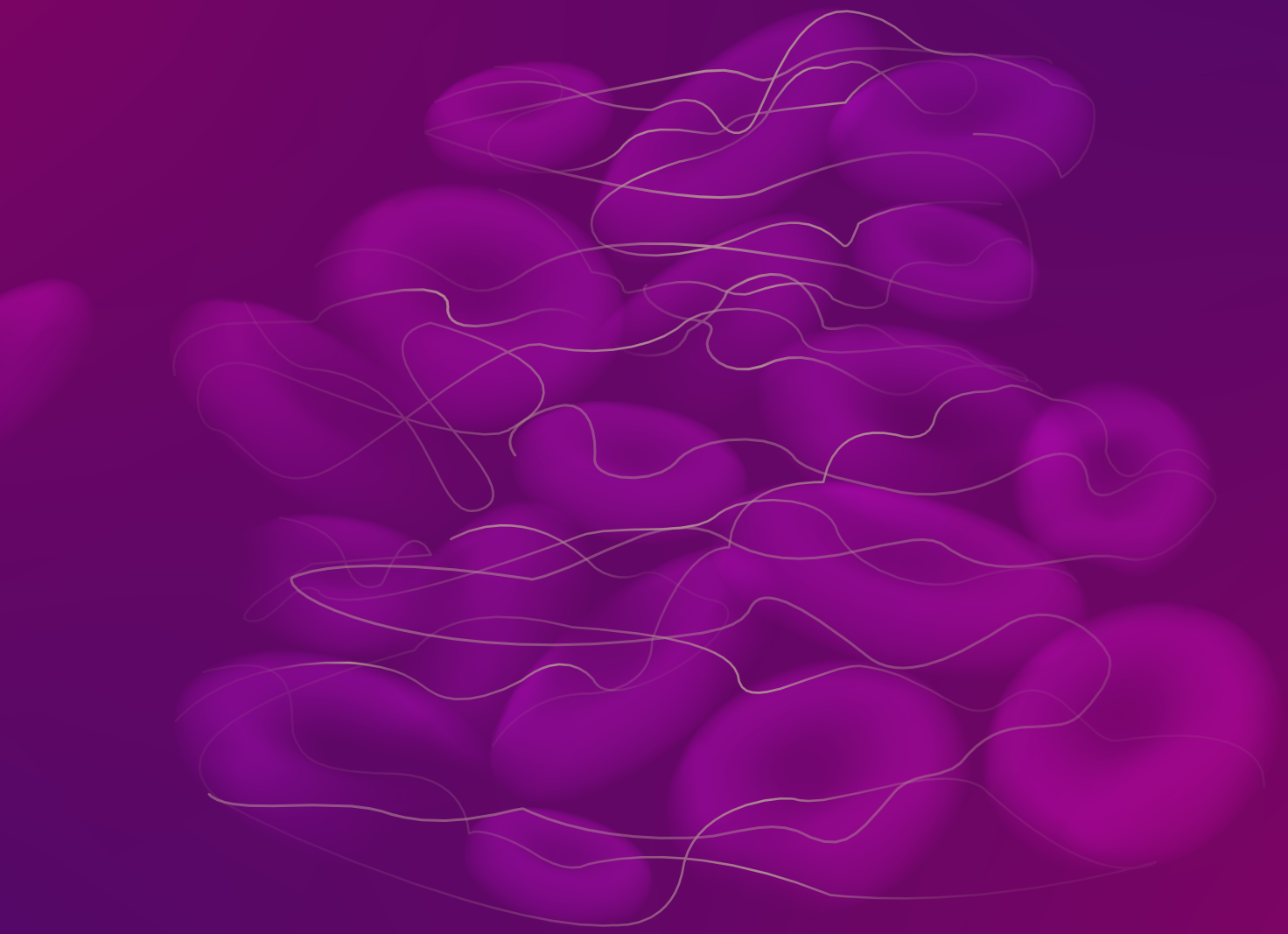
LEARNING GUIDE

COAGULATION



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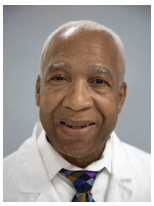

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Dr. Larry Smith received his doctorate in biological sciences from Fordham University, Bronx, New York, and completed his certificate in molecular laboratory diagnostics at Michigan State University, East Lansing, Michigan. He completed his postdoctoral studies in gene therapy and experimental hematopoiesis at Columbia University College of Physicians and Surgeons, New York, New York, and was a research fellow in immunology at Fordham University and at the Laboratory of Lymphokine Biology at the New York Blood Center's Lindsley F. Kimball Research Institute, New York, New York. In addition, Dr. Smith has a Master of Science in medical biology and a Bachelor of Science in French and biology.

