HEMOSTATIC ABNORMALITIES

Introduction

The fluidity of blood and the prevention of bleeding at sites of blood vessel damage is based on the balance of procoagulant and anticoagulant systems. Table 1. presents some selected examples of mechanisms the balance of which maintains the normal hemostasis.

Procoagulant		Anticoagulant
Proteases: thrombin, FVIIa♥	↔	Antithrombin
Co-factors: FVa, FVIIIa	↔	Protein C and S
Tissue factor (TF)	↔	TF pathway inhibitor
Fibrin	↔	Fibrinolytic system
Platelets	↔	Endothelial cells (e.g. PGI_2)

Table 1. Balanced hemostasis (selected factors).

The familial or acquired disorders of the hemostatic balance that result in bleeding tendency are called hemophilias, whereas those that predispose to thrombosis (pathological clot formation within the vascular bed) are named thrombophilias. The incidence of thrombophilias is an order of magnitude higher than the incidence of hemophilias. The most frequent inherited hemophilia (type A; deficiency of factor VIII) occurs in 1 out of 10000 newborn boys, whereas the most frequent congenital thrombophilia (activated protein C resistance, **APC-resistance**) is detected in 5 % of the general population and similar incidence is reported for the most frequent acquired thrombophilia (antiphospholipid syndrome, **APS**).

Objectives

At the end of the consultation the participant will be able to:

1) Define the term thrombophilia

2) Outline the protein C system

3) Discuss the APC-resistance

4) List the phospholipid dependent procoagulant and anticoagulant reactions

5) Discuss the potential molecular mechanisms explaining the thrombotic events associated with APS

6) Formulate a comprehensive approach to the laboratory diagnosis of APC-resistance and APS-syndrome

7) Explain the molecular background of the anticoagulant therapy and its monitoring

♥ **Abbreviations** in the text: **APC**, activated protein C; **APS**, antiphospholipid syndrome; **APTT**, activated partial thromboplastin time; **F**, factor; **Gla**, γ-carboxyglutamic acid; **PT**, prothrombin time; **TF**, tissue factor;

APC-resistance (Factor V Leiden)

Case history

A 42-year-old male internal medicine resident presents with recurrent non-healing left ankle ulcer (post-phlebitis syndrome).

At the age of 27 idiopathic left leg deep-venous thrombosis developed which was treated with heparin for 2 weeks followed by warfarin for 6 months (6 days overlapping of the heparin and the warfarin therapy).

Question: Why should the heparin therapy precede the warfarin? What is the objective of the 6 day overlapping in the two treatment regimens? (Background information: lecture notes, Biochemistry practical manual, Figs. 1. and 2., data on the half-clearance time: for protein C, protein S and factor VII appr. 24 h, for factor IX and X - 40 h, for prothrombin - 60 h)

Two months after stopping warfarin idiopathic right leg deep-venous thrombosis developed and it was treated in the same way for 6 months.

Over the following 8 years 7 additional episodes of deep or superficial venous thrombosis occurred.

Family history: deep-venous thrombosis in the father, paternal uncle and grandfather; no thrombotic history in the sister, mother and two aunts.

