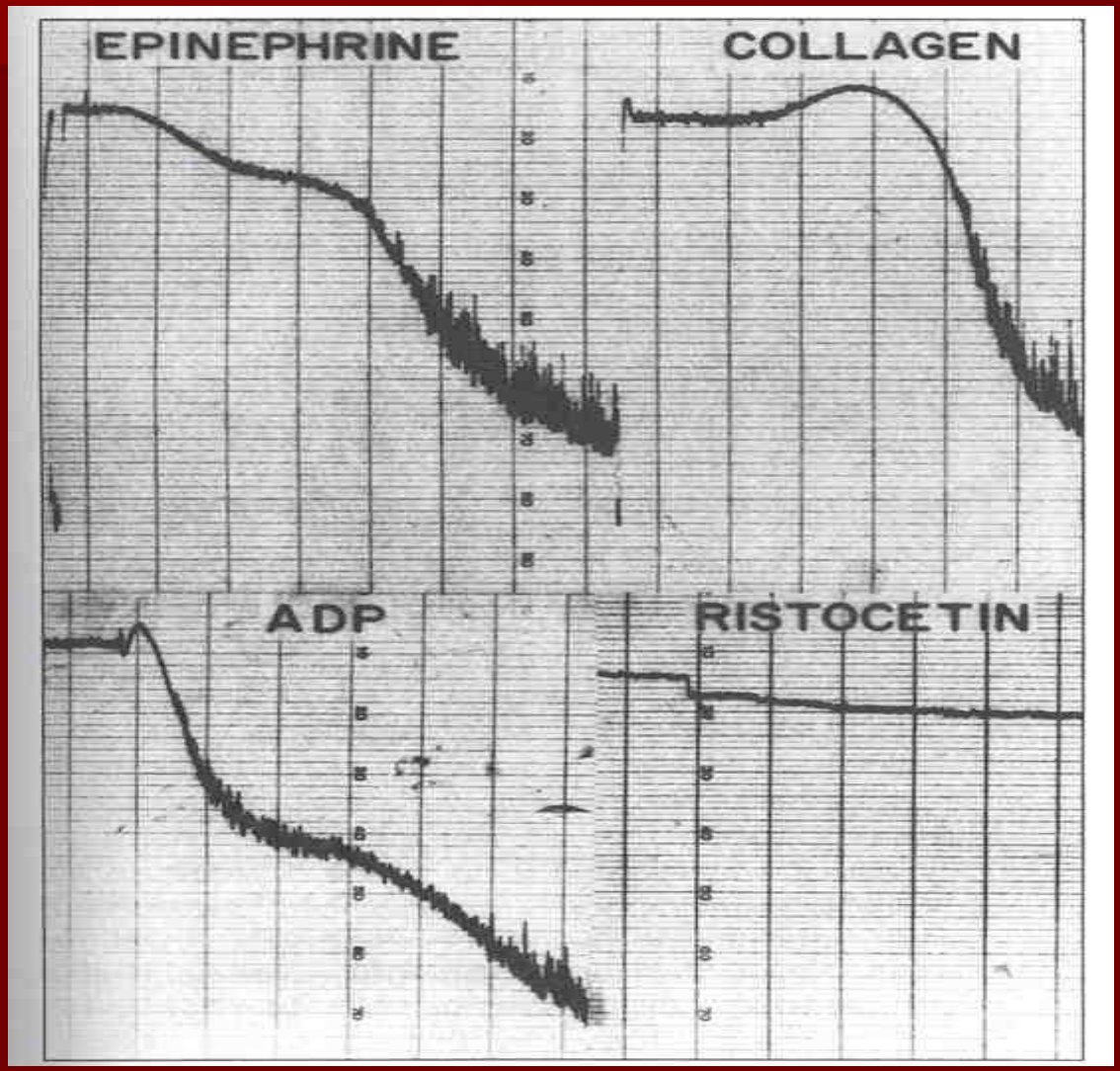
Aggregometer



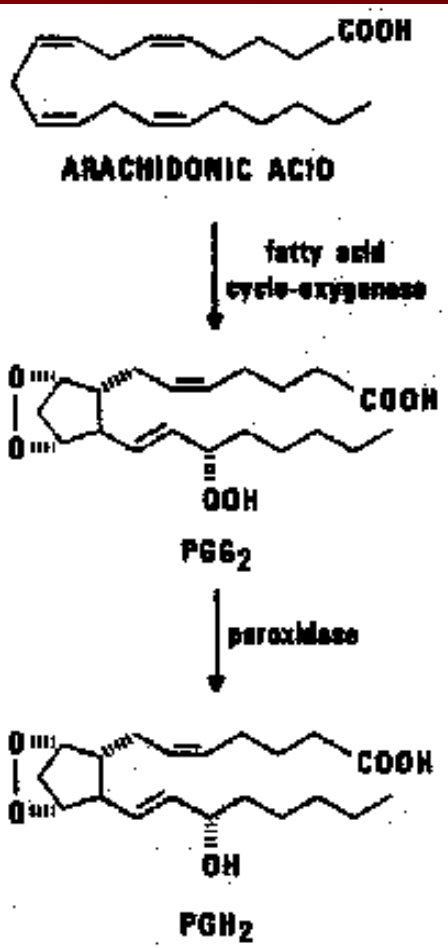
Normal platelet aggregation patterns



