Laboratory Tests of Hemostasis (Part 2)

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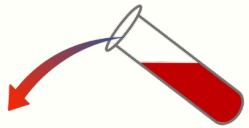
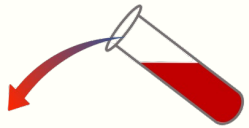
Disclosures

- Research Support (Past 5 years):
  - Amgen
  - Janssen Scientific Affairs
  - Sobi/Dova Pharmaceuticals
  - Anthos Therapeutics
- Advisory Boards (Past 5 years)
  - Janssen Scientific Affairs
  - Sobi/Dova Pharmaceuticals
  - Luzsana (HengruiUSA) Biotechnology
  - Sanofi



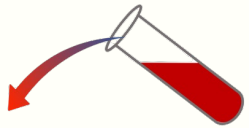
Learning Objectives:
Part 2: Laboratory Tests of Thrombotic Disease

- Describe the pathophysiology of heparin-induced thrombocytopenia and discuss the appropriate use of screening and confirmatory tests.
- Discuss the effect of lupus anticoagulant on coagulation screening tests and describe the test principle and interpretation of confirmatory tests.
- List appropriate laboratory screening tests for the evaluation of patients with inherited thrombophilia.
- Discuss the effect of therapeutic anticoagulants on the reliability of results of clot based, chromogenic, immunologic and molecular tests.



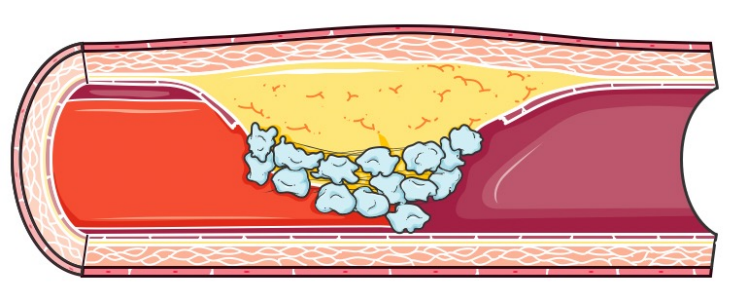
Material To Cover

1. *The Hemostatic Balance*
2. *Overview of The Coagulation Cascade and Testing*
3. *Functional and Immuno Assays*
4. *The Prothrombin and Activated Partial Thromboplastin Times*
5. *Other Tests:*
   - *Anti-Xa Heparin Assay*
   - *Thrombin Time*
   - *Fibrinogen Assay*
   - *D-Dimer*
   - *Thromboelastography (TEG) and Thromboelastometry (ROTEM)*
6. Interpretation of Prolonged PT and/or aPTT Results
7. ***Tests Of Thrombotic Diseases***
8. ***Heparin Induced Thrombocytopenia/Thrombosis (HITT): Pathophysiology***
9. ***Antiphospholipid Antibody Syndrome***
10. ***Laboratory Testing for Thrombophilia (Hypercoagulable State)***
11. ***APC-Resistance—Screening Assay For Factor V Leiden***
12. ***Conditions That Impact Tests for Thrombotic Risk Factors.***
13. ***If/When to Do Hypercoagulable Work-up***

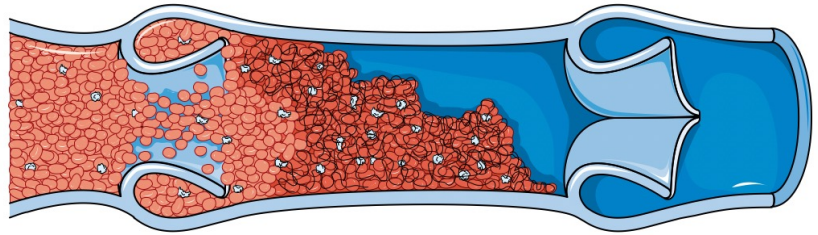


Arterial vs Venous Thrombotic Disease

- **Arterial thrombosis**
  - **Limited laboratory tests available**
  - **Vascular Damage**
  - **Atherosclerotic risk factors**
  - **Platelet activity**

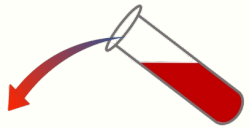


- **Venous Thrombosis**
  - **Laboratory testing available for specific components, but no screening or global tests.**
  - **Decreased regulation of coagulation**
  - **Increased procoagulant activity**
  - **Decreased fibrinolytic activity**
  - **Non0Hematologic parameters**



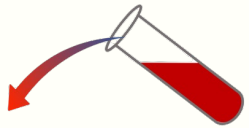
The Hemostatic Balance: Testing

Tests of Hemorrhagic Tendency	Tests of Thrombotic Tendency
➢ PT ➢ APTT ➢ Fibrinogen ➢ Thrombin Time ➢ D-dimer ➢ Factor Assays ➢ VWD testing	➢ HIT ➢ Lupus Anticoagulant ➢ Protein C ➢ Protein S ➢ Antithrombin ➢ FV Leiden ➢ PT G20210A ➢ Homocysteine ?

