Special Coagulation - APC Resistance

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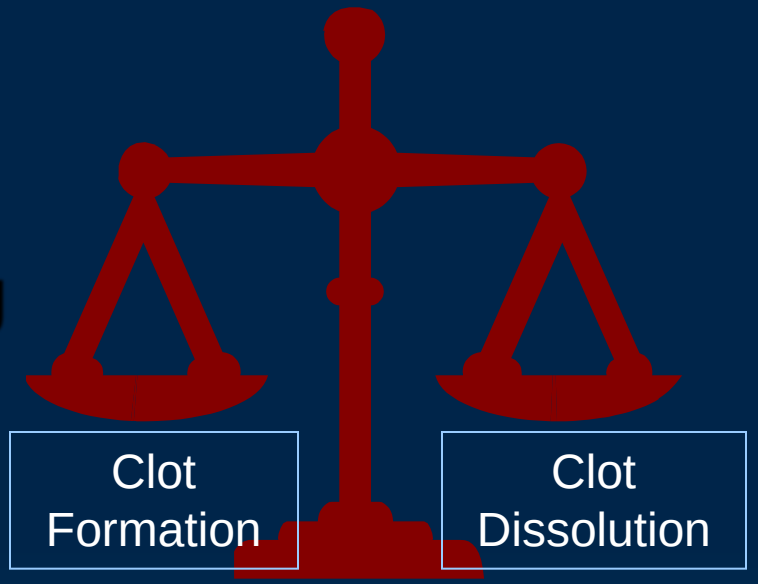
Overview

- Review of Hemostasis
  - Pathways of coagulation, anticoagulation, and fibrinolysis
- Thrombophilia screening
  - Hereditary & Acquired Risk Factors
  - Laboratory Evaluation of Thrombotic Risk
- APC Resistance
  - Phenotype vs. genotype testing
  - Interpreting the results
  - Setting up the test
  - Troubleshooting

Hemostasis

- **Hemostasis:** The balance between clotting and bleeding



Hemostasis

Components of Hemostasis:

- Vasculature
- Coagulation proteins
- Platelets

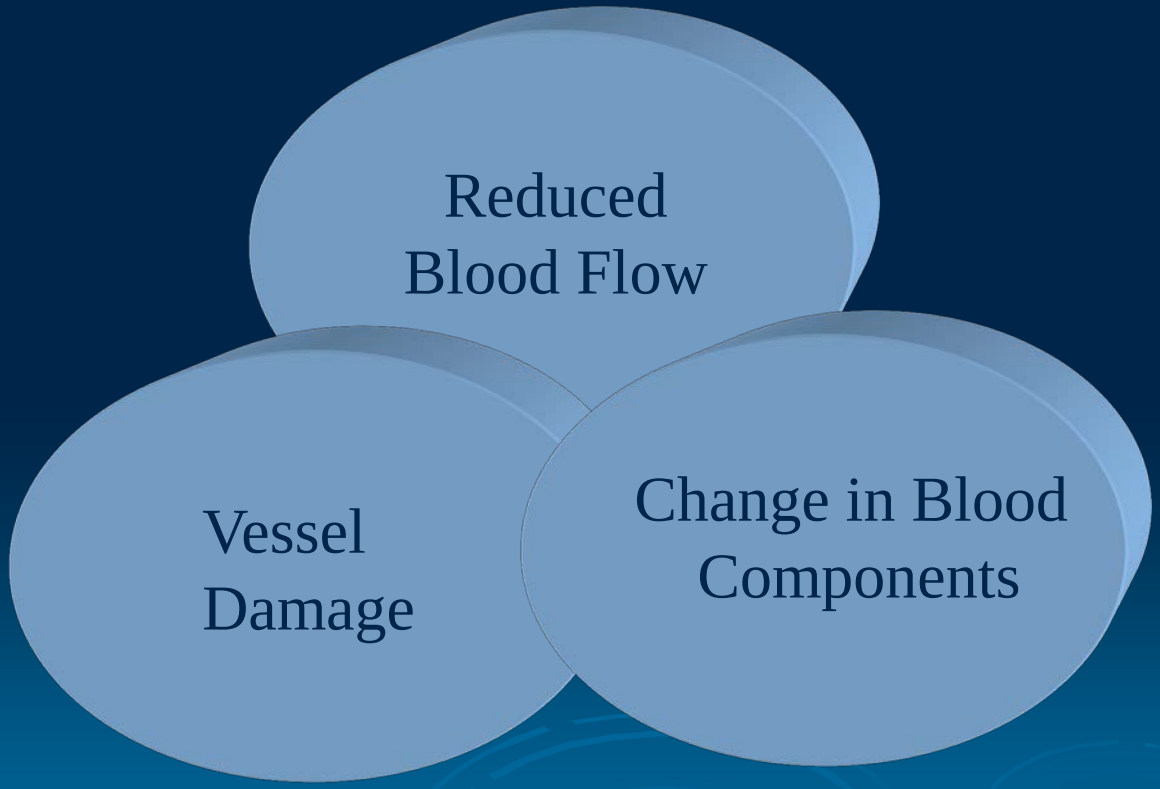
What is Thrombosis?

- Venous Thromboembolism (VTE) comprises DVT & PE
- Deep vein thrombosis (DVT) is a condition in which a blood clot forms inside a deep vein
  - Commonly located in calf or thigh
  - Occurs when the blood clot either partially blocks or completely blocks blood flow in the vein
- Pulmonary Embolism (PE) occurs when a blood clot breaks loose from the wall of a vein and travels to the lungs, blocking the pulmonary artery or one of its branches

What is Thrombosis?

VENOUS THROMBOEMBOLISM

Virchow's triad for venous thromboembolism:



Venous Thromboembolism

- Complex, multi-causal disease
  - Physiological factors
    - Age, hormonal influence (i.e. pregnancy)
  - Acquired risk factors
    - Cancer, surgery, obesity, trauma, immobility
  - Hereditary (genetic) risk factors
    - Deficiencies in anticoagulation proteins
    - Elevated coagulation proteins
    - Gene mutations preventing function of proteins

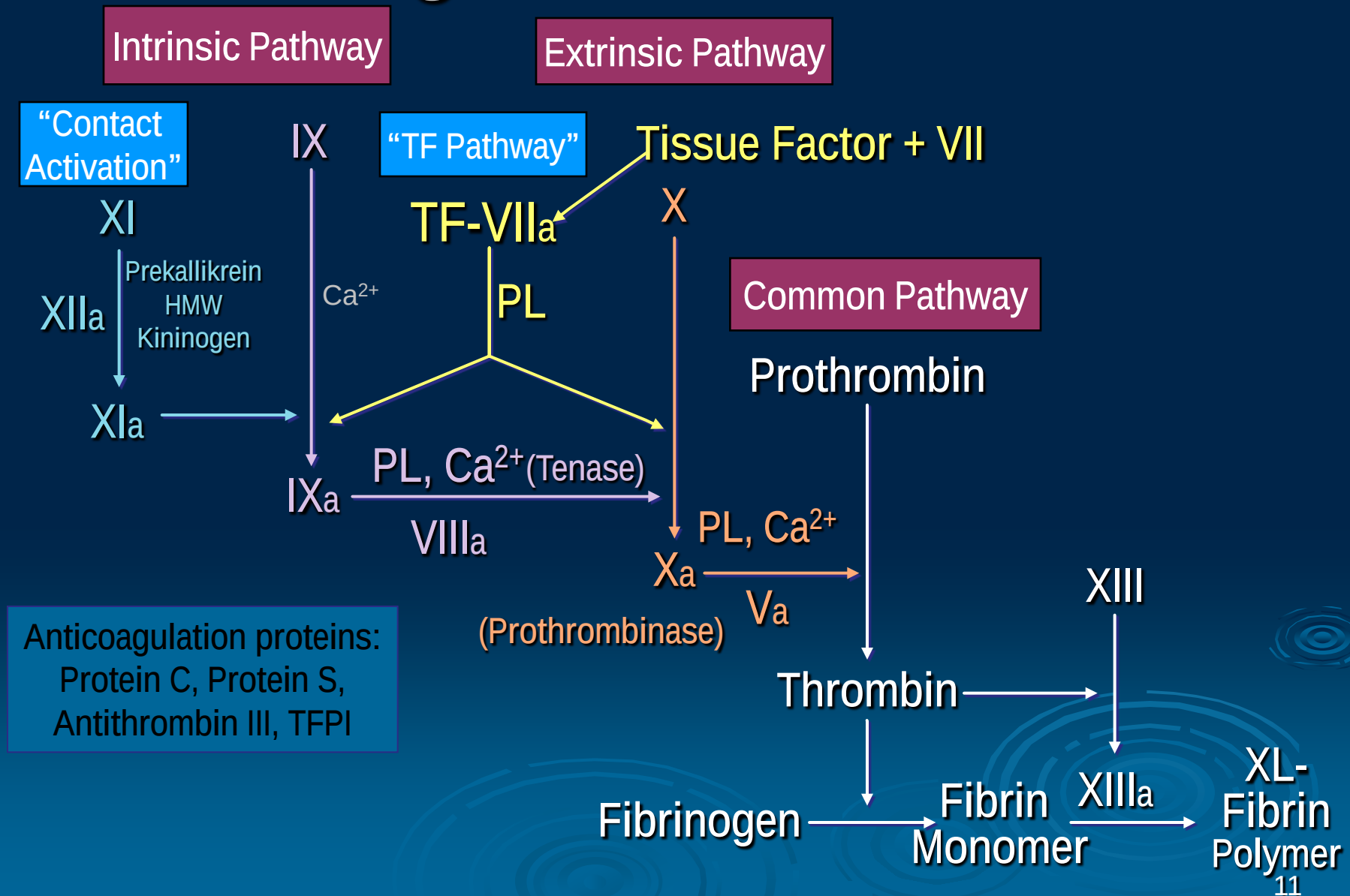
Venous Thromboembolism

- It is important to understand the risk factors associated with VTE in order to better prevent and treat the disease

Coagulation Cascade

- Vascular damage initiates the coagulation cascade.
- Results in the generation of thrombin at the site of injury.
- Thrombin catalyzes the conversion of fibrinogen to an insoluble fibrin (clot) matrix.