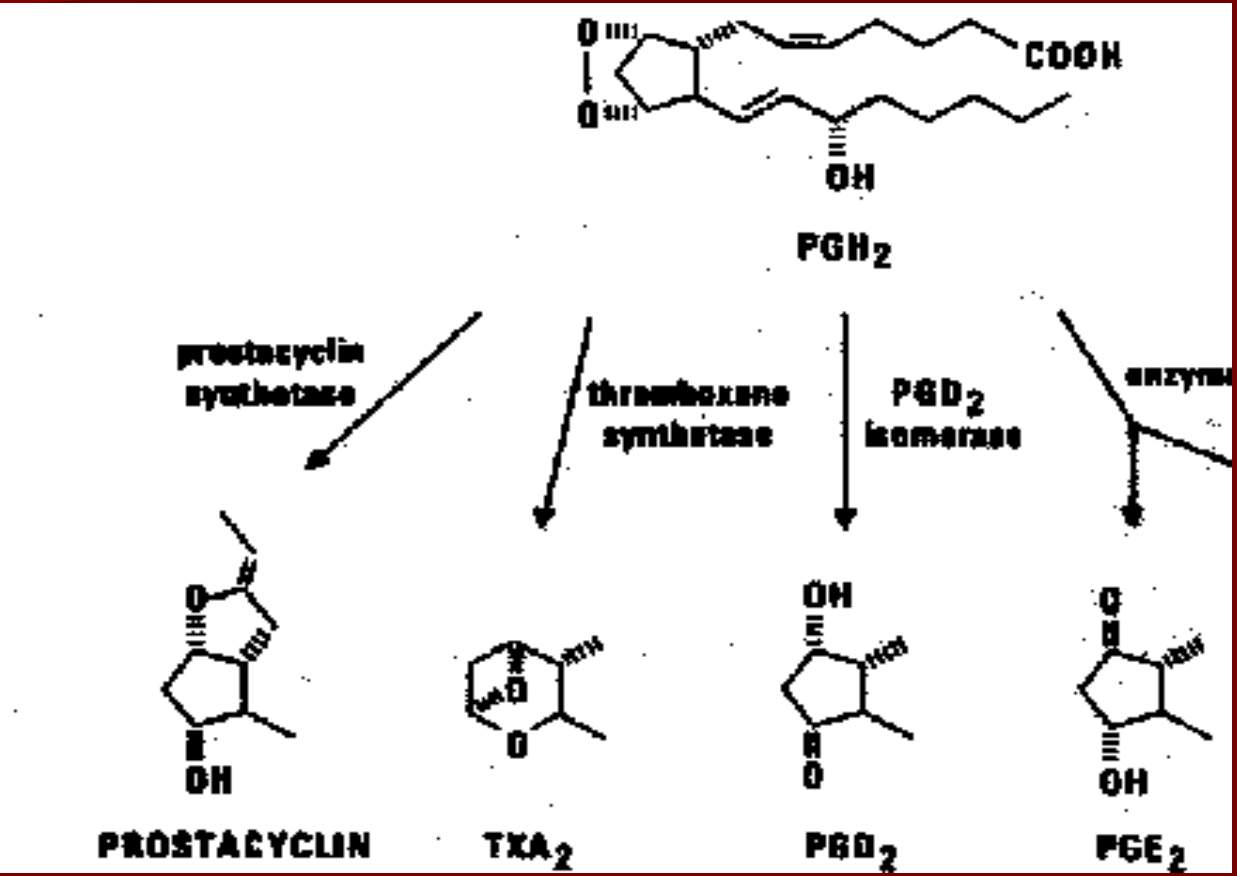
Abnormal platelet aggregation patterns: vWD or Bernard Soulier Syndrome