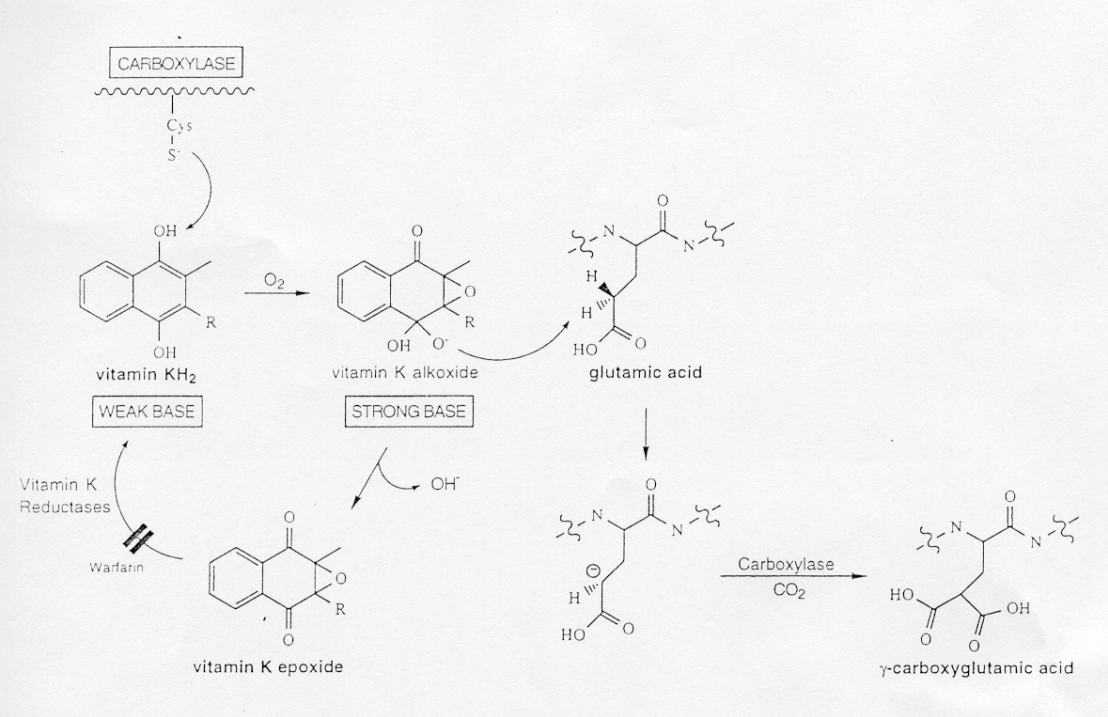

Figure 1. Synthesis of γ-carboxyglutamic acid (Gla).

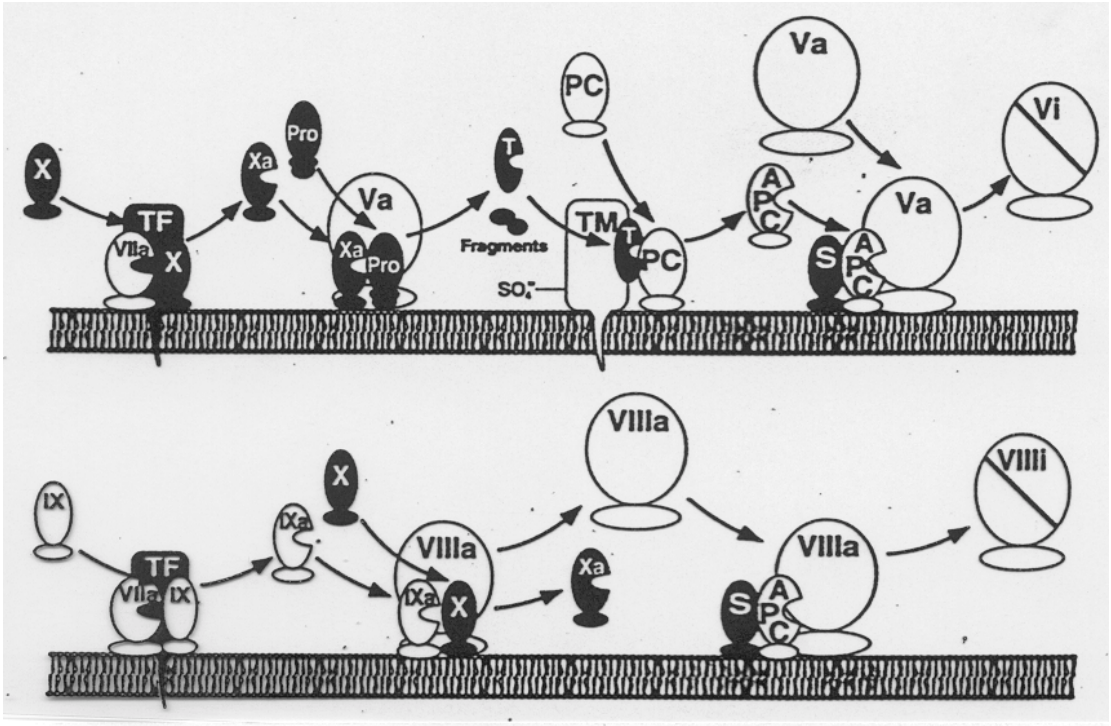

Figure 2. Phospholipid dependent procoagulant and anticoagulant reactions. Abbreviations: Pro, prothrombin; T, thrombin; TM, thrombomodulin; S, protein S; PC, protein C; the numbers follow the conventinal coagulation factor numbering; the 'a' indicates the active form of the factors; the 'i' indicates the inactivated form of the factors.

Laboratory findings

Platelets

- normal number
- no spontaneaous aggregation

Coagulation testing

- prothrombin time: normal
- activated partial thromboplastin time (APTT): normal
- thrombin time (the time required for standard thrombin solution to clot citrated plasma): normal

Fibrinolysis/thrombosis testing

- D-dimer (normal)
- soluble fibrin monomer complex (negative)
- plasminogen (streptokinase activation assay: normal)
- antithrombin antigen (normal)
- protein C antigen (normal)
- protein S antigen (normal)

Question: What is the information content of the above listed tests and what is the rationale for performing all of them?

Diagnosis: APC-resistance (homozygous Factor V^{R506Q}) proved by

- functional assay (Fig.3.)
- PCR assay (Fig. 4.)