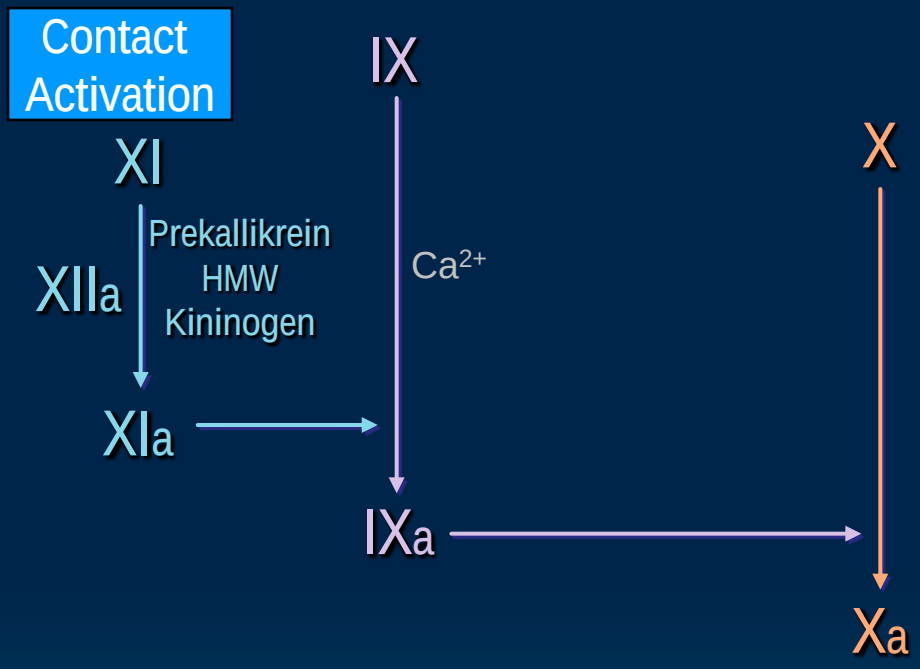
Coagulation Cascade



Coagulation Cascade

- The cascade scheme is organized into the INTRINSIC and EXTRINSIC pathways, converging into the COMMON pathway.

Intrinsic Pathway



Intrinsic Pathway

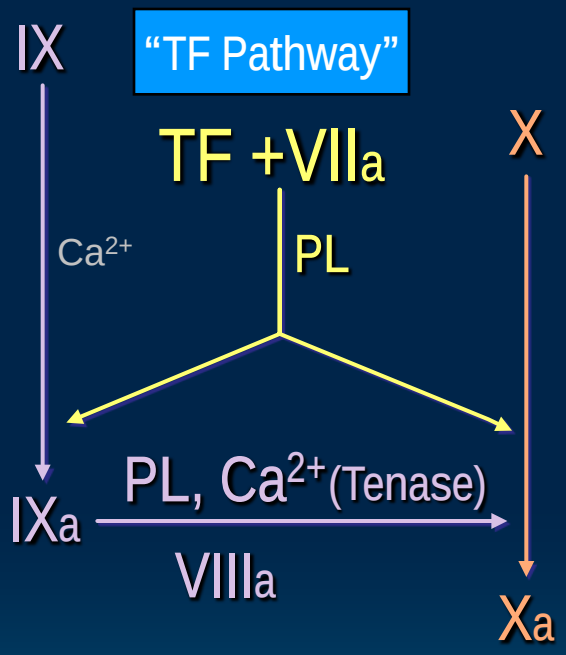
"Contact Activation": Initiated by the activation of FXII involving contact factors on negatively-charged phospholipid surfaces (glass or kaolin in vitro)

- Factors XII, XI, IX, VIII, prekallikrein, HMW kininogen
- Measured with aPTT clotting assay

Intrinsic Pathway - APTT

- The Activated Partial Thromboplastin Time (APTT): The clotting time in seconds of a mixture of citrated plasma, Ca^{2+}, contact activator, and phospholipid
- Tests for deficiencies of pro-coagulant factors in the INTRINSIC and COMMON pathways
- Heparin, Warfarin, Factor Inhibitors, Lupus Anticoagulant can prolong the APTT

Extrinsic Pathway



<u>Extrinsic Pathway</u>
"Tissue Factor Pathway"

Initiated when blood is exposed to TF released from damaged endothelium

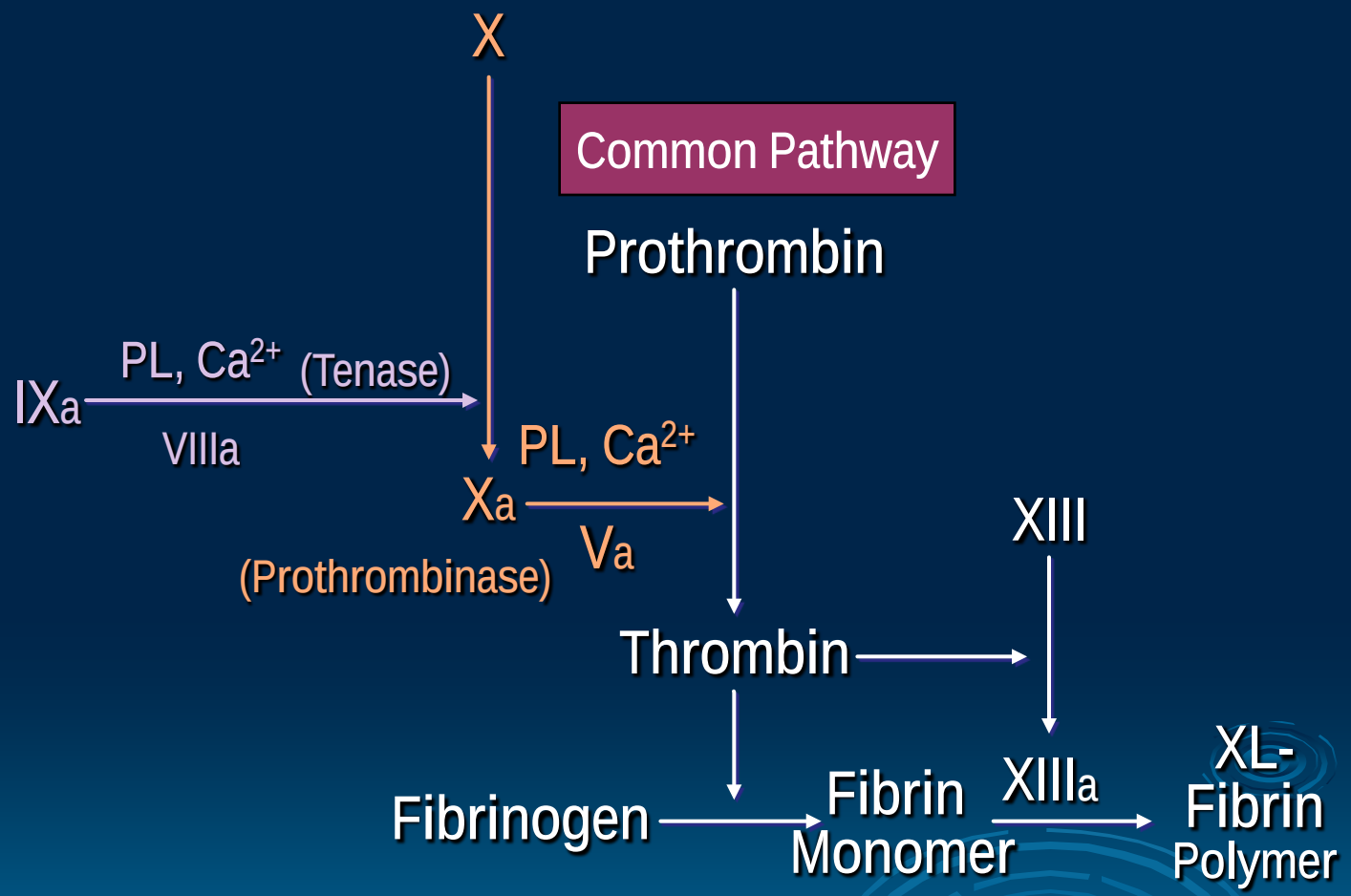
- Tissue Factor (TF), FVII
- Measured with PT clotting assay