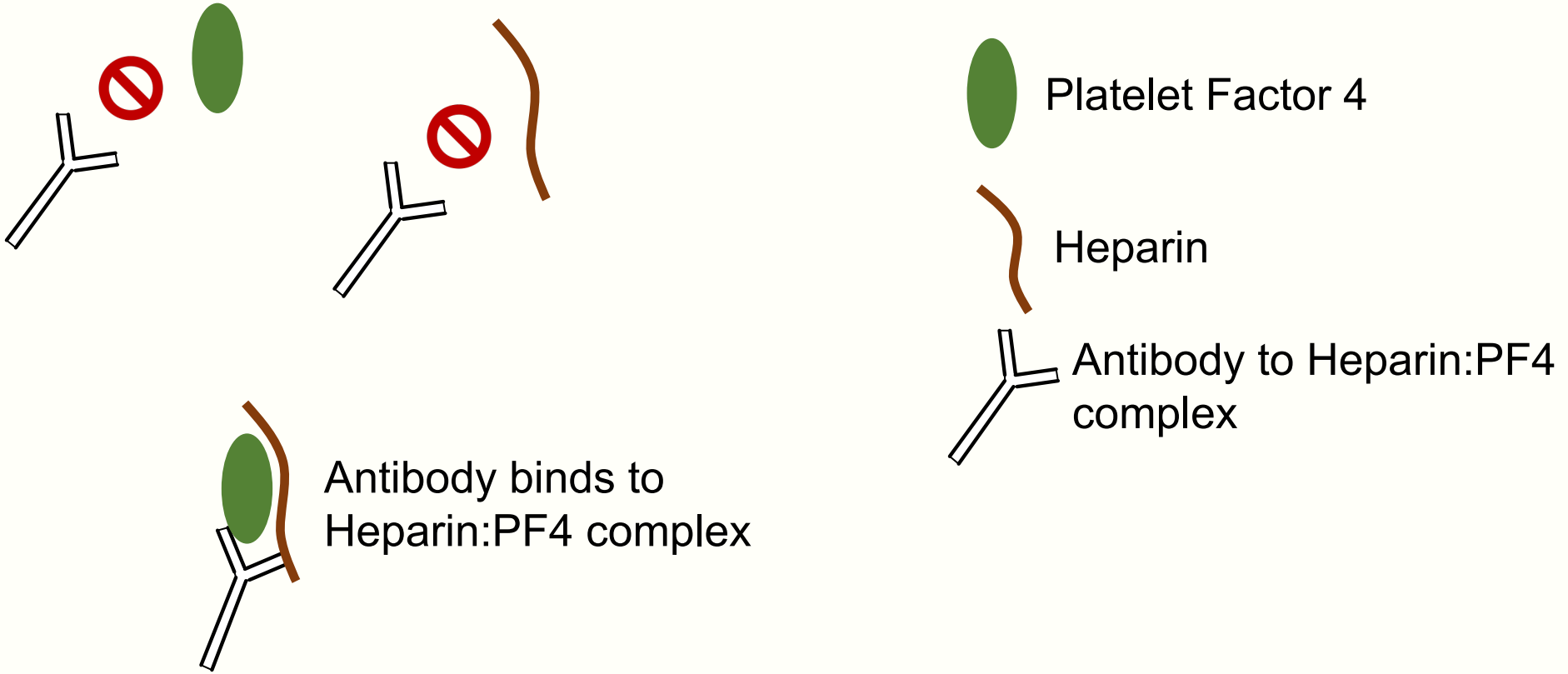


Heparin Induced Thrombocytopenia/Thrombosis (HITT)

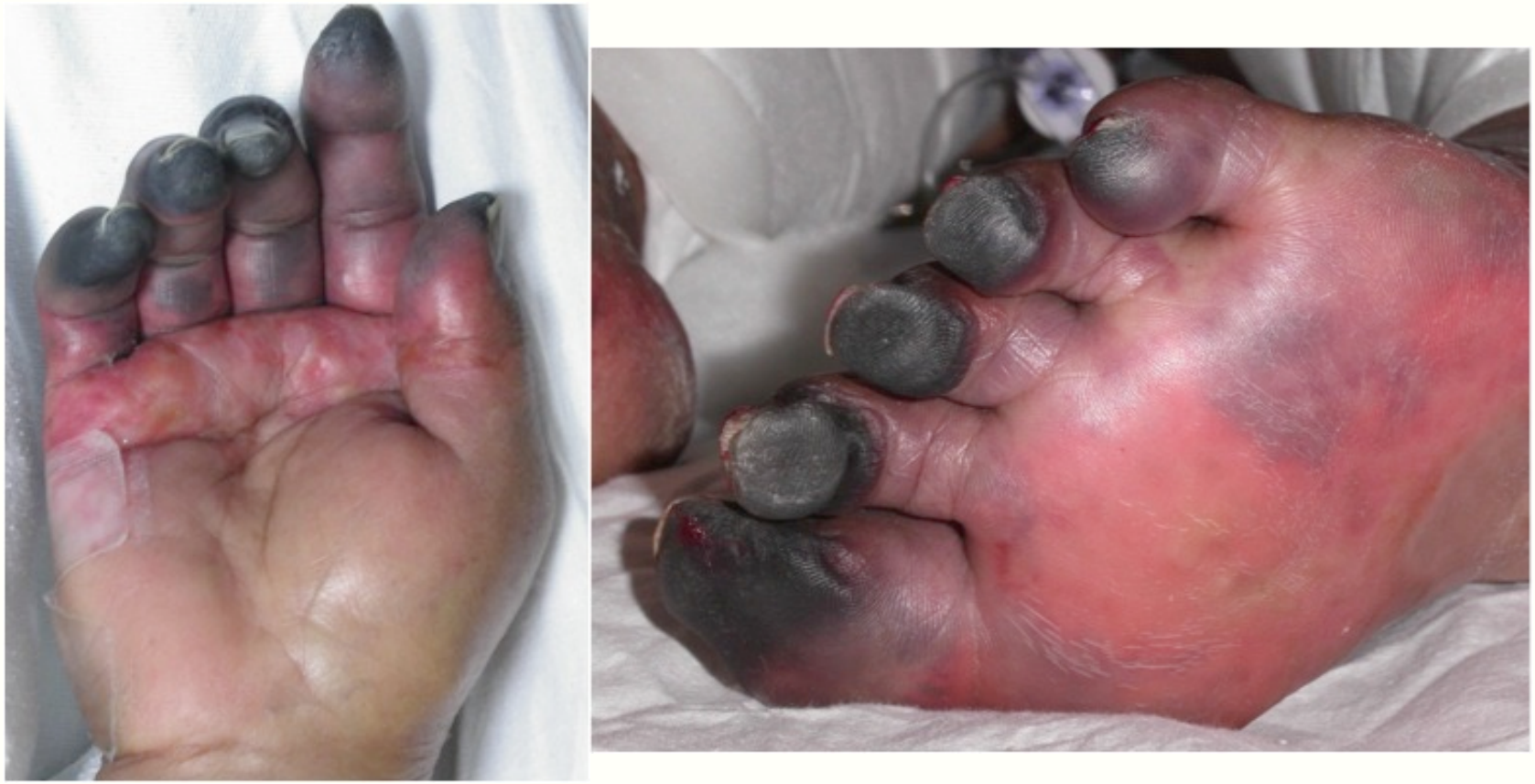
- Full lecture to follow later in the year.
- Here we will focus on testing.



Antibody:Heparin:PF4 Complex Associated With Arterial, Venous, and Microvascular Thrombosis.



AVOID THIS PLEASE!



- Cormack GM & Kaufman LJ. Journal of Medical Case Reports 2007, 1:13.