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Dr. Smith holds multiple board certificates and has published numerous peer-reviewed articles, abstracts, reviews and monographs. He is the co-author of several textbooks on hematology and coagulation and has been an invited presenter at both national and international conferences. His research interests include laboratory test development for hemostasis and coagulation, HPC therapeutic applications, and flow cytometry.

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It is with gratitude that I acknowledge the work of Dr. Smith for making this Coagulation Learning Guide a useful resource for healthcare professionals worldwide. I would also like to thank the Marketing and Communications Department for significant contributions to the design and layout of this guide.

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Diagnostics, Abbott or Diagnostics at Abbott

DIAGNOSTICS AT ABBOTT COAGULATION AND HEMOSTASIS

INTENDED AUDIENCE

This Coagulation Learning Guide is intended to serve the basic educational needs of healthcare professionals who are involved in the fields of laboratory medicine. Anyone associated with coagulation testing will find this monograph of special interest.

The monograph features the basic information necessary to understand and appreciate the importance of coagulation testing in the laboratory, and it is intended for those who use hematology laboratory services, including but not limited to specialists in laboratory medicine, laboratory technicians, laboratory technologists, clinical laboratory scientists, supervisors and managers, nurses, suppliers, and other physician's office and laboratory support personnel.

HOW TO USE THIS LEARNING GUIDE

Each section of this guide begins with a set of learning objectives to help you focus on the key concepts presented. There is also a short section review quiz at the end of each section to help you recall the concepts introduced. If you answer the questions incorrectly, review the appropriate portions of the section before moving to the next one.

A glossary is included at the end of this learning guide, providing a quick reference of commonly used terms in the science of hematology.

CONTENTS

SECTION 1

OVERVIEW OF COAGULATION & FIBRINOLYSIS

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Define coagulation.
2. List the stages of coagulation.
3. List three major components of coagulation

Coagulation refers to the ability of the blood to form a solid clot following vascular injury. Hemostasis is a delicately balanced process that maintains blood in a fluid state within the blood vessels and rapidly results in clot formation when injury occurs that would result in bleeding. Hemostasis is divided into primary hemostasis, whereby an initial soluble platelet plug is formed, and secondary hemostasis, which converts a soluble primary platelet plug into an insoluble secondary clot.

The process of coagulation involves the interaction of three major components **(Table 1)**: (1) platelets; (2) blood vessels (endothelial cells); and (3) specialized circulating plasma proteins, called clotting factors. When vascular injury occurs, circulating platelets are attracted to the damaged endothelial layer, where they form a primary platelet plug to prevent widespread blood loss.

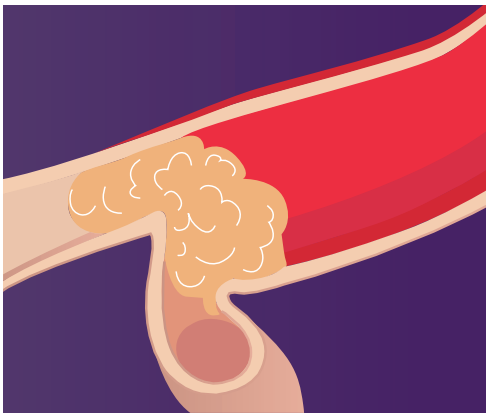


Figure 1: Illustration of a cerebral artery blocked by a blood clot

Table 1: Key Players in Hemostasis

Key player in coagulation
Blood vessels (endothelial cells)
Platelets
Coagulation factors

The initial platelet plug is fragile and soluble. Almost simultaneously, clotting factors located within the blood interact with the tissue factor that has entered the lumen of the blood vessel via the damaged endothelial layer and then are activated. The activation of these clotting factors involves the sequential activation of a series of enzymes that ultimately results in the generation of thrombin. Thrombin converts fibrinogen to fibrin, which leads to the formation of a fibrin clot. Next, fibrin assembles on top of the primary platelet plug, converting it to an insoluble fibrin clot that is far more protective against blood loss since it becomes more stable **(Figure 1)**.