Extrinsic Pathway - PT

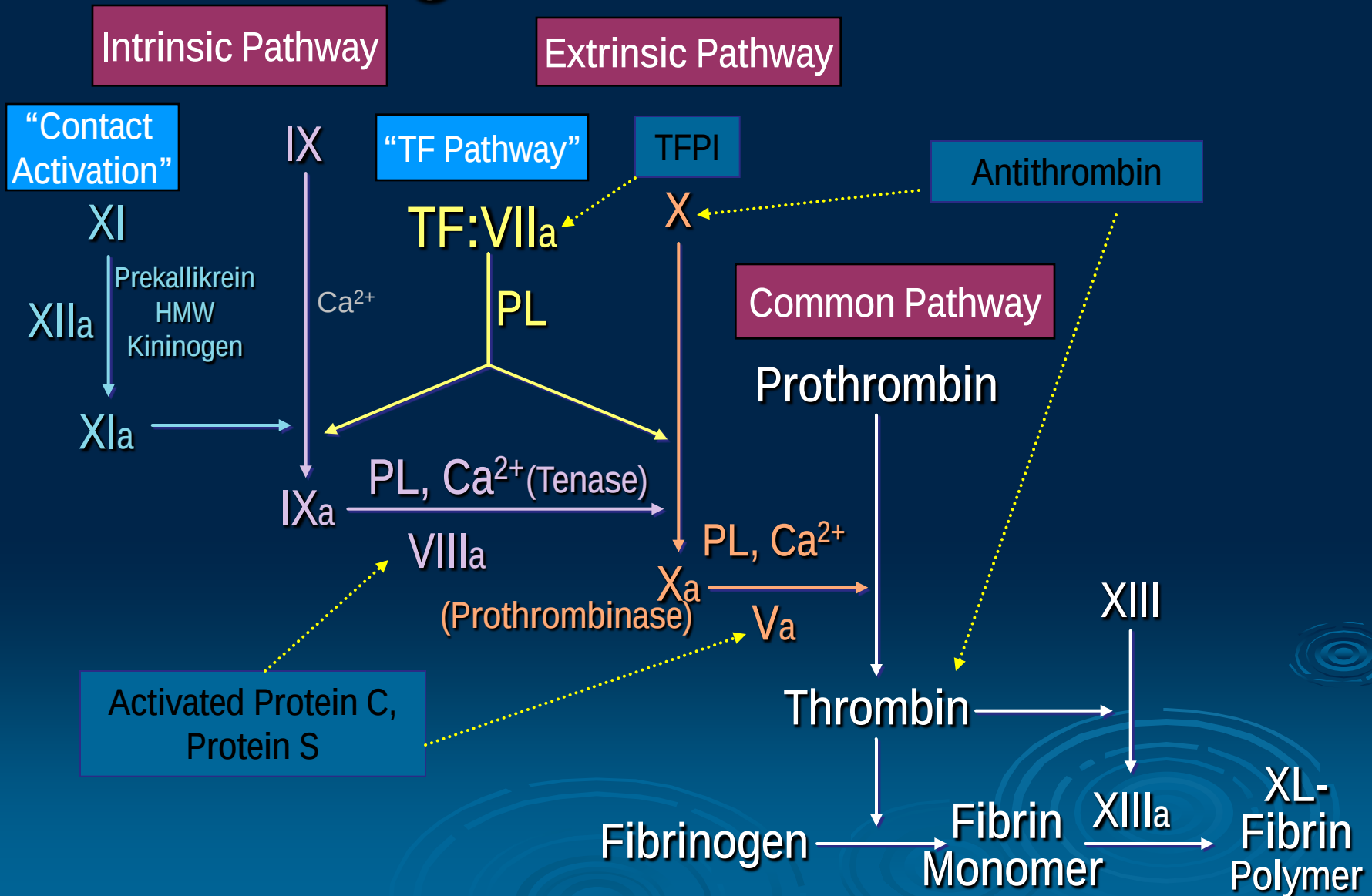
- Prothrombin Time (PT): clotting time in seconds of a mixture of thromboplastin (Tissue Factor) reagent and citrated plasma in the presence of Ca^{2+}
- Tests for deficiencies of pro-coagulant factors of the EXTRINSIC and COMMON pathways