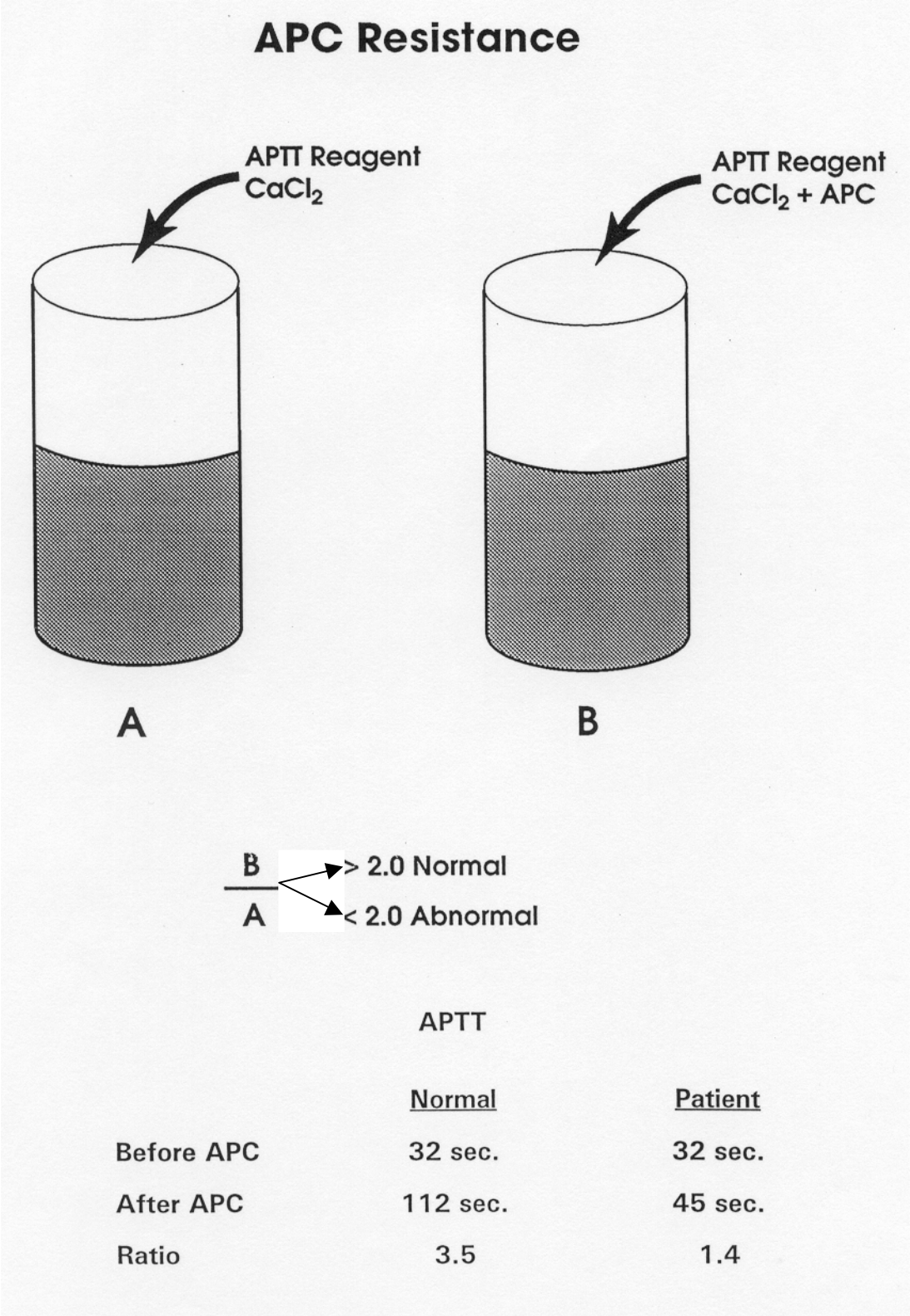

Figure 3. Functional assay of APC-resistance. The APTT is determined twice: A) native citrated plasma; B) citrated plasma treated with APC. The ratio of the two APTT values determines the diagnosis: APC-resistance is diagnosed if the ratio is less than 2.0. (Use your biochemistry practical manual for description of the APTT assay).