4T Scoring System for Pretest Probability

Points	2	1	0
Thrombocytopenia	>50% fall in PLT or PLT nadir of 20K-100K	30-50% fall in PLT or PLT nadir 10K-19K	<30% fall in PLT or PLT nadir of <10K
Timing	5-10 d post heparin [<1 day if previous heparin within 100 days]	Unclear or PLT fall after 10 days	PLT fall <5 days and without recent heparin
Thrombosis	New thrombosis, skin necrosis	Progressive or recurrent thrombosis, some skin lesions e.g., erythema	None
Other causes of Thrombocytopenia	None	Possible	Other causes clearly identified

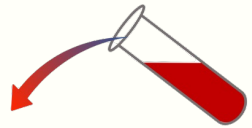
Score ≤3: < 5% chance of HIT
Score 4-5: Intermediate risk
Score ≥ 6: Very high risk of HIT

Cuker, A. et al. Blood 2012, 120(20): 4160–4167.

HIT Testing: Screening ELISA

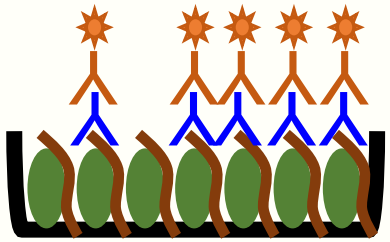
- Antibodies to heparin-PF4 complexes
  - Polyspecific Assay: Combined IgG, IgA, IgM titers
  - Monospecific Assay, IgG only titer (OD) more specific
    - McFarland et al, Am J Hematol. 2012 Aug; 87(8): 776–781.
- High Negative Predictive Value*.
  - If the result is below a pre-specified cutoff, (Typically, <0.4 OD units) can be confident that HIT is not present..
  - If result if >0.4, this does not indicate that HIT is present, but rather it still needs to be considered.

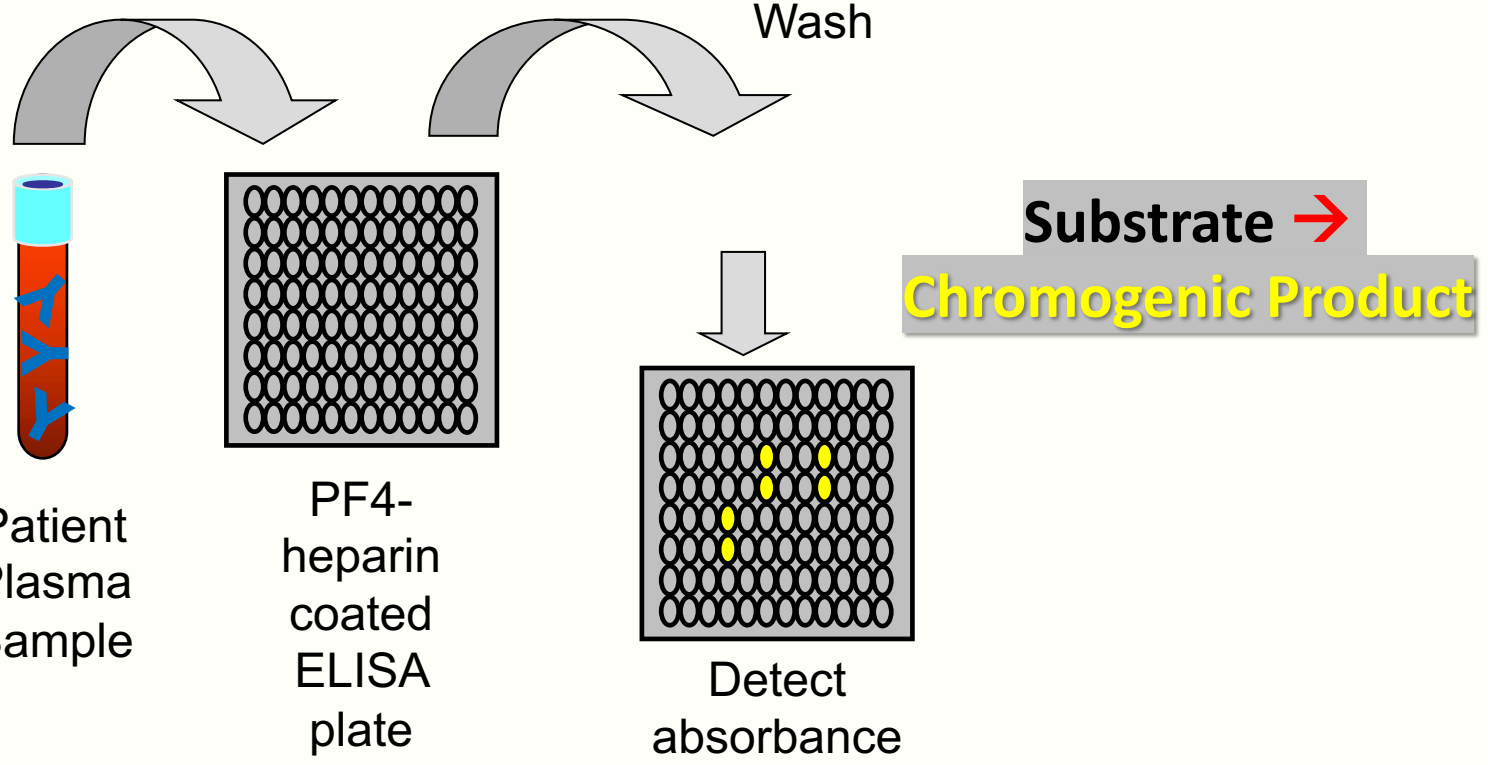
**A negative predictive value (NPV) is the probability that if the test is negative, the subject does not have the disease.*



ELISA-Based Assay

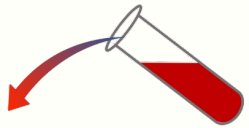
- Anti-human IgG + AP
- Patient Antibody
- Heparin:PF4 complex





HIT/T ELISA Results

- Negative ELISA screen– HIT unlikely
- Positive ELISA screen- consistent with HIT/T in the appropriate clinical setting. Does not mean that HIT/T is confirmed!
- Need confirmatory test (Serotonin Release Assay).

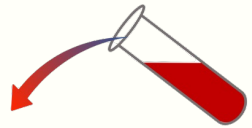


Interpretation of HIT Titers In View of Serotonin Assay Confirmatory Results

HIT Titer (OD)	Probability of Serotonin Assay POSITIVITY
< 0.4	~0 - <1%
0.4 - < 1.00	< 5%
1.00 - 1.50	~ 25%
1.50 - < 2.00	~ 50%
≥ 2.00	>90%

Low titer positive screening test results usually do not require further work-up.