Common Pathway

Common Pathway: Factors V, X, XIII, II (prothrombin), Fibrinogen



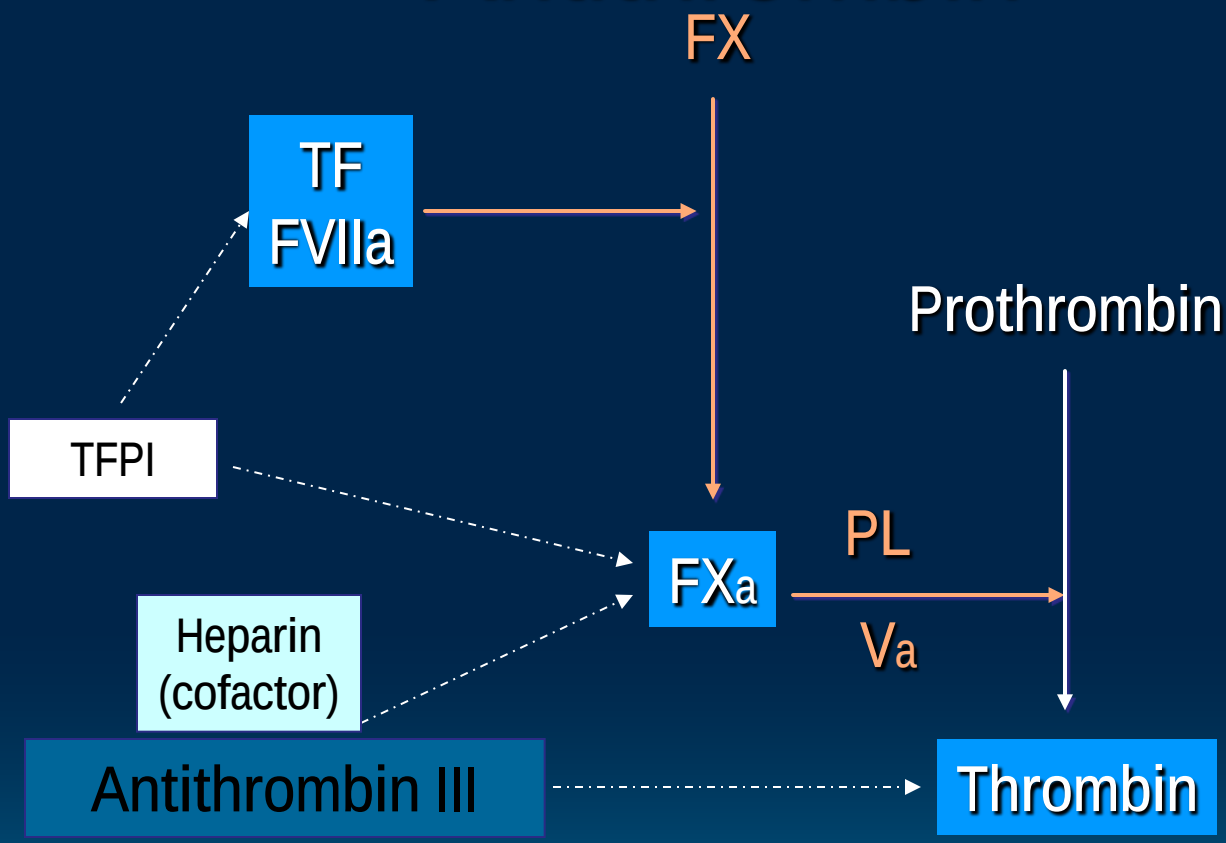
Coagulation Cascade



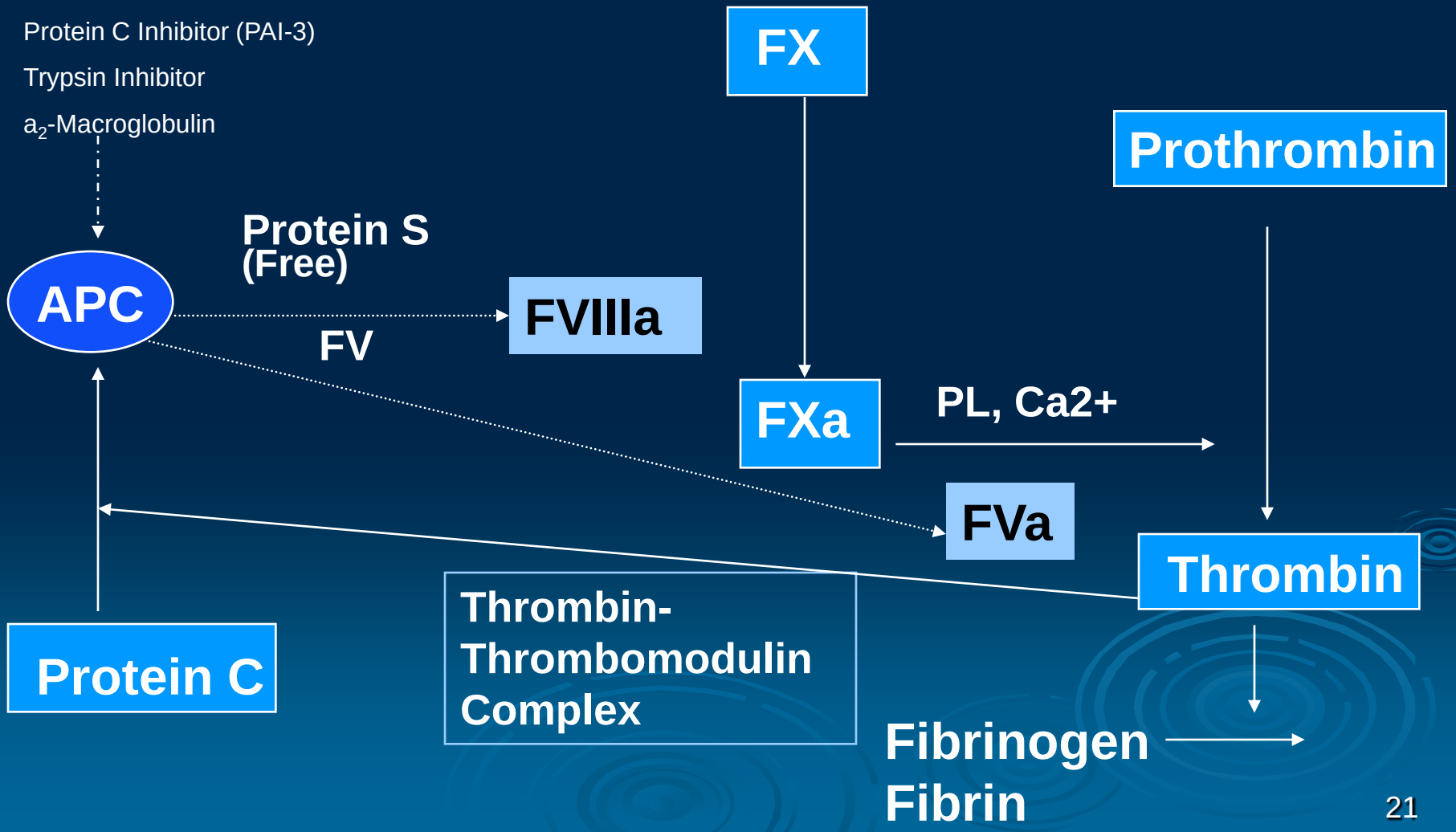
Anticoagulation Pathways - Antithrombin

- Antithrombin is the major inhibitor of thrombin, accounting for approximately 80% of thrombin inhibitory activity in plasma
- Antithrombin primarily inhibits Thrombin and FXa

Anticoagulation Pathways - Antithrombin



Anticoagulation Pathways – Protein C



Activated Protein C (APC) cofactors

APC has two known cofactors: Protein S and Factor V.

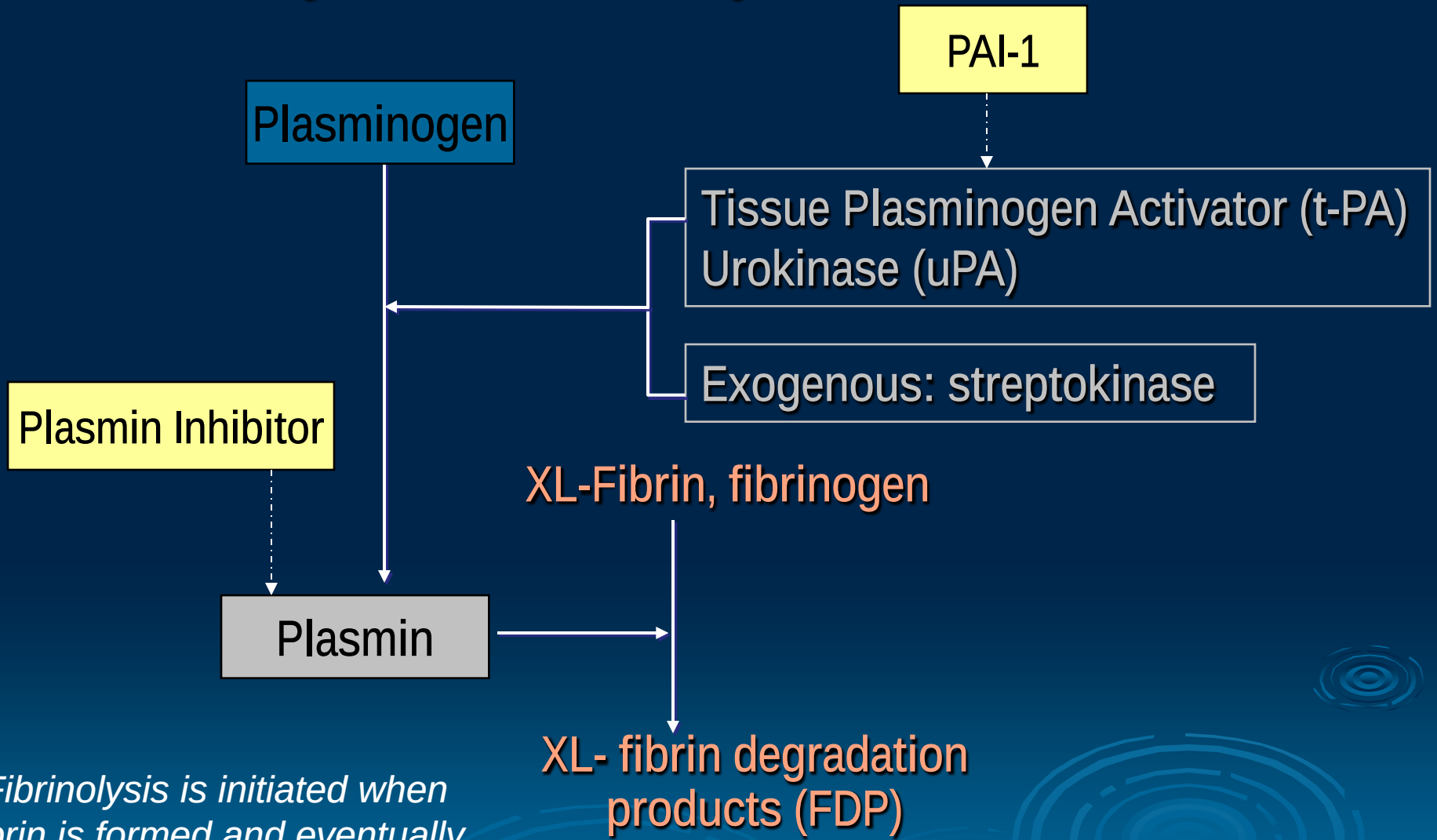
Protein S:

- Protein S enhances binding of APC to the phospholipid of platelets and endothelial cells.
- Only free protein S has a APC cofactor function. 60% of protein S is bound to C4bBP.

Factor V

- Factor V together with Protein S makes APC degrade FVIIIa and FVa more effectively.

Fibrinolytic Pathway



Fibrinolysis is initiated when fibrin is formed and eventually dissolves the clot.