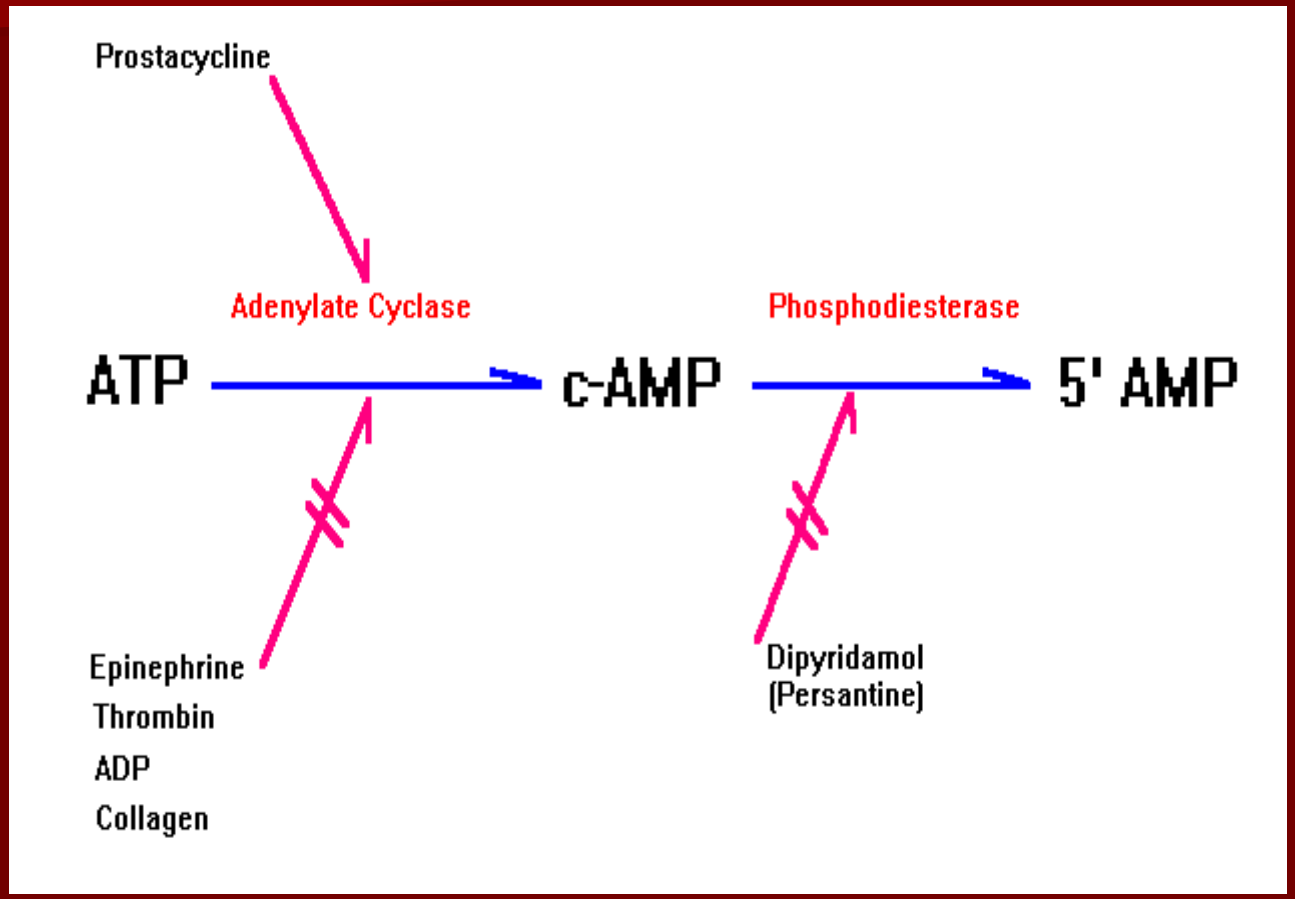
Pathway of platelet activation



Pathway of platelet activation (cont'd)



Pathway of platelet activation (cont'd)



Inherited disorders of platelet function: surface membrane defects

- Glanzmann thrombasthenia: autosomal recessive, defective GP IIb/IIIa
- Bernard Soulier syndrome: autosomal recessive, thrombocytopenia, large platelets, defective GP Ib,V,IX
- Collagen receptor defect: defective thrombospondin
- Platelet-type vWD: autosomal dominant, high affinity for vWF, borderline thrombocytopenia, addition of cryo-> aggregation

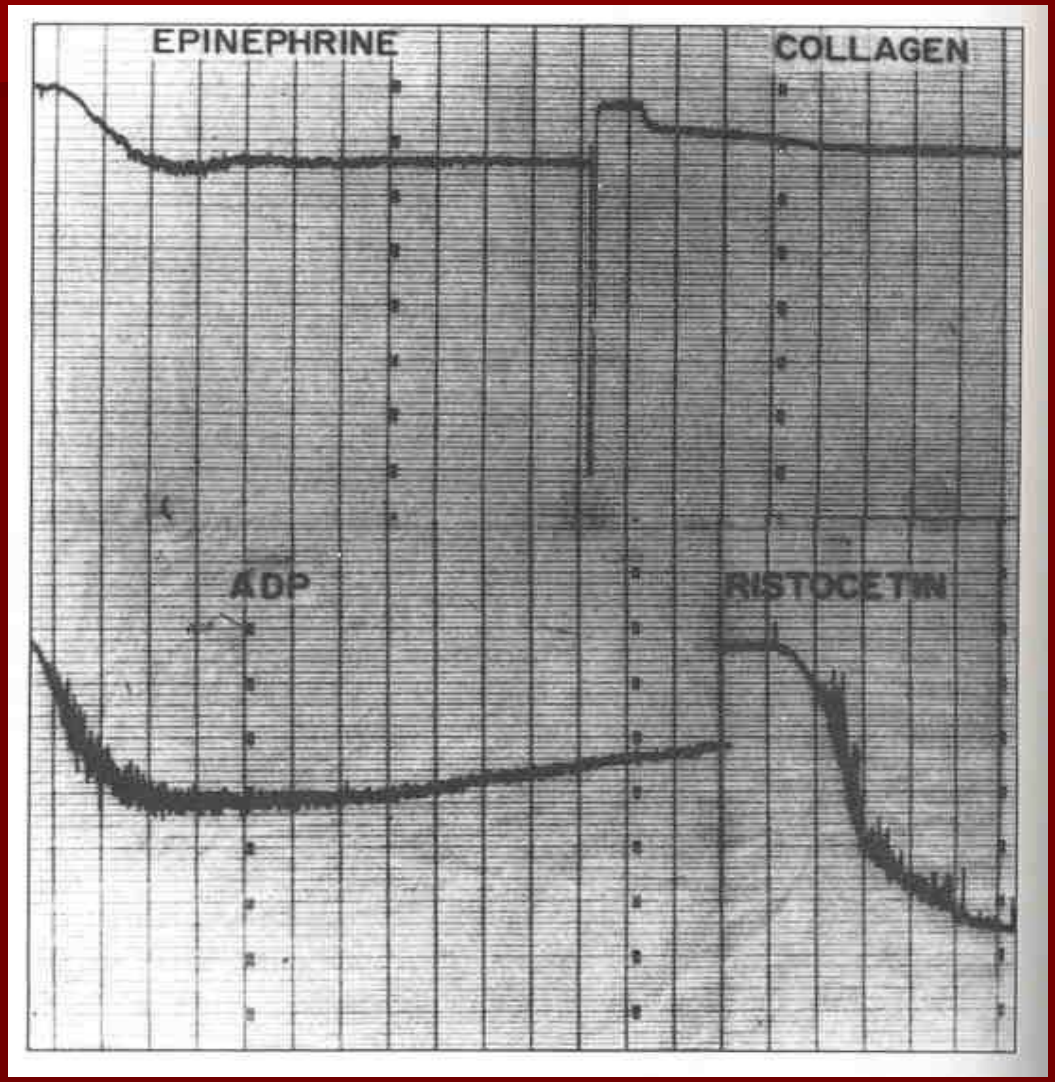
Inherited disorders of platelet function: granule defects