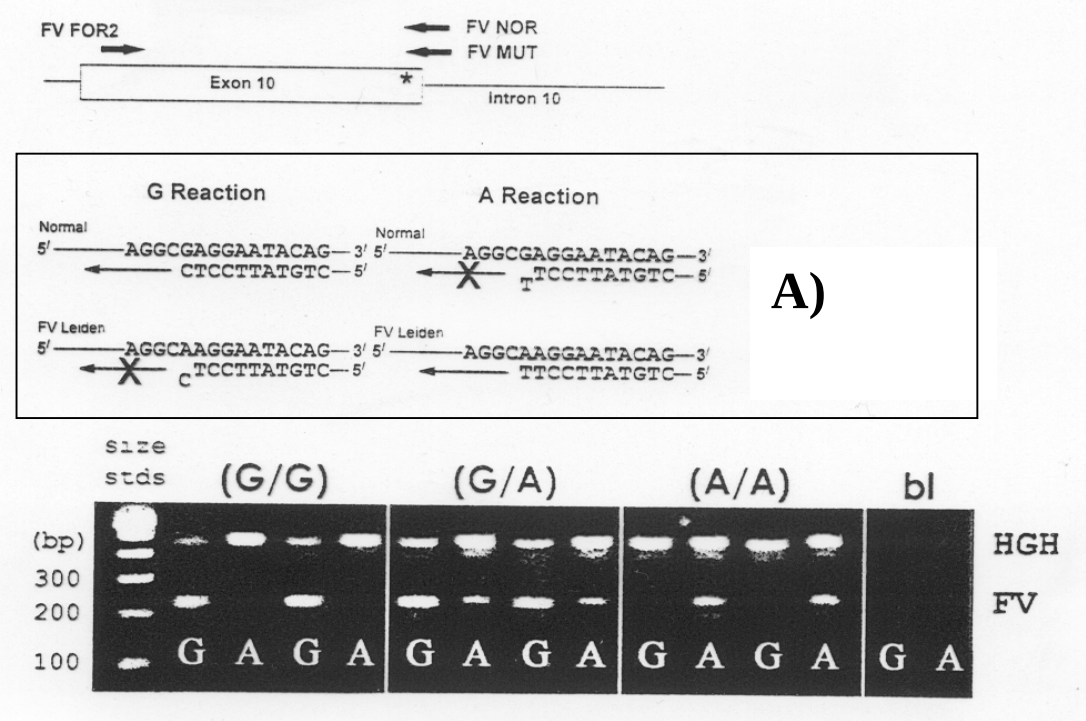

Figure 4. Detection of Factor V^{R506Q} with PCR. DNA samples are isolated from periferal leukocytes and PCR is performed using the primers indicated in panel **A)**, after which the DNA fragments are separated with electrophoresis and visualized. Positive G reaction indicates normal Factor V gene, positive A reaction indicates Factor V^{R506Q} gene. Abbreviations: *, site of the mutation in exon 10 of the Factor V gene; HGH, a DNA fragment gained from a primer complementary to a sequence from the gene of the human growth hormone (control of the PCR performance); FV, Factor V DNA fragment gained from the primers indicated at the bottom of the gel lanes (**G**, primer to the normal sequence; **A**, primer to the mutation).

Comprehension

Fig. 2. summarizes the protein C system (Reference: lectures of professor Raymund Machovich, Semmelweis University, May 2001).

The APC is a serine protease that cleaves three peptide bonds next to arginine residues (at R506, R306 and R679) in FVa with consequent inactivation of this co-factor of blood coagulation. The cleavage of the peptide bond at R506 is a prerequisite for the efficient cleavage of the other two bonds; if the R506 is exchanged for a different aminoacid, the rate of FVa inactivation is decreased by two orders of magnitude. In Factor V Leiden this arginine is replaced by a glutamine, thus following normal activation by thrombin FVa Leiden has a longer lifespan supporting the procoagulant branch shown on Fig. 2.

Usually additional factors are needed to provoke a thrombotic episode in patients with FV^{R506Q}. Table 2. illustrates one such interaction (with oral contraceptives).

	normal Factor V		Factor V^{R506Q}	
	no OC	OC used	no OC	OC used
thrombotic patients	36	84	10	25
subjects examined	437 870	275 585	17 515	8757
thrombosis incidence /10000 persons/year	0.8	3.0	5.7	28.5

Table 2. Clinical manifestation of the APC-resistance: interaction with oral contraceptive (OC) use.

Therapy

Chronic oral anticoagulation with coumarin-derivatives (INR=2.5-3.5, for explanation of INR see below)

- for 6 months: Factor V^{R506Q} heterozygotes after a thrombotic episode
- life-long: Factor V^{R506Q} homozygotes or heterozygotes with additional risk factors

Reporting the results of prothrombin time (PT) assay in INR units

PT test was introduced by Quick in 1935 as an assay for prothrombin in plasma samples.

PT test measures

- Factors VII, X, V, II and fibrinogen, and inhibitor of the enzymes

PT is prolonged and PT test is commonly used

- as screening test for bleeding tendencies due to deficiencies in factors VII, X, V, II and fibrinogen
- as screening test for bleeding tendencies due to inhibitors for the above factors
- to monitor the oral anticoagulant (i.e. warfarin) therapy

PT test is initiated by recalcifying the patient's plasma in the presence of tissue thromboplastin[1]

1. Factor X is activated by factor $VII_{(a)}$ and tissue tissue factor in the presence of phospholipids and Ca^{2+} ions.
2. Factor X_a activates prothrombin in the presence of Factor V_a, phospholipids and Ca^{2+} ions.
3. Thrombin converts fibrinogen to fibrin.
4. Fibrin monomers form fibrin polymers of detectable size.