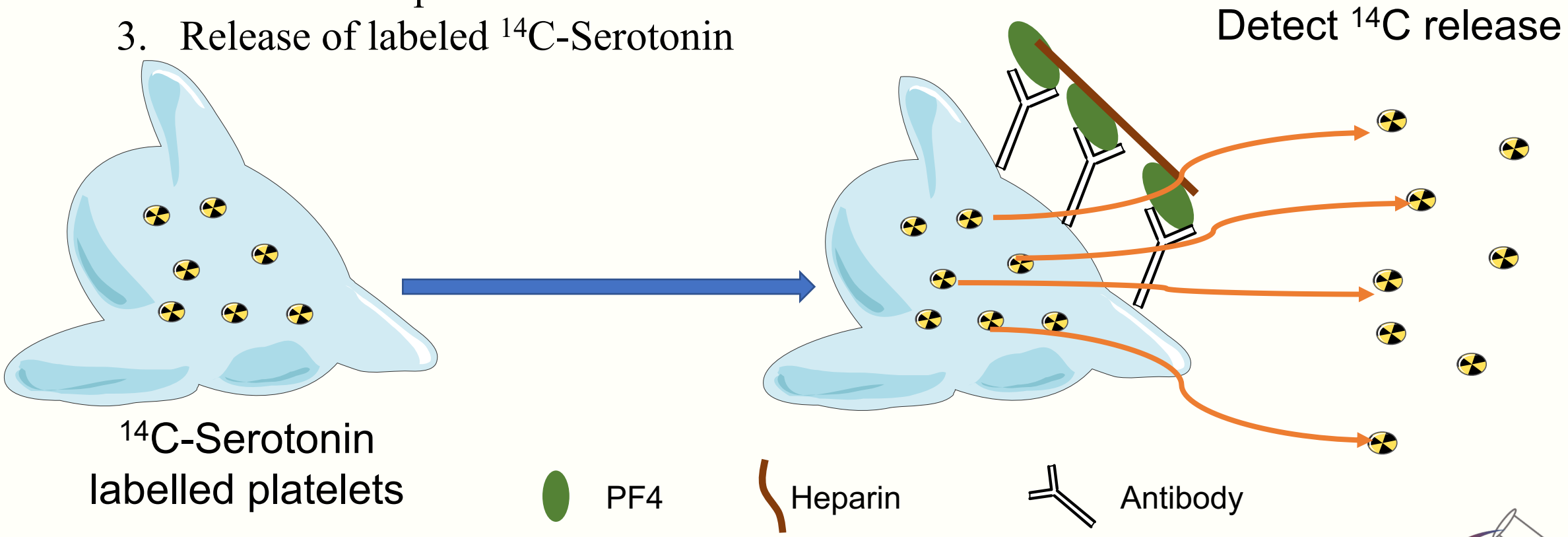
Warkentin et al. Thromb Haemost 2008;6:1304-12.



HIT/T Testing: Serotonin Release Assay

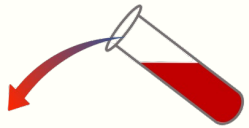
Uses fresh platelets, "loaded with ^{14}C-Serotonin" in dense granules.

1. Exposure to Antibody:Heparin:PF 4 Complex.
2. Activation of platelets
3. Release of labeled ^{14}C-Serotonin



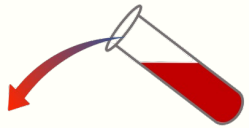
Antiphospholipid Antibody Syndrome: Testing

- Full lecture to follow later in the year.
- Here we will focus on testing.



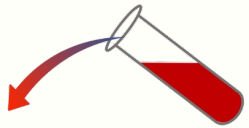
Lupus Anticoagulant

- Heterogeneous antibodies against phospholipids and phospholipid binding proteins, that "usually" prolongs the aPTT.
- Prevalence of 1-4% in the general population. (Increases with Age.)
- A key component of the Antiphospholipid Antibody Syndrome.
- Prolongs Screening aPTT
  - Clinical aPTT reagents are variably sensitive to LA
  - Normal aPTT does not rule out a LA
- Not usually associated with bleeding
- Arterial/venous/small vessel thrombosis
- Pregnancy: Recurrent fetal loss.
  - Rarely patients may also have antibodies against prothrombin
    - Check PT for prolongation



Lupus Anticoagulant Insensitive aPTT Reagents

- aPTT-FS = “Factor Sensitive”
  - May have different names, depending on manufacturer.
- Used to avoid inhibitory effect of LA on clot-based factor assays.
- Used to rule out significant coagulation factor deficiencies in setting of LA.
- Normal APTT Actin FS results rule out a significant factor deficiency.

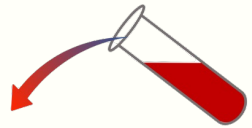


Results in Patient with Lupus Anticoagulant

aPTT: 62”
aPTT- FS: 32.1” (normal)

Mixing Studies

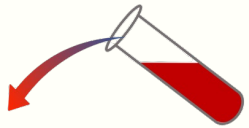
aPTT	Patient	Normal Plasma (22.5-36.5”)	50/50 mix
Immediate	62.6”	29.4”	60.7”
1 Hour Incubation @ 37°C	64.1”	29.3”	67.5”



ISTH Guidelines for Lupus Anticoagulant Testing

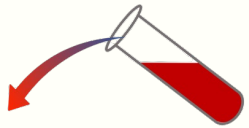
(Pengo V, et al. J Thromb Haemost 2009; 7: 1737–40)

- Specialized testing is required
- **Two tests based on *different principles***
  - dRVVT (activates common pathway)
  - *sensitive* aPTT (low phospholipid and **silica** as activator)
  - A single test will detect only 60 -80% of cases
  - Both tests used together have a 20% false negative rate for low and intermediate titer lupus anticoagulants
- ***LA considered positive if one of the two tests gives a positive result.***
- False positive rate: ~10%
  - (Dembitzer et al, Am J Clin Pathol 2010; 134:764-773)



Lupus Anticoagulant Testing: Interferences

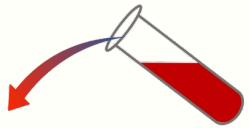
- DOACs (dabigatran, rivaroxaban, apixaban) even at trough levels produce false positive results in 20-40% of patients.
  - (Ratzinger F, et al. Thromb. & Haemost. 2016; 116:235-240)
- Warfarin may produce false positive DRVVT test results
  - Ortel T. Am J Hematol. 2012 May; 87(Suppl 1): S75–S81.
- Heparin may produce false positive aPTT based test results
  - Ortel T. Am J Hematol. 2012 May; 87(Suppl 1): S75–S81.



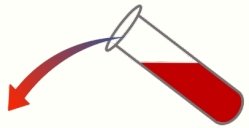
Lupus Anticoagulant Testing:

- Assays may be transiently abnormal in setting of acute thrombosis.
- For diagnosis of APS, tests need to be repeated and confirmed to be persistent after 12 weeks.