- Dense granule deficiency (δ SPD): isolated deficiency or in association with Hermansky-Pudlak, Chediak-Higashi, Wiskott-Aldrich
- Alpha granule deficiency (α SPD): gray platelet syndrome
- Combined granule deficiency (α δ SPD)

Combined granule deficiency (α δ SPD): Clinical Presentation

- Most cases are autosomal-dominant
- Patients may be asymptomatic, mild-moderate bleeding

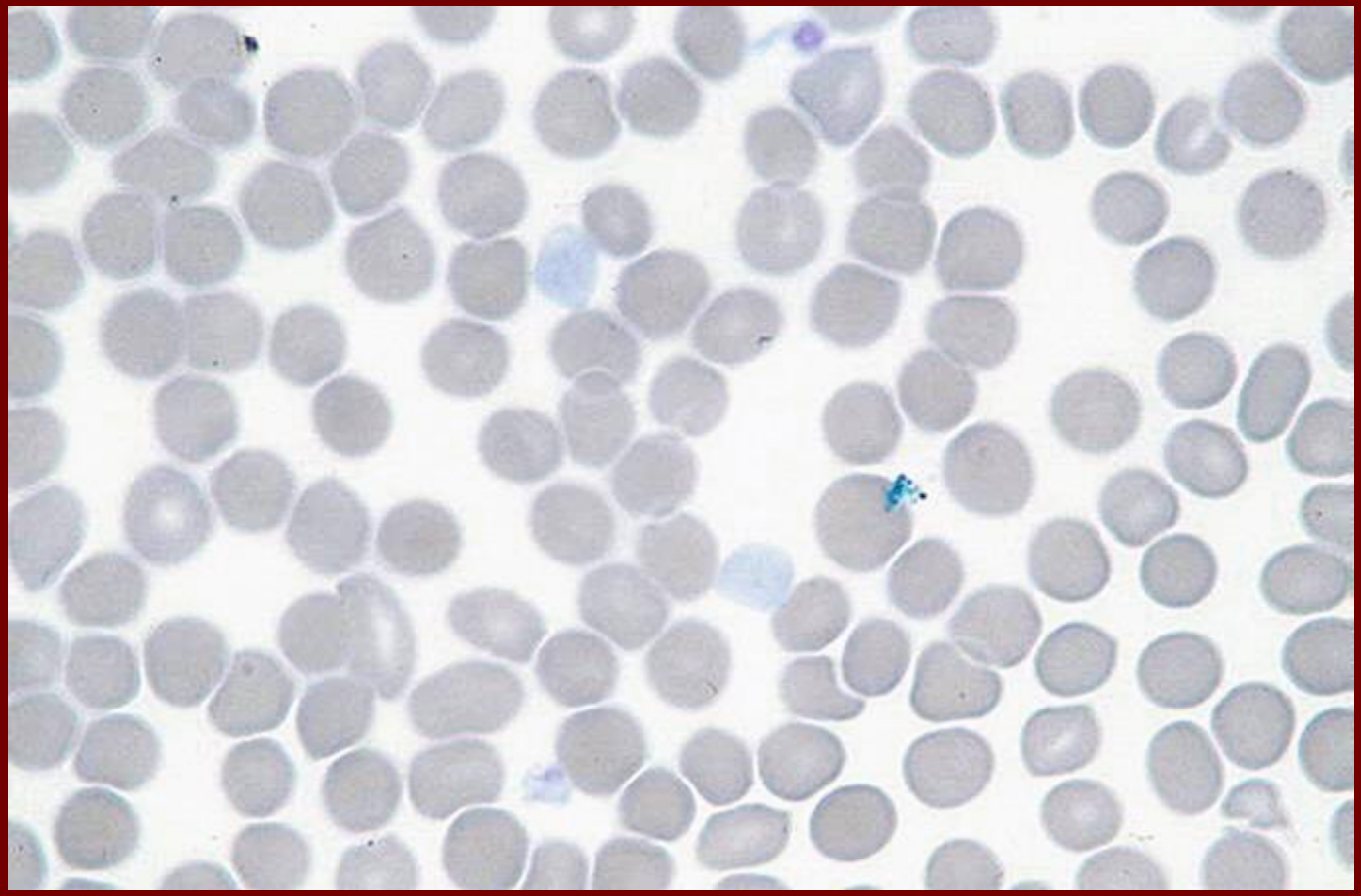
Platelet aggregation study
Platelet storage pool disease