The most thromboplastins used in laboratories are extracts of animal tissues (rabbit brain, lung) but now human recombinant thromboplastins in standardised phospholipid mixture are also available.

Manual techniques (tilting or hooking methods) are supplanted by coagulometers, detecting clot formation as mechanical or optical change.

[1] Thromboplastin: prothrombin time test reagent containing tissue factor and phospholipids

PT value depends on both the type of thromboplastins and on the principle of clot detection. The PT test should be ideally determined by the same method and with the same reagent, but this is not possible. Interlabor variability is increased by the variety of reagents and methods used to detect clot formation of patient's plasma. For most reagents/methods the reference interval is a few seconds around a mean PT that is between 11 and 15 sec.

Interpretation of PT results

PT - for diagnostic screening test:

Patient's plasmas of outside of reference range are investigated with further specific tests (beyond the scope of the present tutorial)

PT - for therapeutic monitoring of oral anticoagulation drugs:

The international normalized ratio (INR) was devised to standardize prothrombin time (PT) reporting on oral anticoagulant users. The system takes into account the varying degree of sensitivity of commercial thromboplastins to warfarin-induced clotting factor deficiency by assigning each an international sensitivity index (ISI).

$$INR = PTR^{ISI}$$

$$PTR = \frac{PT_{patient's\ plasma}}{PT_{normal\ pool\ plasma}}$$

INR: international normalized ratio

PTR: prothrombin time ratio

ISI: international sensitivity index

The ISI of a given thromboplastin reagent is obtained by comparing the sensitivity of the test thromboplastin with that of a reference thromboplastin[2] through a calibration system

PT: prothrombin time

Normal pool plasma: mixture of plasmas of 40 healthy patients stored at -80 ^{o}C.

Current recommendations for anticoagulant prophylaxis:

	INR
Preoperative prophylaxis	1,3-1,5
For the most anticoagulant therapies	2,5-3,5
In patients with mechanical prosthetic heart valves*	3,5-4,5

*Some authors disagree with these recommendations, insisting that lower INR are more appropriate.

[2] The first international reference thromboplastin is the Manchester Comparative Thromboplastin of rabbit brain.

Antiphospholipid syndrome (APS), lupus anticoagulant coagulopathy (LAC)

Clinical case

A 30-year-old woman presents in the early second trimester of her fourth pregnancy with complains of intermittent aching of the knees and swelling of the left leg. The only positive physical findings were a uterus compatible with a five-month gestation and mild edema of the left leg.

History:

- at age of 21 deep-venous thrombosis in the left iliofemoral area while taking oral contraceptives
- a year later superficial thrombophlebitis in the right leg which was treated with heat
- over the next 2 years two pregnancies and each time fetal death occurred during the 25^{th} to the 29^{th} week of gestation
- at age of 28 she carried a fetus to term and delivery was uncomplicated; during this pregnancy she was treated with heparin and aspirin
- no history of bleeding diathesis (normal menses, uneventful extraction of all four wisdom teeth).

Laboratory findings

Test	Patient	Normal range
Blood platelets (count/μl)	135 000	130 000-370 000
Bleeding time (min)	2	1-6
Prothrombin time (s)	19	17-19
Thrombin time (s)	22	20-22
APTT (s)	47	25-40
+ equal vol. normal plasma	43	
+ 1/10 vol. normal plasma	45	

Questions to the case

What is the likely diagnosis?

Why is the APTT prolonged and what is the interpretation of the mixing studies?

Would the patient be likely to experience abnormal bleeding if surgery were required (e.g. Caeserian section)?

Should the patient be treated with an anticoagulant or other relevant medication during the remainder of pregnancy?

Before you answer the questions recall the lectures of professor Raymund Machovich (Semmelweis University, May 2001) and read the supplementary background information below.

Dynamics of the membrane phospholipid bilayers in blood coagulation

Classical representations of phospholipid organization in membranes (Figure 5) focus on lamellar or bilayer structures such as the ordered gel state (Lβ) or fluid liquid crystalline state (Lα). However, some natural phospholipids can assume nonbilayer structures such as the reversed hexagonal (HII) phase, as has been documented with X-ray crystallography and nuclear magnetic resonance (NMR) in liposomes and with HII specific antibodies in cultured human cells. Although an extensive treatment of the physical chemistry of lipid polymorphism is beyond the scope of this tutorial, the dominant structural feature allowing nonbilayer structures for lipids is a small polar head group associated with a large hydrophobic domain, i.e. a cone shape for the lipid. Extensive and permanent amounts of such nonbilayer structures would compromise the barrier function of membranes. However, bilayer lipids have a different intrinsic radius of curvature from nonbilayer lipids, so changes in the proportion of these two types of lipids will change the collective physical properties of the membrane. Every biological membrane system has at least one nonbilayer- forming lipid component (phosphatidyl-ethanolamine, phosphatidyl-serine), so lipid polymorphism is the rule, not the exception, in biological membranes. Phosphatidyl-choline is generally considered a bilayer-forming lipid that only forms nonbilayer structures under extreme conditions or in the presence of nonbilayer-forming lipids.