- In addition to Lupus Anticoagulant, one can test for Antiphospholipid Antibodies by ELISA for:
  - Anti cardiolipin antibodies
  - Beta 2 glycoprotein 1 antibodies
  - (To be discussed at later date, in lecture on APS.)



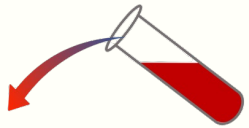
Tests for Other Thrombophilias



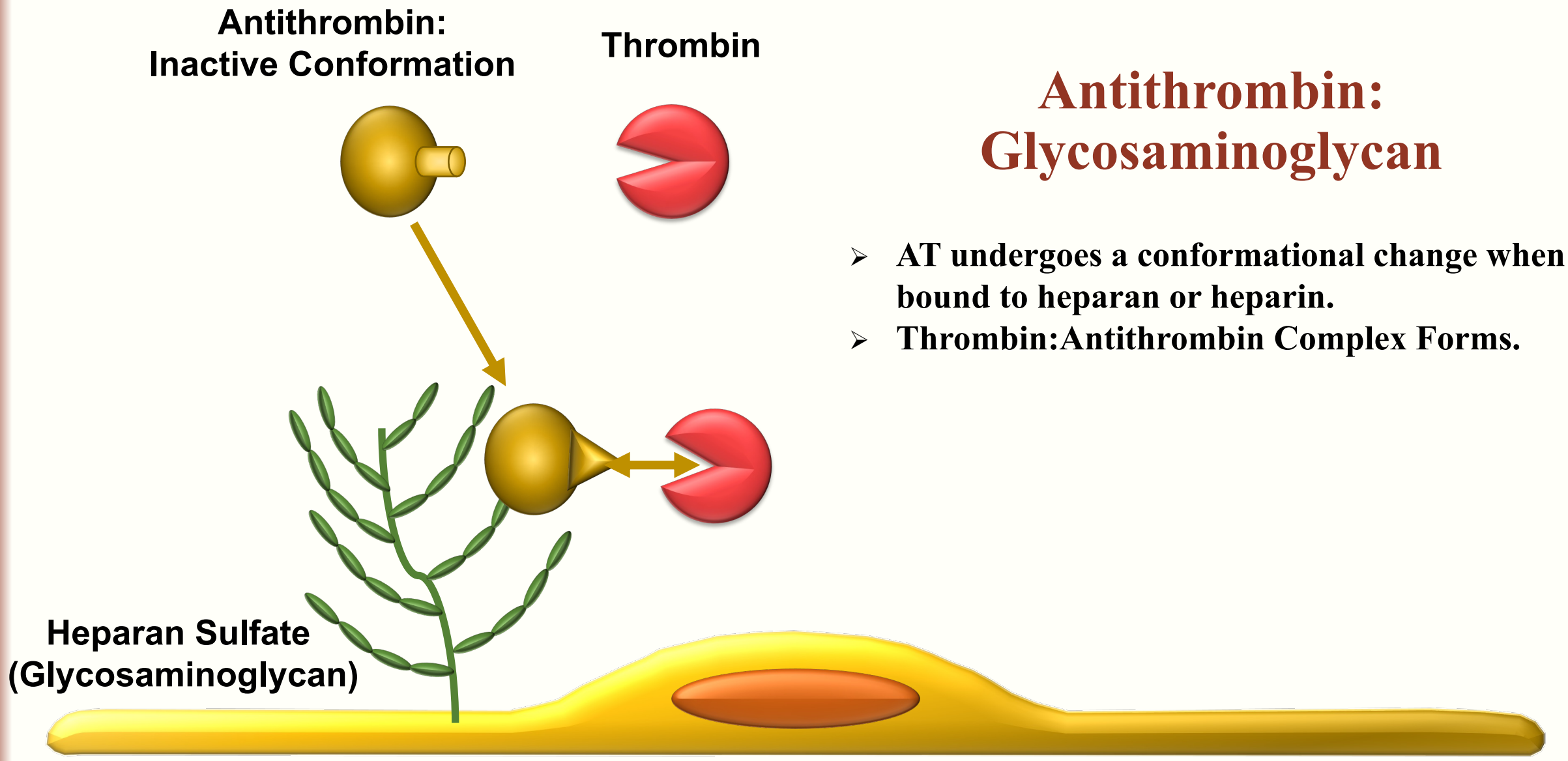
Physiologic Anticoagulants

Antithrombin (AT)*	Protein C/Protein S
➢ With heparin/heparan as a cofactor, AT inactivates the activated serine protease enzymes of the coagulation system. ➢ *Previously referred to as Antithrombin 3. But there is no antithrombin 1 or 2, so it is now referred to as Antithrombin.	➢ Inactivates the activated cofactors of the coagulation system. ➢ Factors Va, VIIIa ➢ Activate Protein C also has anti-inflammatory activity.

- **Can be measured functionally and antigenically.**
- **Deficiencies of AT, Protein C and Protein S are autosomal dominant. i.e. ~50% levels are associated with thrombotic tendency.**
- **Rare cases of homozygous Protein C deficiency: Purpura Fulminans**