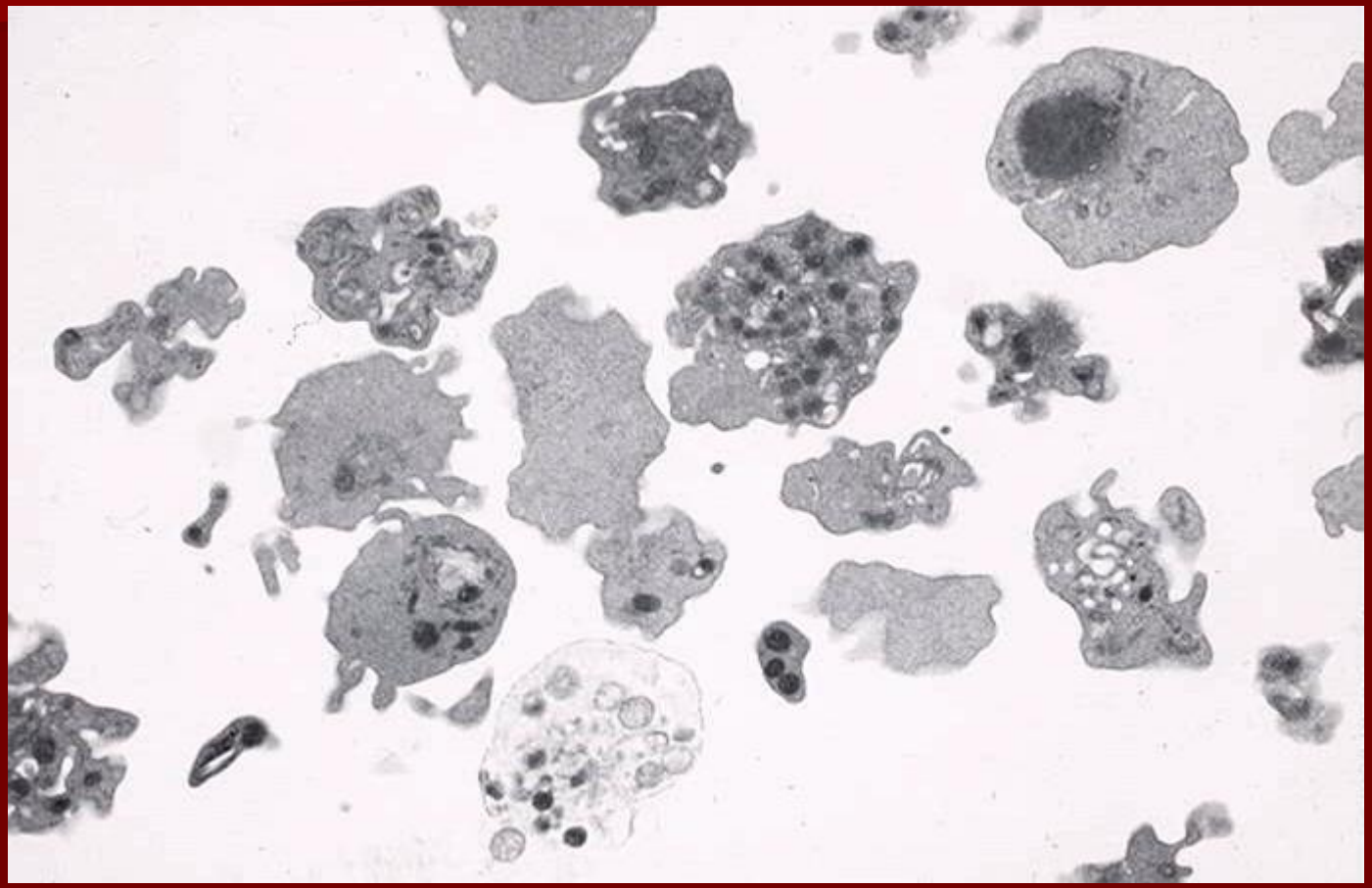
Ruled out:
- **vWD**
- **Bernard Soulier Syndrome**
- **Glanzmann Thrombasthenia**
- **Plavix**