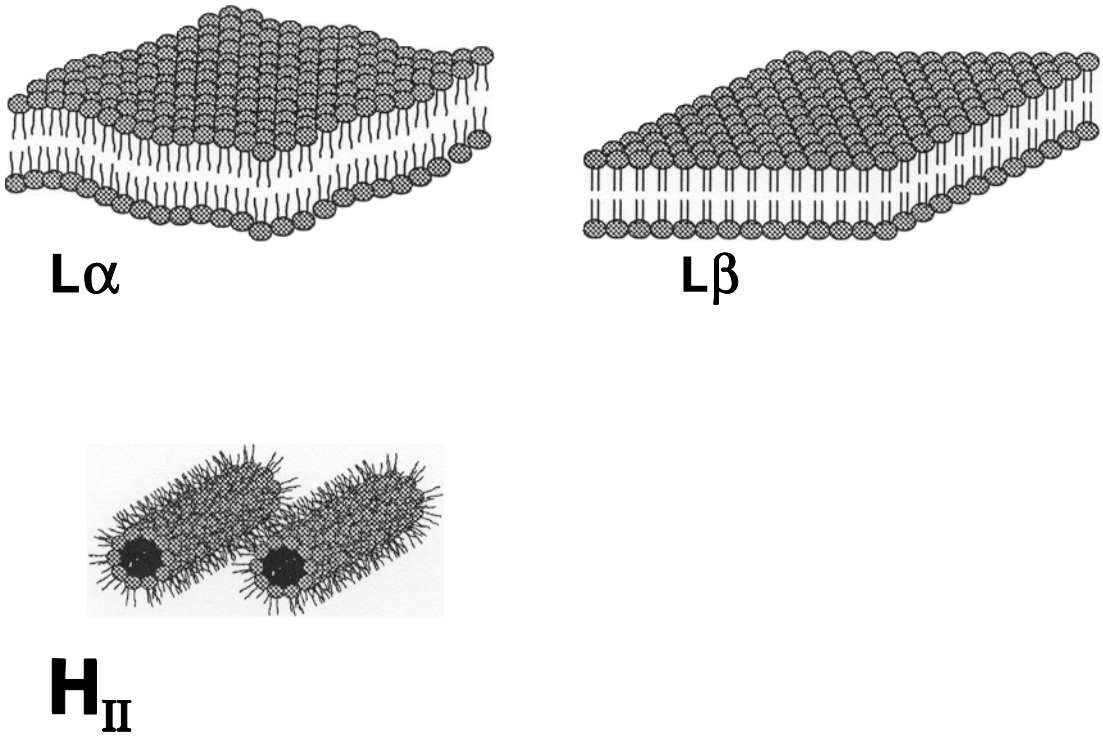

Figure 5. Structural representation of the arrangement of phospholipids. Arrangement of the head-group and fatty-acid domains of phospholipids in the fluid liquid crystalline state (Lα), the ordered gel state (Lβ), and the reversed hexagonal state (HII).

The nonbilayer-forming lipids are usually present in the inner layer of the cellular plasma membranes. At this site the potential to form nonbilayer structures may provide nonleaky discontinuity in the bilayer structure of membranes for the following important biological functions: membrane fusion and membrane vesicle formation during cell division and vesicle-mediated protein trafficking; integration of nonlipid components into the membrane bilayer; movement of macromolecules through the membrane; lateral movement of macromolecules within the bilayer; stabilization of specific membrane protein complexes; and conformational interconversions necessary to protein function. The regulated transfer of anionic phospholipids to the outer layer of plasma membranes is part of the physiological function of certain cells (among these are the cellular elements of the hemostatic system; platelets and endothelial cells) and thus hexagonal areas can be formed on the extracellular surface of the membranes.