Hereditary & Acquired Risk Factors

- There are several well-established risk factors and corresponding assays to test for them
  - Most of these risk factors can be hereditary or acquired

Hereditary & Acquired Risk Factors

- **Inherited Risk Factors**
- APC resistance-Factor V Leiden
- AT deficiency
- Protein C deficiency
- Protein S deficiency
- Prothrombin Mutation
- Dysfibrinogenemia (rare)

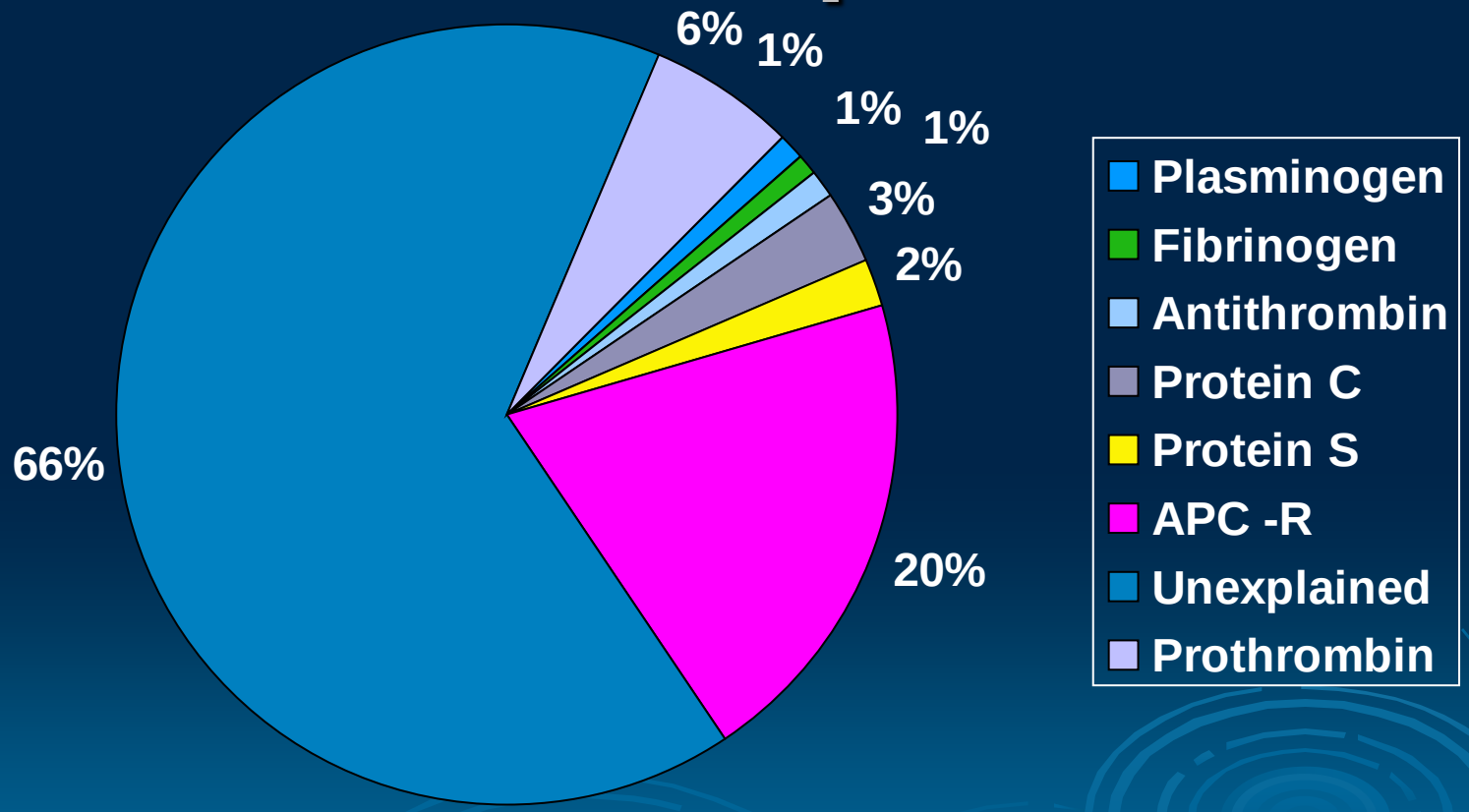
Inherited or Acquired Risk Factors:

•Hyperhomocystenemia

•Elevated levels of FVIII, IX,XI

- **Acquired Risk Factors**
- Age
- Malignancy
- Immobilization
- Trauma, Post-op
- Pregnancy
- Estrogen use
- Antiphospholipid Antiboides
- Long distance flights
- Hematologic Diseases

Genetic risk factors in unselected thrombosis patients



Laboratory Evaluation of Thrombotic Risk

- Laboratory Screening for thrombophilia is appropriate only in certain circumstances, as it is cost-prohibitive.

- There is no global assay currently available to determine thrombotic risk, so a panel of assays is performed.

Laboratory Evaluation of Thrombotic Risk

What is the role of the coag lab in evaluating patients with thrombosis?

- Laboratory personnel have an important role in discussing with clinicians:
  - Diagnostic tests available
  - Which assays are optimal and appropriate
  - Sample collection & timing

Laboratory Evaluation of Thrombotic Risk

- The quality of blood sample is of major importance
- Evacuated tubes with 3.2% trisodium citrate should be used for blood draws
- An improperly drawn sample may be activated, interfering with measured levels of coagulation factors…AVOID CONTACT ACTIVATION
- Samples drawn from lines may contain heparin, interfering with clotting assays

Laboratory Evaluation of Thrombotic Risk

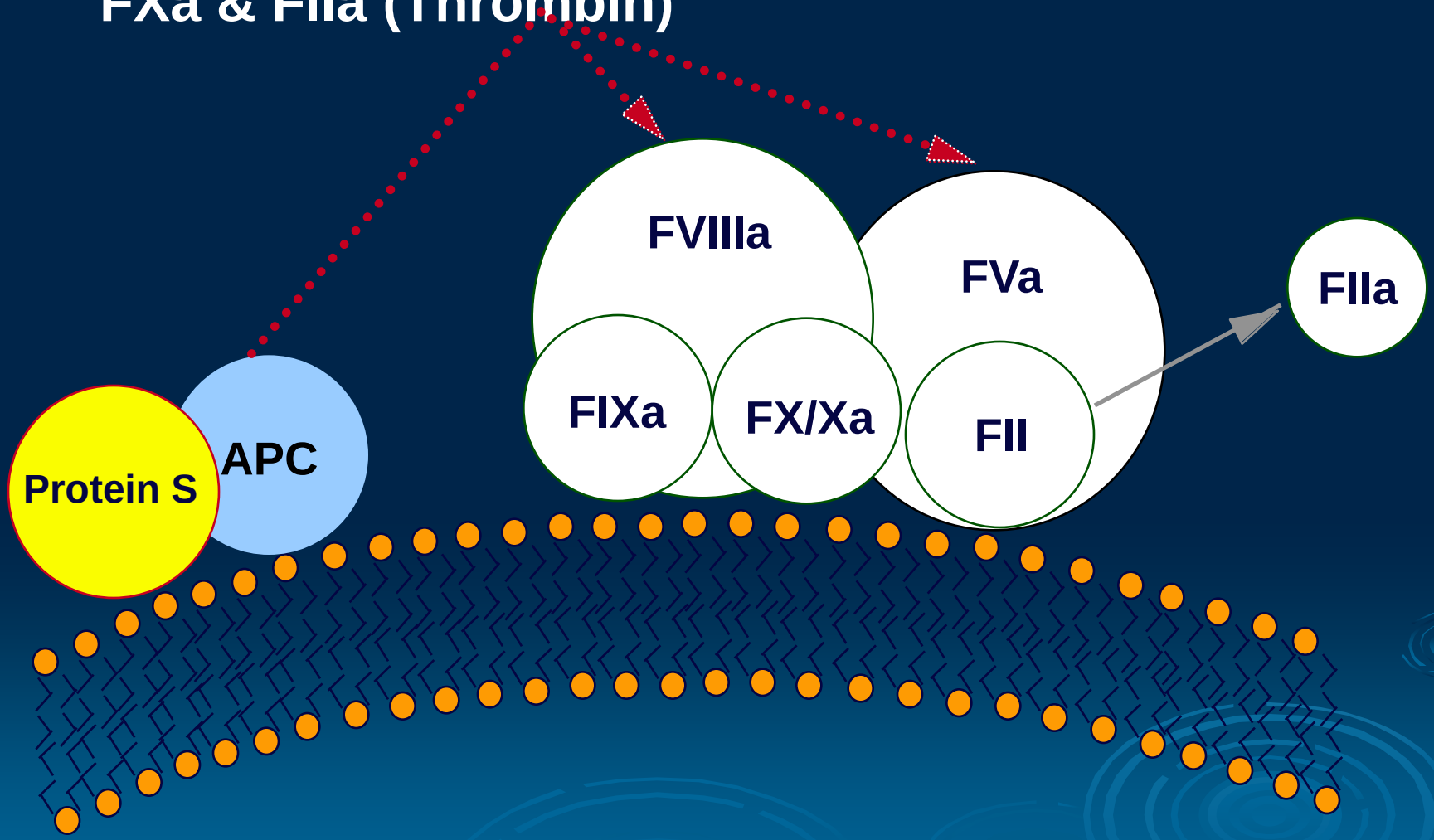
Types of Assays

- Functional Activity Assays
  - Clotting
  - Chromogenic
- Immunological / Antigenic Assays
  - ELISA
  - LIA

Laboratory Evaluation of Thrombotic Risk

Risk Factor	Laboratory Assay
Antithrombin Deficiency	AT activity
Protein C Deficiency	Protein C Deficiency PC activity (clotting or chromogenic)
Protein S Deficiency	Protein S Free Antigen (ELISA, LIA)
APC Resistance / Factor V Leiden Mutation	APC Resistance (aPTT); FV Leiden genetic test if abnormal
Prothrombin Mutation G20210A	Genetic Test
Hypherhomocysteinemia (elevated homocysteine)	EIA, HPLC
Elevated Factor VIII Activity	Factor VIII activity (clotting or chromogenic)
Lupus Anticoagulant	DRVVT Clotting Assay
Anticardiolipin Antibody, IgG / IgM	aCL IgG/IgM Antigen ELISA