Could not rule out:
- **NSAIDs**
- **Platelet storage pool disease**

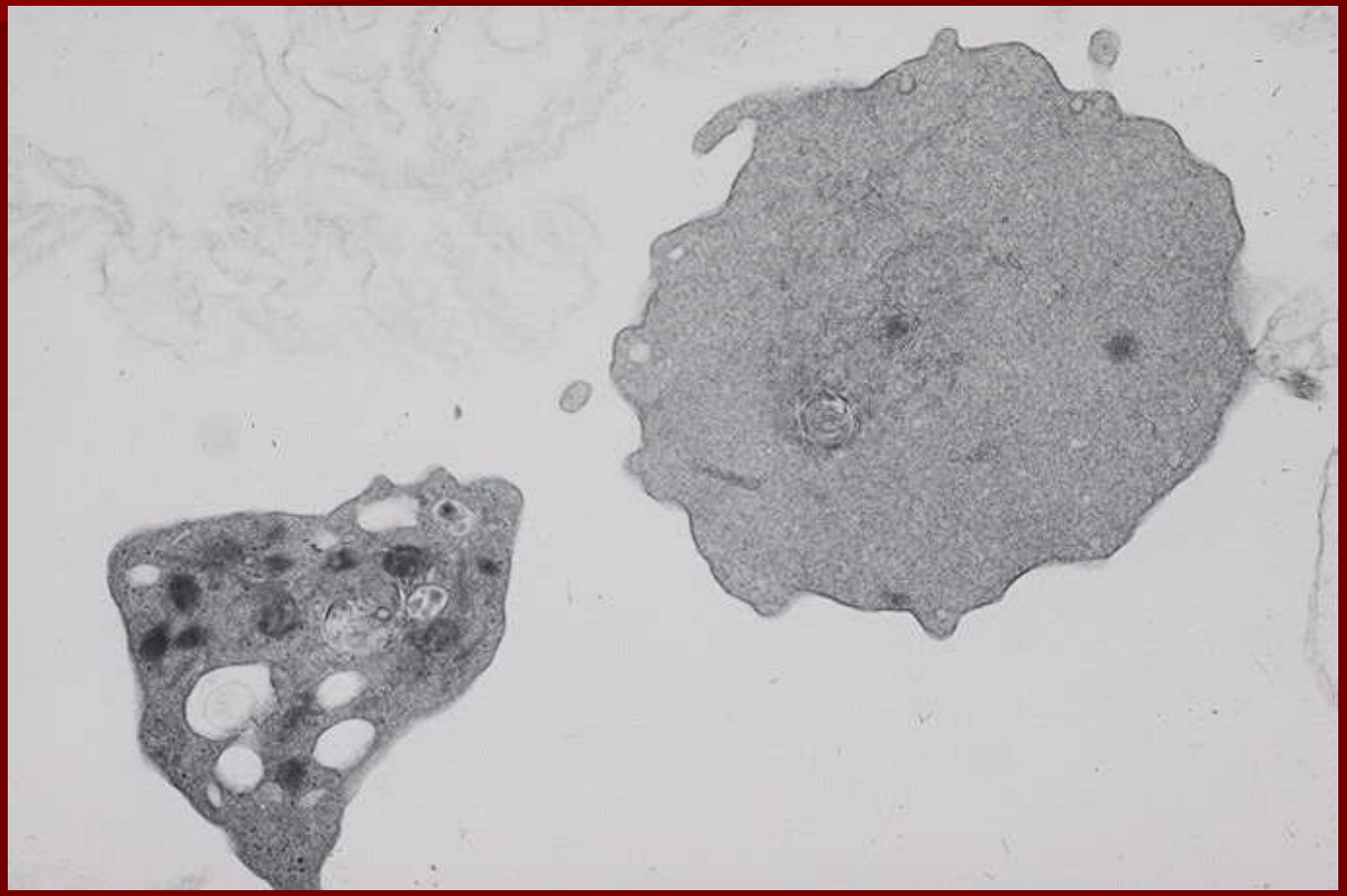
Platelet granule deficiency: blood smear



Deficiency of alpha granules and delta granules : EM



Deficiency of alpha granules and delta granules : EM



Combined granule deficiency (α δ SPD): Treatment

- Platelet transfusion for symptomatic patients

Topic 7
ADAMTS-13 Testing

Collagen-Binding Assay

- Gerritsen, et. al.
- Principle: small vWF fragments do not bind collagen; large forms do
- Dilutions of patient's plasma, incubation for 2 hours
- ELISA – Microtiter plates coated with collagen type III
- Collagen-bound vWF quantified using labeled antibodies: detection of (large) vWF bound to collagen by ELISA indicates poor ADAMTS-13 activity
- ADAMTS-13 activity inhibited by EDTA (purple-top)
  - Must use citrate (blue-top) instead

Bethesda Inhibitor Assay

- Mixing studies
  - Normal human plasma mixed with patient's plasma
- Residual activity measured via ADAMTS-13 assay
- One Bethesda Unit = quantity of inhibitor that neutralizes 50% of the ADAMTS-13 activity in normal plasma
  - Increase in Bethesda units is exponential
  - Normal is ≤ 0.3 Bethesda Units