Whereas the tissue factor complex can provide the necessary phospholipid surface for triggering the coagulation process as depicted in the lectures of professor Machovich, the surface of the activated platelets in the platelet plug is considered to be of major importance for coagulation in hemostasis. This directs the fibrin formation to the site of damage and enforces the sealing effect of the platelet plug. Factor V is derived from either plasma or platelets. Platelet factor V is found in the α-granules and is released or translocated to the membrane during secretion as a partly proteolyzed active molecule. It has been calculated that the platelets contribute approximately one fifth of the total factor V of whole blood. Except for tiny amounts of factor Xa from intracellular platelet stores, factor X and factors VIII and IX are exclusively found in plasma. The catalytic subunit of factor XIII, the α chain, is present in significant amounts in platelets, surprisingly enough not in the α-granules, but in the cytosol. Factors IXa and Xa, which bind calcium ions with high affinity because of their content of γ-carboxyglutamic acid, demonstrate a strongly enhanced binding on platelet activation. This is directly related to the breakdown of the phospholipid asymmetry of the platelet surface membrane. The inner leaflet of the nonactivated platelet membrane contains most of the aminophospholipids and almost all of the phosphatidylserine, but a considerable amount of this is found in the outer monolayer after platelet activation. The negative charge of the phosphatidylserine, hydrophobic interactions, and the calcium-binding properties of the vitamin K-dependent coagulation factors are essential for the catalytic activity at the activated platelet surface.

Two phospholipid transport-mediating proteins, or systems, are postulated to maintain the steady-state phospholipid asymmetry of the cell membrane, whereas a third one has been implicated in the fast breakdown of the phospholipid asymmetry (scrambling) observed on platelet activation (Fig. 6.). The fIrst one, usually called the aminophospholipid translocase is specific for aminophospholipids (phosphatidylserine and phosphatidylethanolamine). This protein is considered responsible for the transport of aminophospholipids against a gradient from the outer to the inner membrane leaflet in a process that is dependent on ATP and is inhibited by calcium ions. A slow, outwardly acting phospholipid- nonspecific "floppase," supposed to counterbalance the aminophospholipid translocase, has also been postulated. A bidirectional phospholipid-nonspecific "scramblase" has been isolated from erythrocytes, cloned, and sequenced. This protein is believed to be identical or similar to the one responsible for the fast breakdown of the phospholipid asymmetry during platelet activation. Its biological activator is believed to be calcium ions, the cytosolic level of which is significantly increased on platelet activation triggered by thrombin, thromboxane A_2 or

collagen. Knowledge of the scramblase activity was to a large extent obtained by use of platelets, erythrocytes, and lymphocytes from patients suffering from Scott syndrome characterized first of all by a reduced ability to expose aminophospholipids on the surface of the cells in response to an increased cytosolic calcium ion concentration. Thus, upon platelet activation and consequent increase in the cytosolic Ca^{2+} the active inward translocation of anionic phospholipids against the concentration gradient is blocked (inhibited translocase) and their facilitated outward translocation is stimulated (active scramblase).

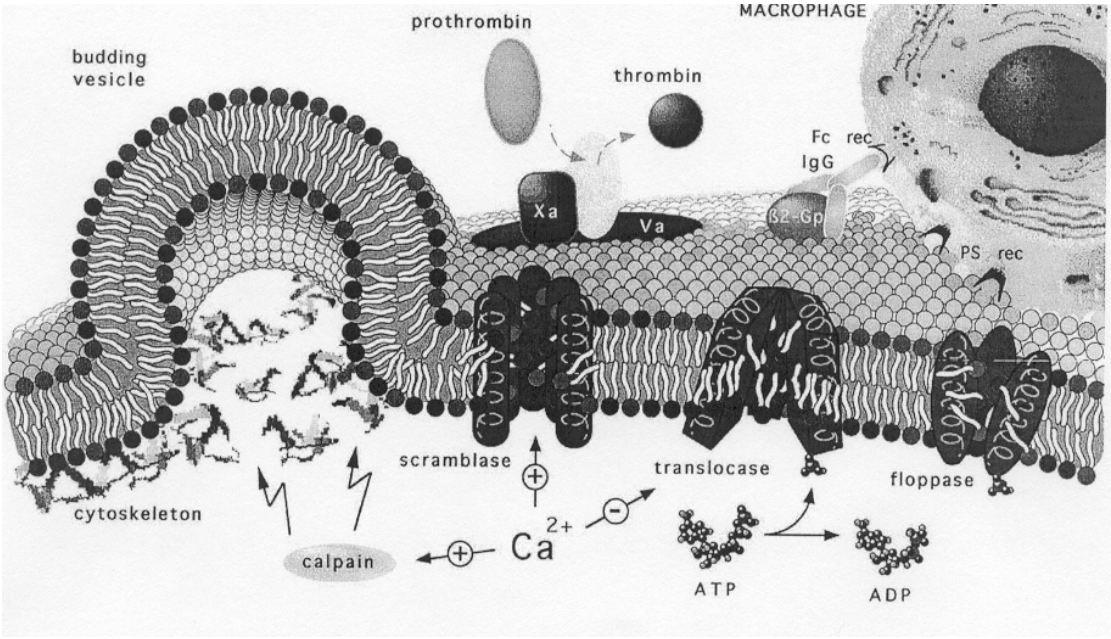

Figure 6. Maintenance and breakdown of the phospholipid asymmetry in platelets.