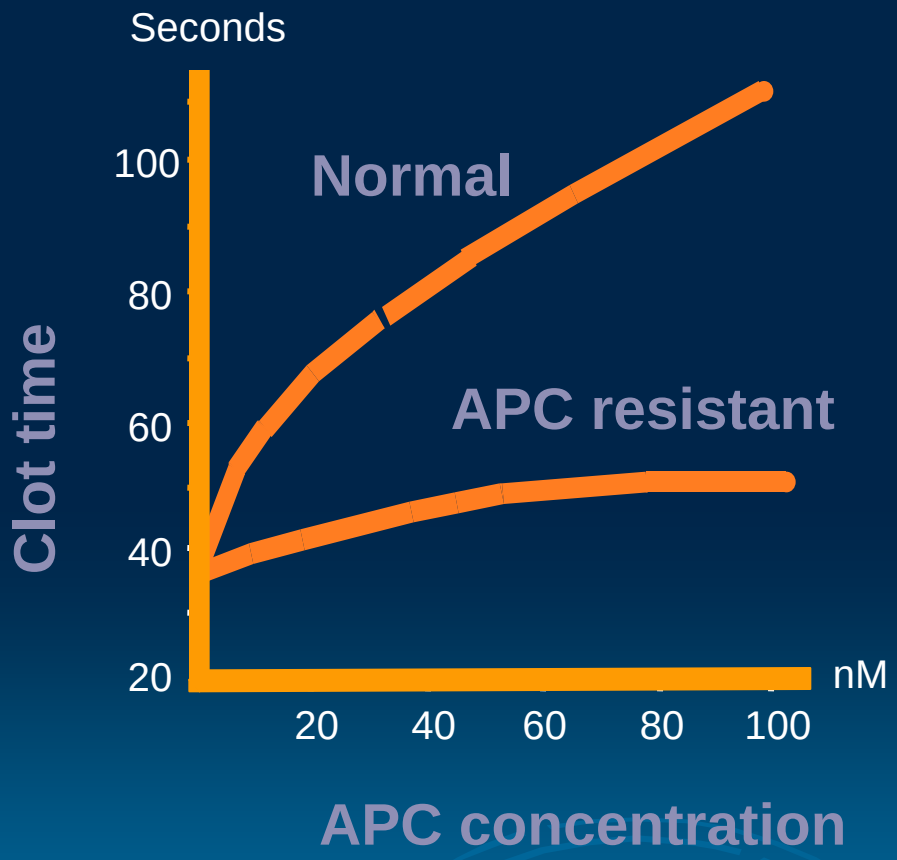
Protein S / APC Complex: Inactivates FVa & FVIIIa, inhibiting generation of FXa & FIIa (Thrombin)



APC Resistance

- Common in the general population
- Most common cause of hereditary thrombophilia
- Can be hereditary or acquired
- APC Resistance alone is not a significant risk factor. Having APC Resistance combined with other risk factors, however, greatly increases risk of thrombosis

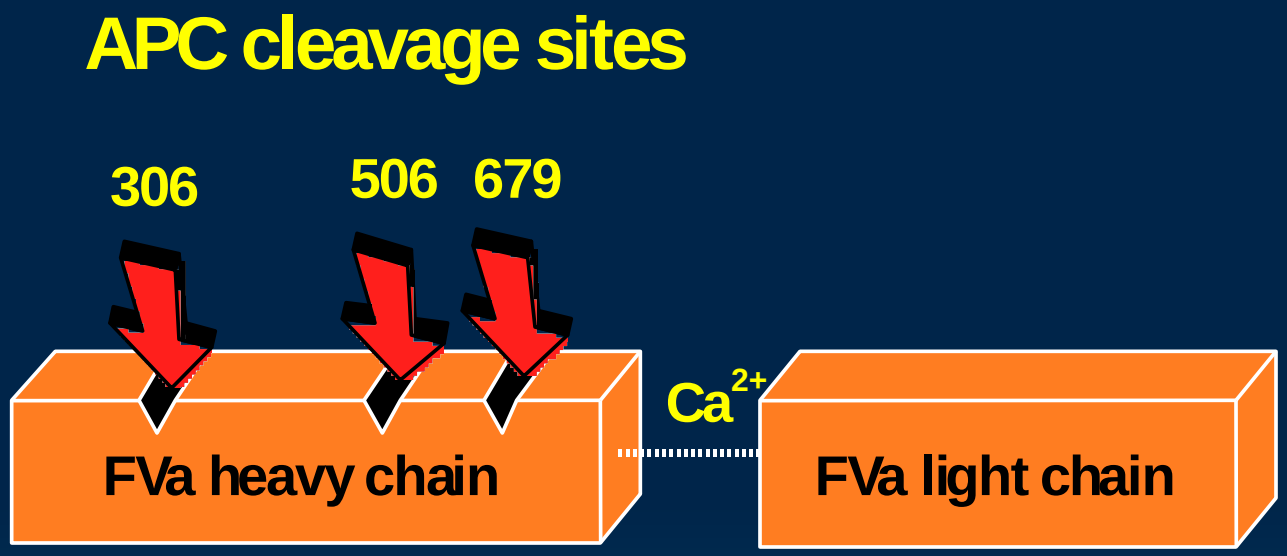
ANTICOAGULANT RESPONSE TO APC



APC resistance phenotype

- A poor anticoagulant response to activated protein C (APC).
- In an APC R patient, there is not as much inactivation of coagulation

INACTIVATION OF NORMAL FVa



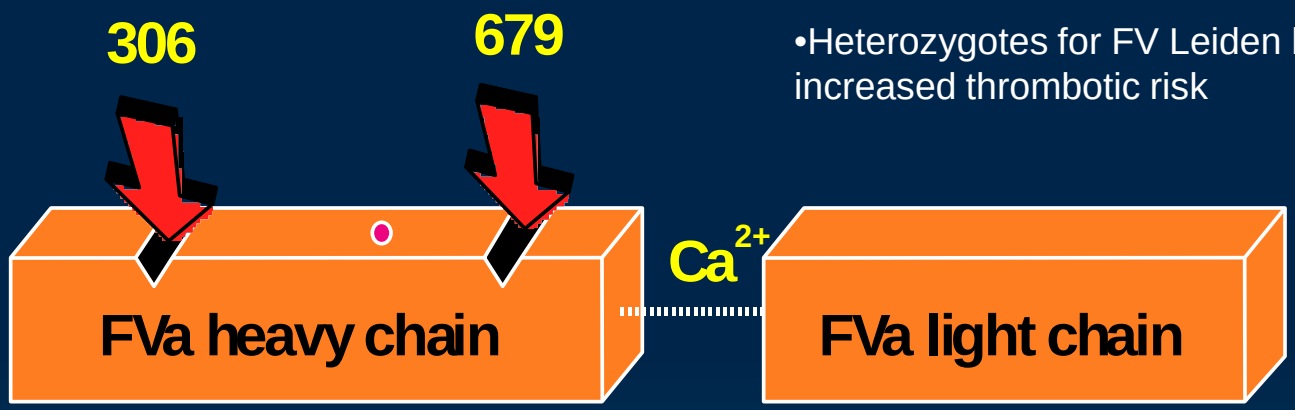
Normal

- APC cleaves sites on the heavy chain, inactivating FVa and helping to prevent too much thrombin activation.
- Cleaves at the 306, 679 and **506** positions.

INACTIVATION OF MUTANT FVa:Q^{506}

FV Leiden Mutation

- Accounts for approx. 90% of APC Resistance
- Prevalent in about 2 – 13% of general population
- Accounts for about 20 – 60% of VTE cases
- Heterozygotes for FV Leiden have 2 – 5 fold increased thrombotic risk



- **Mutant**

Arg to Glu Mutation results in a 10-fold lower inactivation rate of FVa

i.e. FVa molecule isn't allowing APC to do its job of inactivating FVa and ultimately inhibiting thrombin generation.