Topic 8
Thromboelastograph

Thromboelastograph (TEG): principles

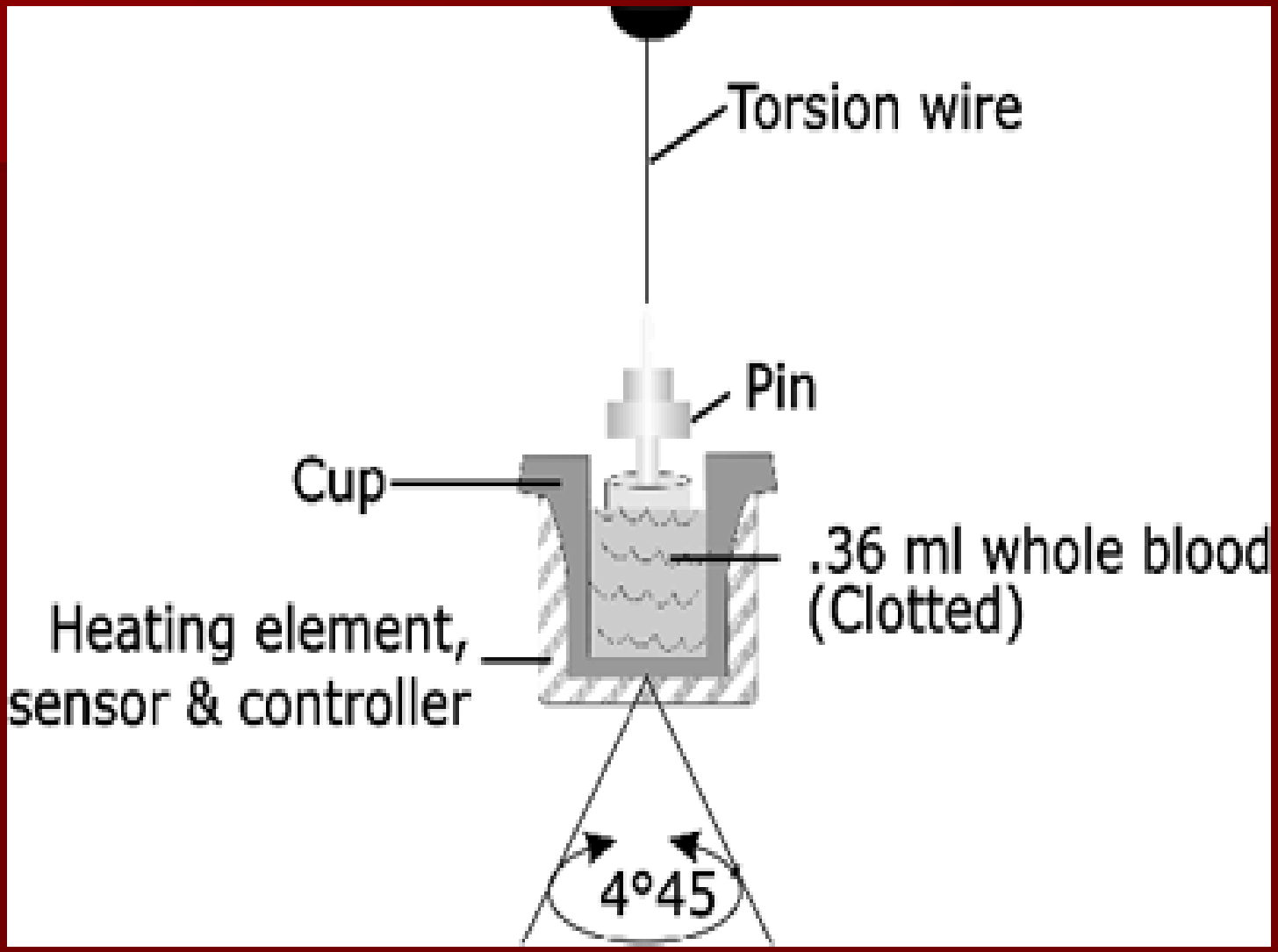
Measuring the mechanical properties of the developing clot:

- The time it takes until initial fibrin formation.
- The kinetics of the initial fibrin clot to reach maximum strength.
- The ultimate strength and stability of the fibrin clot, ie. its ability to mechanically impede hemorrhage without permitting inappropriate thrombosis.

TEG® 5000
Thrombelastograph®
Hemostasis Analyzer

TEG

- The TEG analyzer has a sample cup that oscillates back and forth constantly at a set speed through an arc of 4°45'. Each rotation lasts ten seconds. A whole blood sample of 360 ul is placed into the cup, and a stationary pin attached to a torsion wire is immersed into the blood.
- When the first fibrin forms, it begins to bind the cup and pin, causing the pin to oscillate in phase with the clot. The acceleration of the movement of the pin is a function of the kinetics of clot development.


Torsion wire
Pin
Cup
.36 ml whole blood
(Clotted)
Heating element,
sensor & controller
4°45