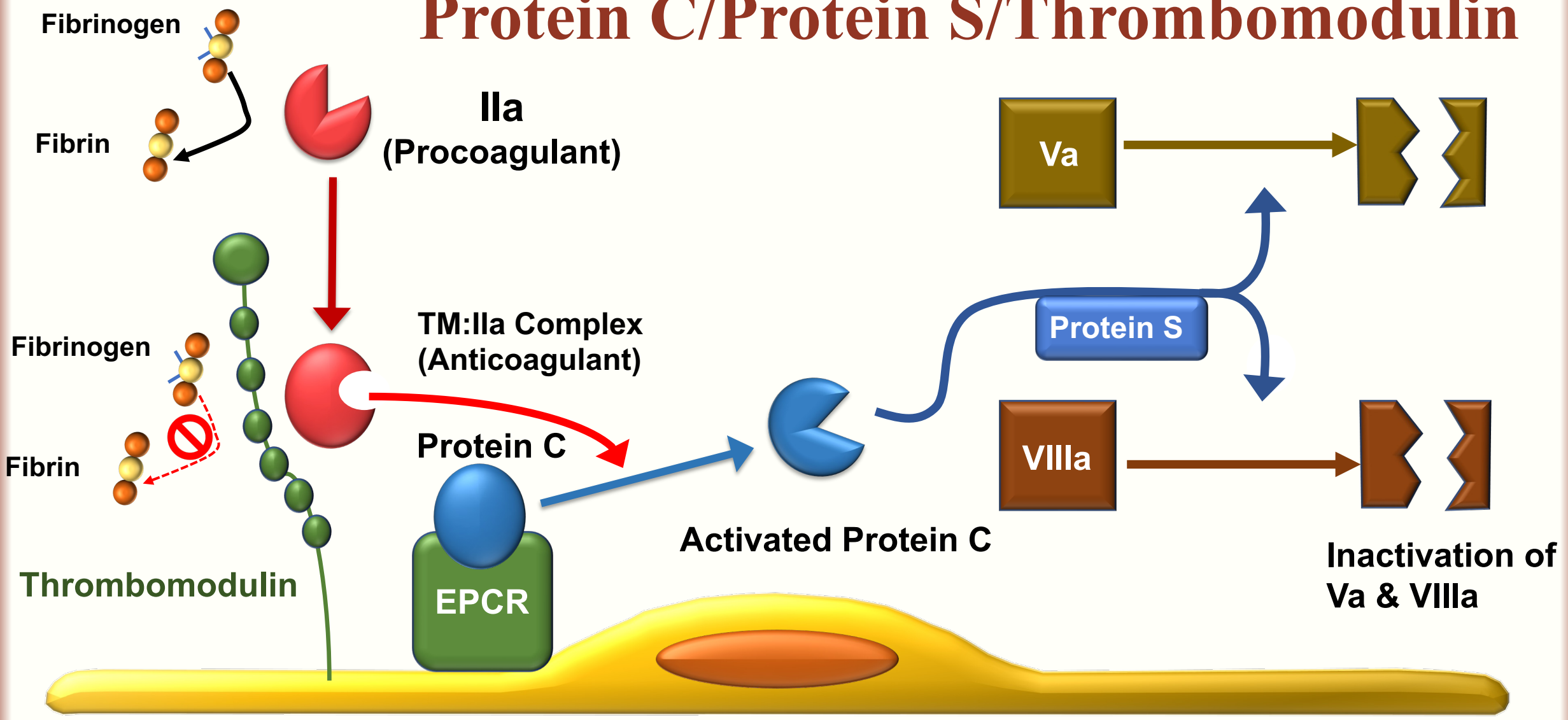


Antithrombin: Glycosaminoglycan



- AT undergoes a conformational change when bound to heparan or heparin.
- Thrombin:Antithrombin Complex Forms.

Protein C/Protein S/Thrombomodulin



EPCR: Endothelial Cell Protein C Receptor

Antithrombin Functional Assay

The assay measures functional AT levels in plasma.

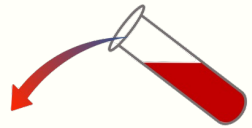
Plasma (source of AT) + Heparin ⟶ **[AT:Heparin] Complex**

[AT:Heparin] + FXa or F IIa ⟶ **[AT-FXa- Heparin] + *FXa or F IIa (residual)***

FXa/IIa (residual)

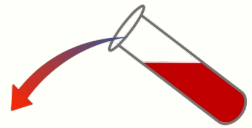
Chromogenic substrate ⟶ **Peptide + *pNA***

- Plasma (source of AT) is incubated with heparin and excess of Xa (or IIa).
- Residual Xa (or IIa) is determined by the rate of cleavage of the chromogenic substrate.
- The amount of product inversely proportional to the AT activity in the plasma sample.

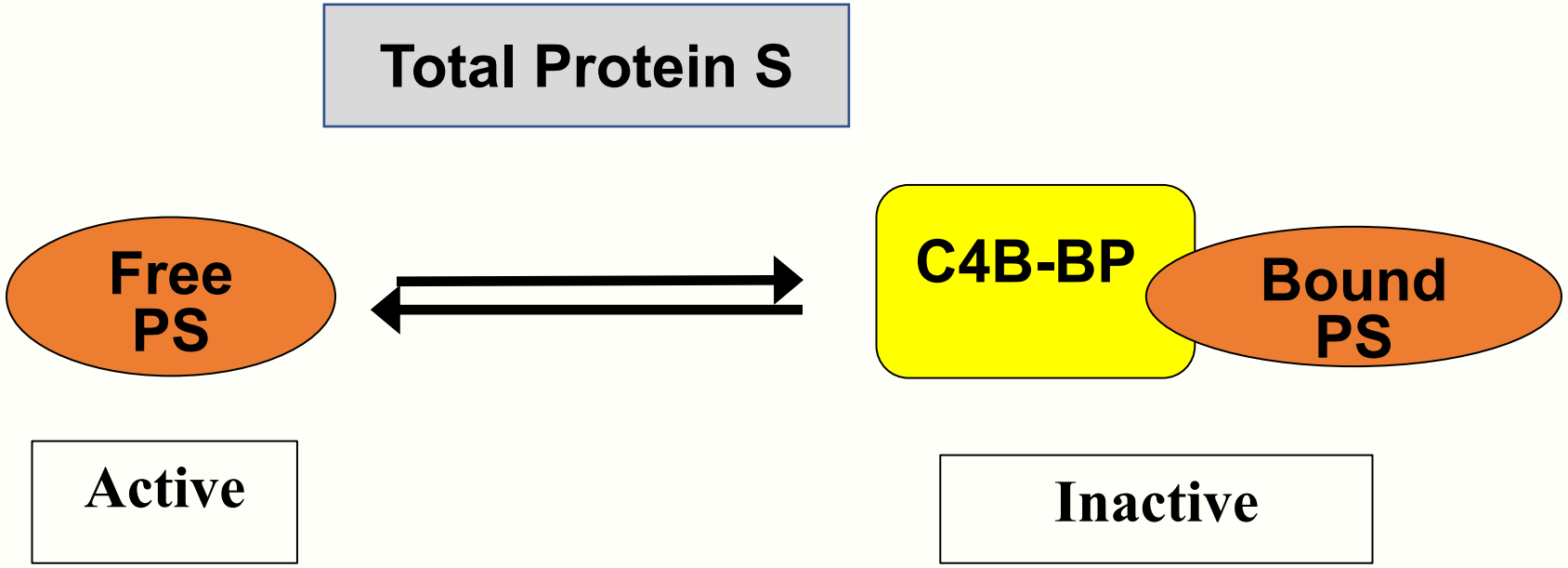


Protein C Functional Assays

Clot-Based Assay	Chromogenic Assay
➢ Preanalytical variables ➢ False low levels ➢ FVIII ➢ FVL ➢ Hyperlipidemia ➢ False normal or high results ➢ DOAC ➢ Heparin ➢ Lupus Anticoagulant	➢ Subject to **fewer** preanalytical variables ➢ Detects **most** functional defects but not all



Protein S Circulates in Two Forms



- Equilibrium between bound and free Protein S.
- Normally, ~60% of total Protein S is bound to C4b Binding Protein.
- Increase in C4B-BP reduces levels of free Protein S.