GENETIC AND ACQUIRED RISKS

Genetic risk factors:

APC resistance (FV:Q^{506}, FV Leiden)

Acquired risk factors:

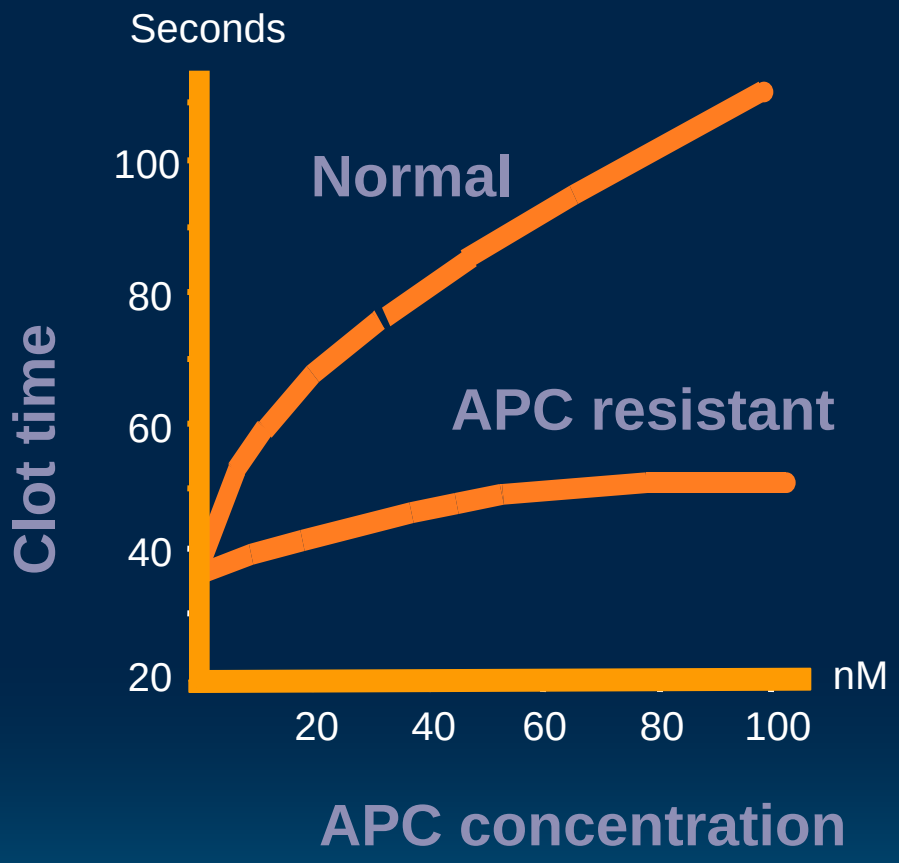
Surgery, Pregnancy and Oral Contraceptive Pills / Patch

Account for about 5 – 10% of APC resistance

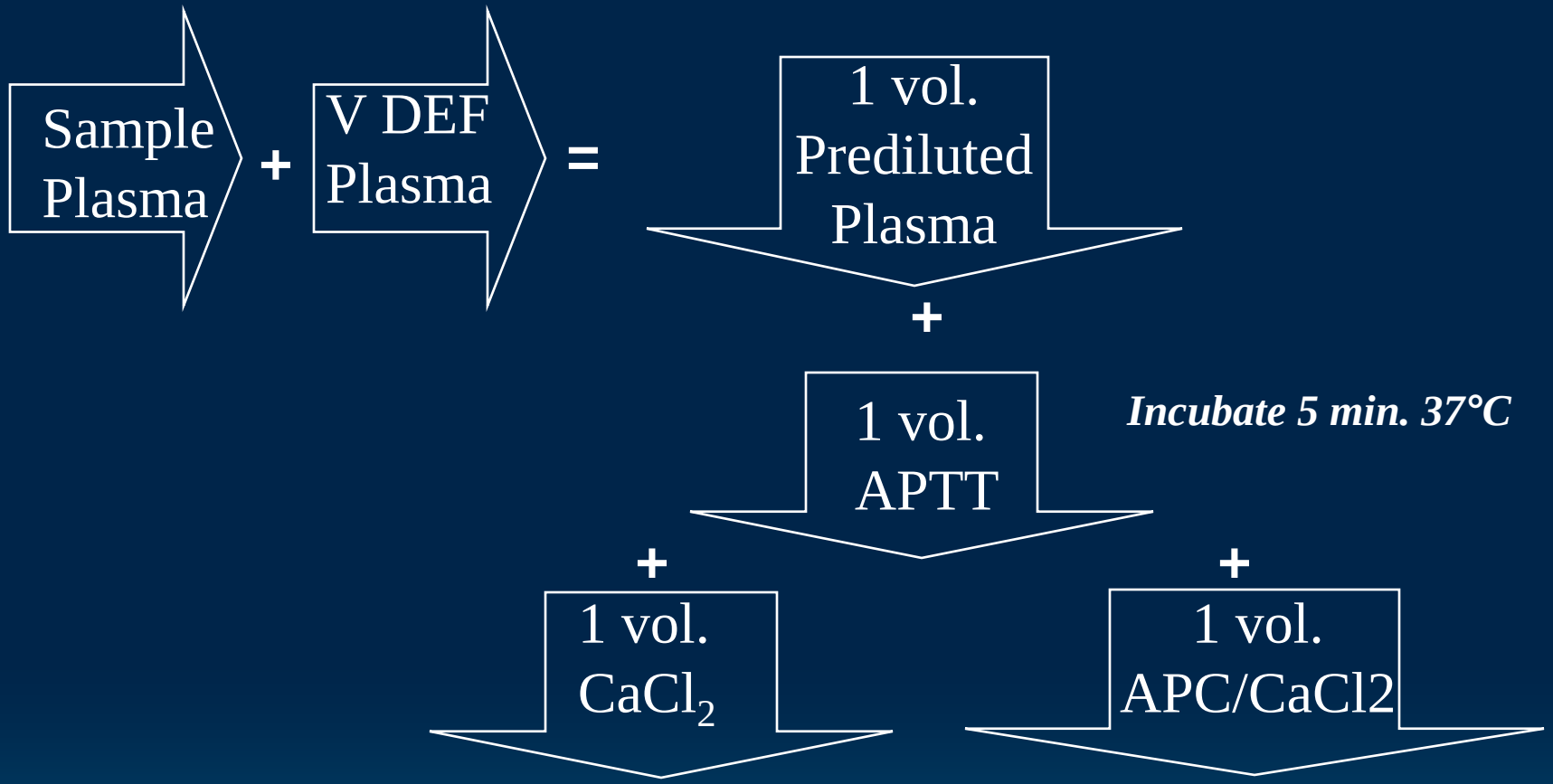
TESTING FOR APC RESISTANCE

- "Gold standard" is an APTT based clotting assay.
- Two APTT tests are run: one with CaCl2 ("Baseline clotting time") and one with an excess of APC and CaCl2 ("Activated clotting time").
- Record the clotting times and calculate the ratio.
- In a normal patient, this excess APC will cause inactivation of FVa at a higher rate, meaning less thrombin generation, prolonged clotting time, and higher ratio between basal and APC clotting times.
- In an abnormal patient, however, even if you add that excess APC, FVa is not being inactivated as much, so you don't see that prolongation of APC clotting time.
- *Therefore, the ratio between basal and APC clotting times is not as high as it would be in a normal patient.*

- By diluting the sample 1:4 in FV-deficient plasma, you test for FV Leiden. This also allows testing of samples containing heparin or warfarin.


Seconds
100
80
60
40
20
Normal
APC resistant
Clot time
nM
20
40
60
80
100
APC concentration

APTT-based APC Resistance Assays



Record time for clot formation

APC RESISTANCE: INTEPRETATION OF RESULTS

- APC- ratio = $\frac{\text{Clot time APC/CaCl}_2}{\text{Clot time CaCl}_2}$

- APC Resistance is indicated when the APC ratio is below or equal to the calculated cut-off value.

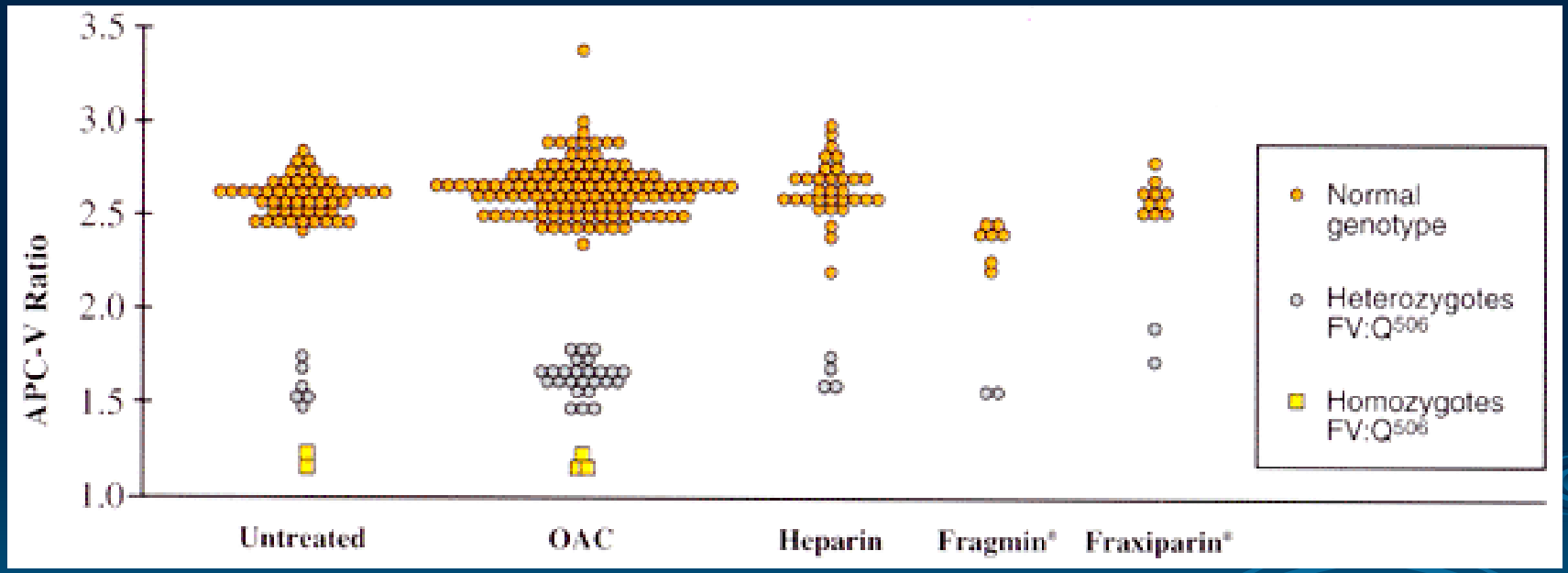
- APC R V ratio below the calculated cut-off is due to presence of the factor V:Q506 mutation