TEG

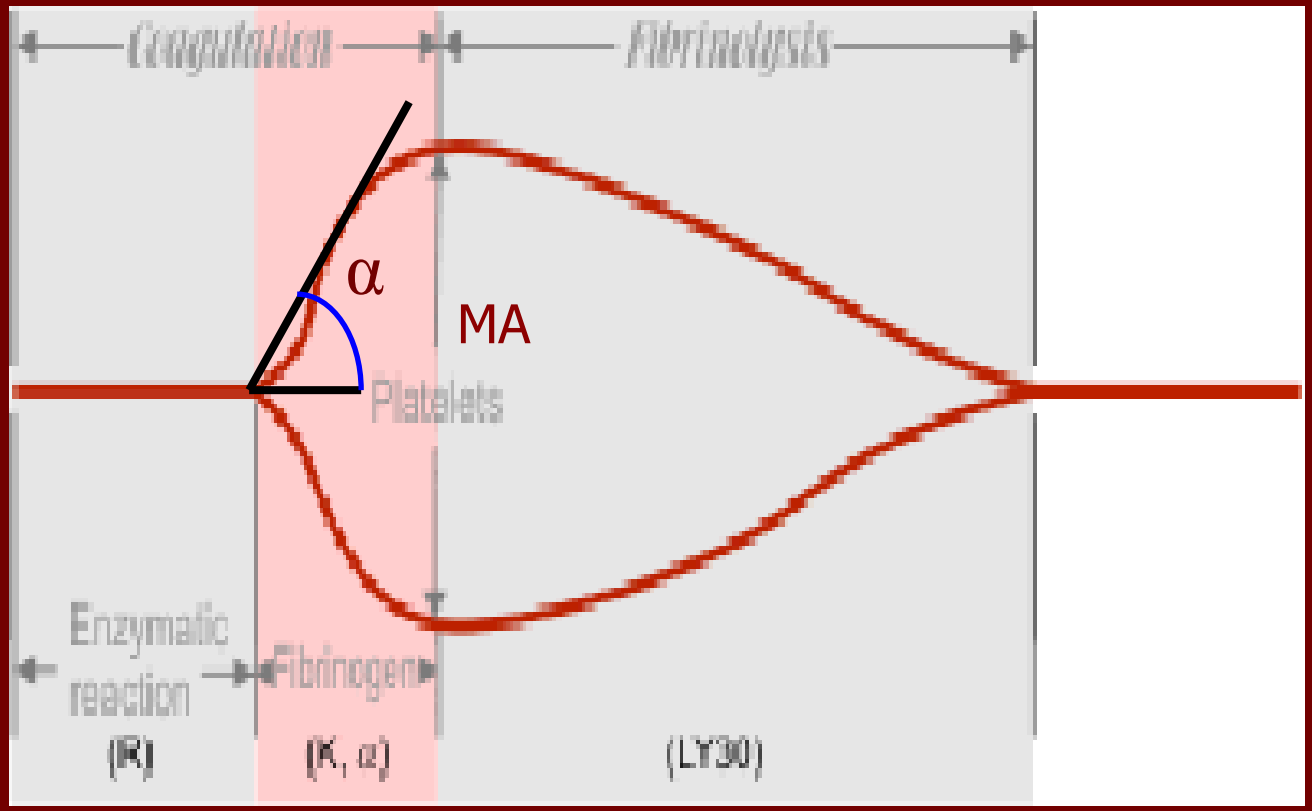
- The torque of the rotating cup is transmitted to the immersed pin only after fibrin-platelet bonding has linked the cup and pin together. The strength of these fibrin-platelet bonds moves the pin directly in phase with the cup motion. Thus, the magnitude of the output is directly related to the strength of the formed clot.
- As the clot retracts or lyses, these bonds are broken and the transfer of cup motion is diminished. The rotation movement of the pin is converted by a mechanical-electrical transducer to an electrical signal which can be monitored by a computer.

TEG

- The resulting hemostasis profile is a measure of:

  -The time it takes for the first fibrin strand to be formed,
  -The kinetics of clot formation,
  -The strength of the clot (in shear elasticity units of dyn/cm2), and
  -Dissolution of clot.

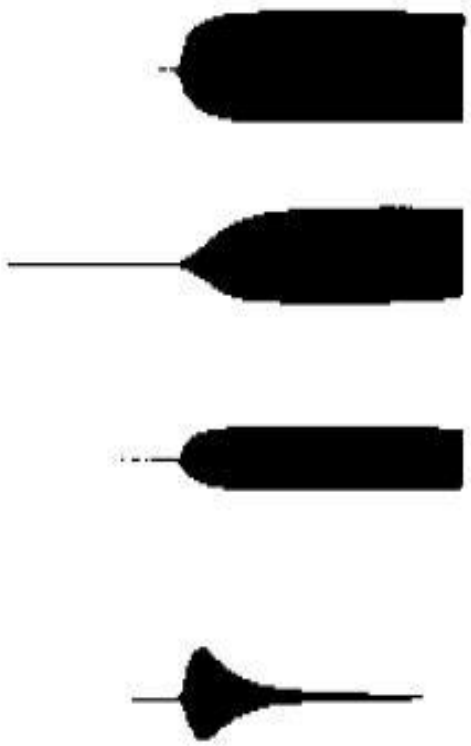
Parameters of clot dynamics



Parameters of clot dynamics

Clotting time	R	**The period of time of latency from the time that the blood was placed in the TEG analyzer until the initial fibrin formation (MA= 2 mm).**
Clot kinetics	**K**	**A measure of the speed to reach a specific level of clot strength (MA= 20 mm).**
	alpha	**Measures the rapidity of fibrin build-up and cross-linking (clot strengthening)**
Clot strength	**MA,G**	**A direct function of the maximum dynamic properties of fibrin and platelet bonding via GPIIb/IIIa and represents the ultimate strength of the fibrin clot.**
Hemostasis profile	**CI**	**Coagulation Index, which is a linear combination of the above parameters.**
Clot stability	**LY30**	**Measures the rate of amplitude reduction 30 minutes after MA.**

Patterns of TEG Tracings



- **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**
  R ~ Normal;
  MA = Continuous Decrease

Patterns of TEG Tracings

- **Hypercoagulation**
  R;K = Decreased
  MA;Angle = Increased
- **D.I.C.**
  Stage 1- Hypercoagulable state with secondary fibrinolysis
- Stage 2 - Hypocoagulable state