Three Types Protein S Assays

1. **Clot-based functional PS assay—"activity" assay**

   Based on APC inactivation of FVa and FVIIIa

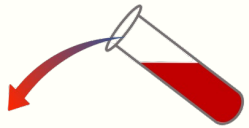
2. **Antigenic - Free PS assay (represents functional PS)**

   Free PS is adsorbed on the C4BP latex particle → triggers an agglutination reaction with the second latex reagent which is sensitized with a monoclonal antibody directed against human Protein S

   The degree of agglutination is directly proportional to the free PS concentration

3. **Antigenic - Total PS assay**

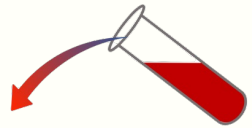
   Immunologic assay that measures PS bound to C4BBP + free PS



Three Types of Protein S Deficiencies

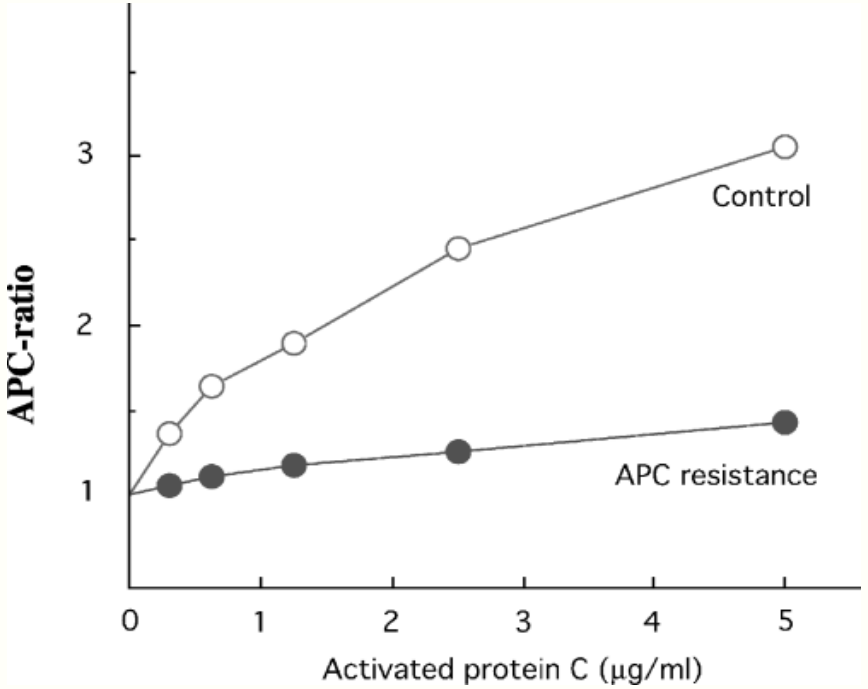
Type	PS (Activity)	PS (Free)	PS Total	C4B-BP
I	Decreased	Decreased	Decreased	Normal
II	Decreased	Normal	Normal	Normal
III	Decreased	Decreased	Normal	Elevated

- Type I protein S deficiency is a reduction in the level of free and total protein S.
- Type II deficiency is a reduction in the cofactor activity of protein S, with normal antigenic levels.
- Type III deficiency is a reduction in the level of free protein S only, due to increase in C4B-BP.
  - (Acquired due to pregnancy, oral contraceptives).



Factor V:Leiden: Activated Protein C Resistance

- Reduced anticoagulation response to added aPC when added to plasma in aPTT assay.
- Originally hypothesized a novel cofactor to aPC.
- Now understood as substrate (Factor Va) resistance to aPC.
- First described in 1993: Dahlbäck B, et al. *Proc Natl Acad Sci USA* 1993; **90**: 1004 - 8.



DahlbäckB. J of Thrombosis Haemost, Volume: 1, Issue: 1, Pages: 3-9, First published: 03 January 2003, DOI: (10.1046/j.1538-7836.2003.00016.x)

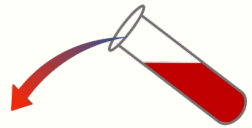
APC-Resistance—Screening Assay For Factor V Leiden

- Ratio of aPTTs (+/- APC)