APTT-based APC Resistance Assays

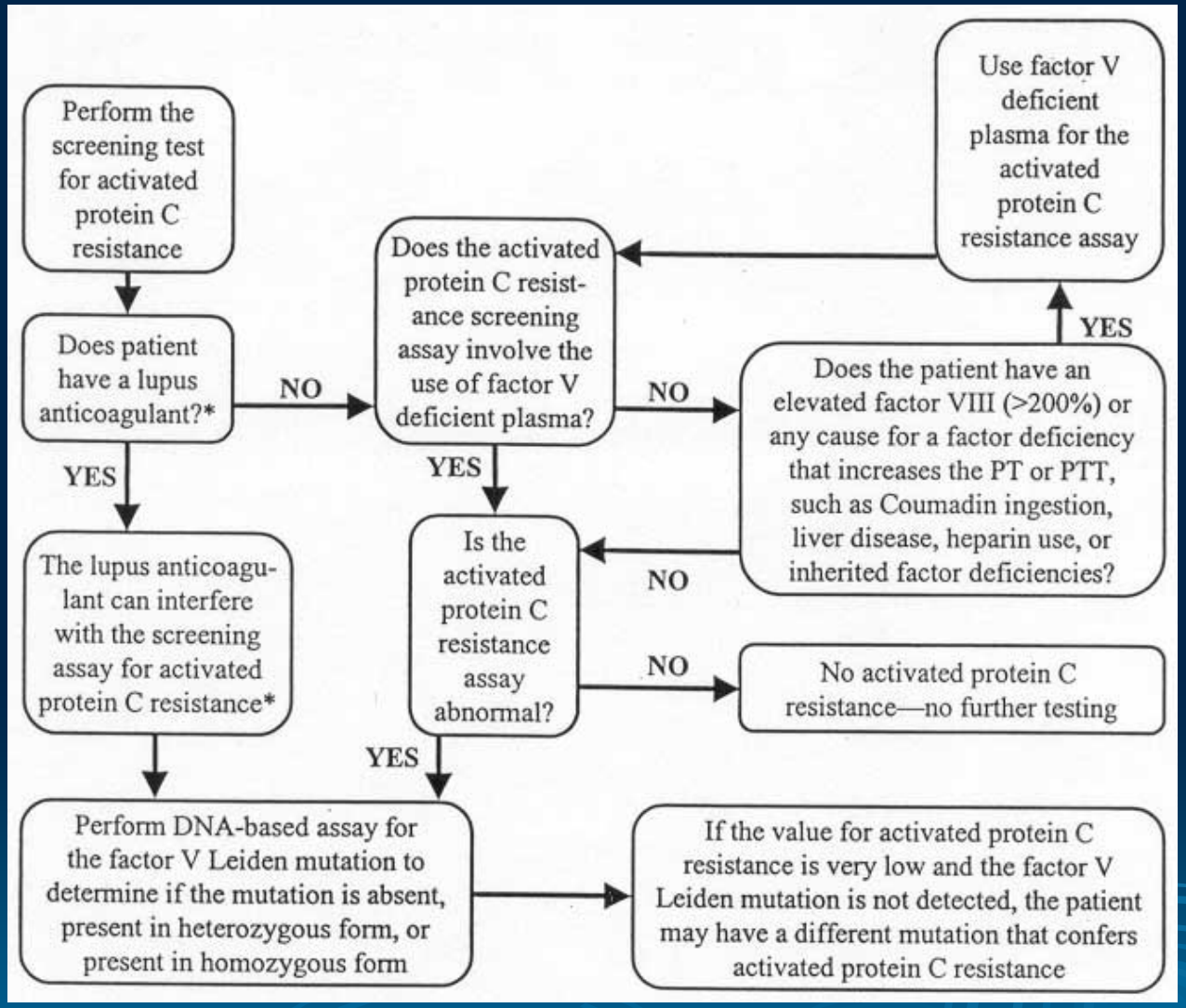
- Benefit:
  - Offers genotypic information for clinical decision-making
- Utility:
  - For factor V:Q^{506} mutation *screening*
  - Ratio at or below cut-off may be confirmed with genetic test
- Features:
  - Unsurpassed sensitivity for the factor V:Q^{506} mutation and close to 100% specificity
  - Applicable to anticoagulant treated patients
  - Economical alternative to genetic testing

APC Resistance

Clear discrimination between normals, heterozygotes, and homozygotes is achieved with the APTT-based screening assay.



Algorithm for APC R Testing



APC Resistance Testing – Tech Tip

- When performing Coatest® APC Resistance and Coatest® APC Resistance V/VS, pay attention to the clotting times as well as your ratio.
- The baseline APTT time (only CaCl2), which is the bottom number in your ratio, should be within the range of APTT times you observed when you established when determining your cut-off.
- Typical values are about 30 - 45 seconds, and will vary between labs and instrumentation.

APC Resistance Testing – Tech Tip

- Although the 1:4 pre-dilution with Factor V Deficient Plasma strongly decreases interferences, prolonged baseline APTT may occur in patient plasmas with high inhibitor activity (e.g. phospholipid antibodies).
  - In that case, increasing the dilution (e.g. 1:9 or 1:19) may correct the result. If the additional dilution with Factor V Def Plasma does not correct the clotting time, then the calculated APC ratio is not valid, and it is recommended to use PCR results to determine APC resistance.

APC Resistance Testing – Tech Tip

- It’s important to flag any results from patient plasmas with a baseline APTT time that lies outside of your normal distribution to avoid misleading APC ratio results

APC Resistance Testing – Tech Tip

- Calculating the Cut-off:
  - Use at least 30 patient plasma samples from healthy individuals
  - Include Control Plasma Level 1 (normal) and Level 2 (heterozygous) for QC
  - Determine the MEDIAN ratio…not the average…you may have an outlier in that supposedly normal population
  - Multiply the median ratio by 0.8 if the ratio is below 2.8 OR by 0.75 times the median ratio when 2.8 or higher.

APC R - Troubleshooting

- Some lot-to-lot variation is expected due to the nature of the assay
  - APC is added to activator to adjust the APC/$CaCl_2$ clotting time to keep ratio within about +/- 5% of prior lots

APC R - Troubleshooting

- Recalculation of cut-off may be needed periodically
  - How do you know if you have to?
    - Look back at last 30 normal patients, find the median ratio, and recalculate the cut-off as described in the package insert
    - Compare this cut-off to your current cut-off, and decide whether it needs to be changes using new normal plasma.

APC R - Troubleshooting

- Quality Control:
  - Chromogenix Control Plasma Level 1: Normal
    - Ratio should be in normal range
  - Chromogenix Control Plasma Level 2: Heterozygous for FV Leiden
    - Ratio should be BELOW the cut-off

APC R - Troubleshooting

- What if my controls don't come in range?
  - Are you using the Chromogenix controls (normal and heterozygous)?
  - Chromogenix controls are tested to have specific clotting times and ratios
  - If using a different brand of controls, test the kit with the Chromogenix controls and see if they are within range

APC R - Troubleshooting

- What role does the coagulation analyzer play?
  - Kits are validated on the ACL line of instruments
  - Clotting times can vary depending on the instrument, especially with optical vs. electromechanical clot detection principles
    - Sometimes baseline clotting times are more prolonged on electromechanical vs. optical instruments
  - Cut-offs should be calculated for each analyzer

- Changing analyzers = validate the assay

APC R - Troubleshooting

Reagents

- Reagents are carefully standardized
- Use only the Factor V deficient plasma supplied in the kit
  - This is not a reagent to be used in FV mixing studies, but is specifically formulated with concentrations of FV and FVIII to work with the aPTT reagent in the Coatest APC R-V kit
- Don't mix reagents from different lots
- Proper storage matters
  - Don't freeze the aPTT
  - Mix the aPTT well; don't let it settle
  - APC/$CaCl_2$, FV-def plasma, control plasmas can be frozen, but thaw rapidly at 37°C and don't refreeze (only 1 freeze/thaw cycle)

Questions?

To learn more about hemostasis, visit www.diapharma.com

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