During regular platelet activation, 3 phenomena are observed, namely, secretion, surface exposure of aminophospholipids, and microvesiculation, which means the formation of small membrane vesicles containing cytoplasmic material. These are also called microparticles. In the vesiculation process, small areas of the surface membrane are shed in a true budding process. However, even if the microvesicles are formed in an outside-out configuration, they possess a procoagulant surface. This is explained by the hypothesis that the phospholipid scrambling occurs before the shedding of the microvesicles. Physiologically, the surface expression of phosphatidylserine is probably the most important feature of the microvesicles, because this means that they are shed with a procoagulant surface. In platelets the budding is preceded by reorganization of the cytoskeleton initiated by the Ca^{2+}-dependent protease calpain. Activation of calpain means degradation of the cytoskeletal proteins filamin, talin, and myosin, resulting in a reorganization of the cytoskeleton. During these dynamic events of membrane rearrangement and budding the outer leaflet of the membrane is enriched in HII-preferring lipids with consequent formation of areas of intermediate HII phase. Similar changes triggered by increase in the cytosolic Ca^{2+} occur in the endothelial cells following stimulation by inflammatory mediators (interleukin-1, TNF) or endotoxin.

If the exposure of the HII-phospholipid domains and the attachment of Gla-proteins (e.g. prothrombin) is not followed by fibrin formation (due to lack of trigger, tissue factor) these phospholipid-protein complexes present neoepitopes for the immune system. The immune response is seen as high blood levels of antiphospholipid-prothrombin immunoglobulin G or antiphospholipid-β_2-glycoprotein I immunoglobulin M (the β_2-glycoprotein I is a phospholipid-binding plasma protein with a poorly defined anticoagulant function). Such antibodies are transiently detected in patients after common viral infections, but if these persist (due to unknown defect in the immune response), the thrombotic complications and spontaneous abortions of the antiphospholipid syndrome develop (formerly it was named lupus anticoagulant coagulopathy, because it was first described as thrombosis in patients with systemic lupus, in whom antibodies prolonging the APTT were detected).

Molecular basis of the thrombosis in APS

The shift in the hemostatic balance in the direction of thrombosis can be explained with the interference of the antiphospholipid antibodies with the phospholipid-dependent hemostatic reactions (Fig. 2. and Table 3.)

	Procoagulant reactions			
Process	FIX activation	FX activation	FX activation	FII activation
Enzyme	FVIIa	FIXa	FVIIa	FXa
Cofactor	Tissue factor	FVIIIa	Tissue factor	FVa
Surface	Parenchymal cells/ endothelium	Platelets/ endothelium	Parenchymal cells/ endothelium	Platelets/ endothelium
Substrate	FIX	FX	FX	prothrombin
	Anticoagulant reactions			
Process	Protein C activation		Cofactor inactivation	
Enzyme	thrombin		Activated protein C	
Cofactor	thrombomodulin		Protein S	
Surface	endothelium		Platelets/ endothelium	
Substrate	Protein C		FVa, FVIIIa	

Table 3. Phospholipid dependent reactions in hemostasis.

Binding of the antibodies to the anionic phospholipid surface prevents the formation of the above listed enzyme-substrate complexes. In the *in vitro* assays (APTT), in the absence of the anticoagulant cofactor thrombomodulin only the effect on the procoagulant reactions that do not require cellular components is detected (compare the PT and APTT values of the clinical case). However, *in vivo* the effects on the anticoagulant reactions dominate.

Diagnosis of APS

1. Screening: prolonged APTT despite thrombotic history (**Question:** Which factor deficiency is consistent with such a finding?)
2. Establishing an inhibitor as explanation for abnormal APTT (**Question:** Which of the laboratory findings in the presented clinical case supports the presence of an inhibitor of blood coagulation? What kind of inhibitors should be considered?)
3. Proving that the inhibitor is phospholipid dependent

- use an APTT reagent with hexagonal phase phospholipid component (a better target of the aPL antibodies with consequent more expressed prolongation)
- platelet neutralization procedure: the examined plasma sample is mixed with washed freeze-thawed platelets (the freeze-thawing activates the platelets and exposes the anionic phospholipids which bind and neutralize the aPL antibodies) and the APTT is determined again (normalization of the value is expected)

4. Testing the titer of antiphospholipid antibodies (ELISA with cardiolipin or phosphatidylserine as target).

Treatment of APS

Asymptomatic patients are not usually treated (approximately 30 % of the anticoagulant antibody positive patients develop thrombosis). After thrombotic events long-term oral anticoagulation is necessary: the coumarin dose should be adjusted to yield INR of 2.5-3.5. In pregnancy complicated with aPL antibodies heparin and low dose aspirin is the therapy of choice. (**Question:** What is the mechanism of action of these drugs? Why should the coumarin therapy be avoided in pregnant women?)