$$\frac{\textbf{(aPTT with APC)}}{\textbf{(aPTT without APC)}}$$

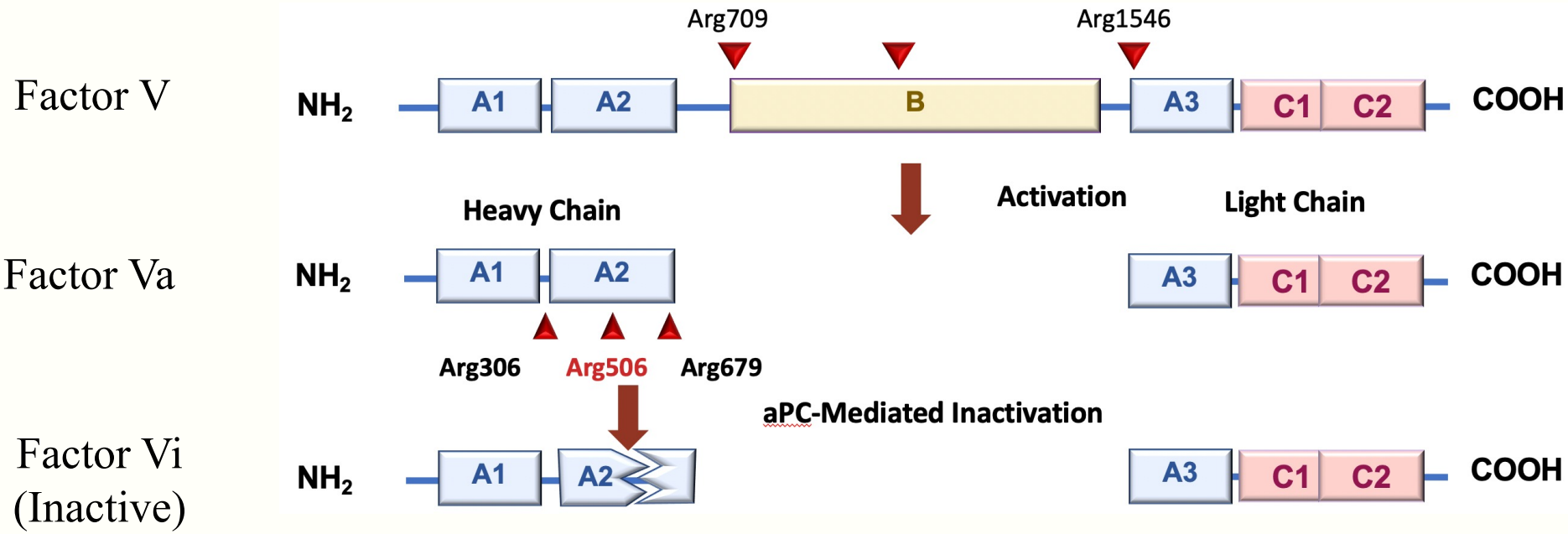
- Normal Ratio >2.0

- Assay is affected by
  - Lupus anticoagulant
  - DOAC
  - Cancer, pregnancy, inflammatory states
  - Elevated Factor VIII levels.
  - Other Factor deficiencies



Factor V:Leiden: Activated Protein C Resistance

- Inactivation of Factor Va involves four sequential cleavages of the membrane-bound procofactor at Arg306, Arg506, Arg679, and Lys994.
- Factor V:Leiden polymorphism (G1691A (R506Q) is associated with activated protein C resistance, prolonged/enhanced activity of Factor Va, and thrombotic tendency.
- Now routinely tested by PCR/molecular tools.



Prothrombin Gene Mutation: (Prothrombin G20210A)

- G-to-A point "polymorphism" at position 20210 in the 3' untranslated region of the prothrombin (factor II) gene.
- The polymorphism does not affect the protein-coding region (exons) of the gene.
- Increases mRNA half-life and Prothrombin levels.
  - Poort et al Blood. 1996;88(10):3698.

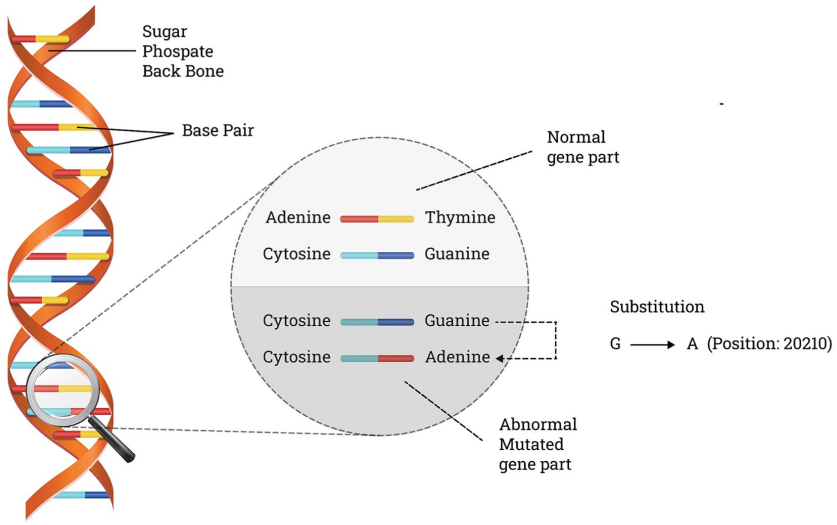


FIGURE 2: Prothrombin G20210A Mutation

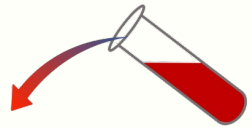
G- Guanine & A- Adenine

Adapted from: www.coagulationconversation.com/medical/risk-factors-thrombophilia-prothrombin-20210-mutation

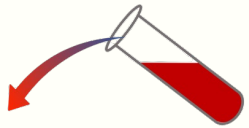
Poudel S, Zeb M, Kondapaneni V, et al. (December 08, 2020) Association of G20210A Prothrombin Gene Mutation and Cerebral Ischemic Stroke in Young Patients. Cureus 12(12): e11984. DOI 10.7759/cureus.11984

Molecular Assays: PCR Assays

Factor V Leiden	F II Polymorphism
➢ Caused by single point mutation in the FV gene ➢ Substitution of adenine for guanine at 1691 – G1691A ➢ Changes arginine to glutamine at 506 – R506Q ➢ Molecular mechanism of most cases of APC Resistance	➢ Single nucleotide substitution G20210A in the 3' UT regions of the prothrombin gene ➢ G → A substitution at nucleotide 20210 in prothrombin gene ➢ Results in elevated levels of prothrombin (~30% increase) ➢ No screening test available



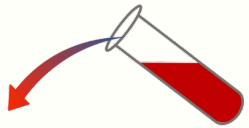
Laboratory Testing for Thrombophilia (Hypercoagulable State)



Laboratory Testing for Thrombophilia (Hypercoagulable State)

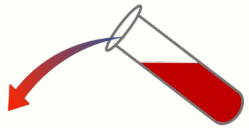
- No Screening test exists
- Requires a panel of tests
- Diagnosis of an abnormality can be made in ~50% of patients, depending on family history and presence/absence of provoking factors for the index venous thromboembolism.

1. Antiphospholipid Antibody Syndrome.
   A. Lupus anticoagulant
   B. Anti cardiolipin antibodies
   C. Beta 2 glycoprotein I antibodies
2. Antithrombin (AT)
3. Protein C
4. Protein S
5. F V Leiden
6. Prothrombin G20210A
7. Homocysteine
   (Controversial if should be tested)



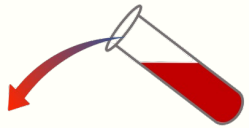
When to Evaluate for Thrombophilia?

- Assays may be impacted due to anticoagulants, acute venous thromboembolism, inflammation, acute illnesses.
- THEREFORE, testing in setting of an acute thrombosis is not typically indicated.
- No consensus on if thrombophilia testing is indicated in most situations.



Hypercoagulable Work-up

- Recurrent VTE rates weakly associated with thrombophilia. (Especially if provoked VTE).
- Why work-up?
  - Avoidance of oral contraceptives
  - Family knowledge
- Growing Consensus in Hematologic Community is to not routinely do hypercoagulable workup during acute episode of thrombosis.
- If one is going to do testing, wait until the thrombus has been treated.



Routine Testing for Hereditary Thrombophilias in Patients With a First VTE ?

- "Routine testing for hereditary thrombophilias in patients with a first VTE is not helpful in predicting risk of recurrence or altering initial therapy."

- Galioto et al, Am Fam Physician. 2011 Feb 1;83(3):293-300
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