Following the events leading to primary and secondary hemostasis, the naturally occurring inhibitors of coagulation are activated to downregulate the action of thrombin. This process involves the activation of a series of specific proteins that targets procoagulant clotting factors in the coagulation cascade and inhibits their ability to generate thrombin. The final step in coagulation is fibrinolysis. This system is responsible for dissolving the fibrin clot and any unwanted fibrin material that might interfere with blood flow.

Defects in hemostasis can occur at a number of levels and lead to either bleeding or thrombosis. Failure of primary hemostasis, due to the inability to form a stable primary platelet plug, leads to bleeding. Clinically, this is manifested as petechiae and ecchymoses. Failure of secondary hemostasis, due to absent or decreased levels of coagulation factors that stabilize the primary platelet plug, leads to an increased risk of bleeding, as well. Bleeding from a secondary hemostatic defect is often more serious and may involve bleeding into tissue, muscle and joints. Thrombotic complications result from decreased function of the inhibitors of coagulation that regulate thrombin generation.

QUIZ QUESTIONS

1. Define coagulation.

2. List three major components of coagulation.

   A. _____

   B. _____

   C. _____

SECTION 2

PRIMARY HEMOSTASIS

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Describe the structure and function of platelets.
2. Recognize platelet glycoprotein receptors.
3. Name four major zones of platelets.
4. Define key biochemical reactions involved in platelet function.
5. Explain the contribution of endothelial cells to coagulation.
6. List the three major components of coagulation.
7. Recognize the role of platelets in coagulation.
8. Explain the contribution of endothelial cells to coagulation.
9. Describe the sequential interaction of the coagulation proteins.

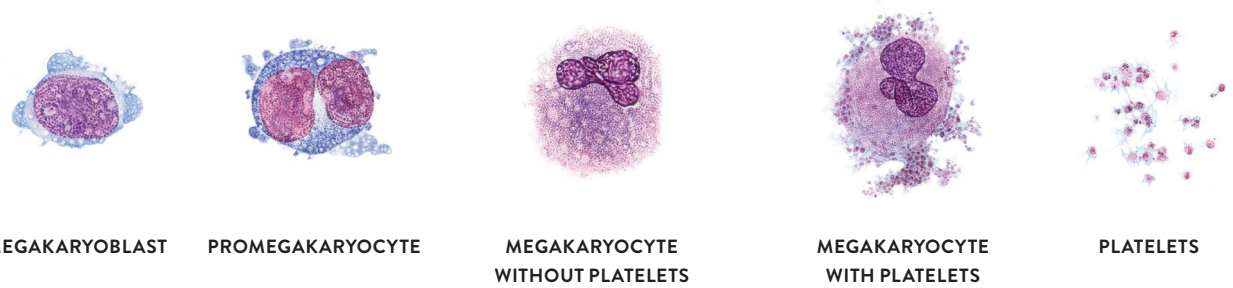


Figure 1

Primary hemostasis involves the interaction of platelets and endothelial cells (ECs). Platelets, derived from megakaryocytes in the bone marrow, are circulating anucleated cells approximately 2–4 μm in diameter **(Figure 1)**.[1] Platelets have a circulating life span of 7–10 days. Platelet structure is divided into four arbitrary zones: (1) peripheral zone, (2) structural zone, (3) organelle zone, and (4) membranous zone. The peripheral zone consists of an amorphous, spongy exterior layer, called the glycocalyx. The glycocalyx contains a number of adsorbed coagulation factors, glycolipids and glycoproteins. The glycocalyx also contains an open canalicular system (OCS), consisting of invaginations of the platelet membrane **(Figure 2)**. The OCS allows for products stored within the platelet to be released to the outside of the platelet. The OCS also provides increased surface area of the platelet membrane for interaction with the coagulation factor complexes that form to generate thrombin. A number of glycoprotein receptors that play roles in platelet adhesion and aggregation are also found in the glycocalyx.

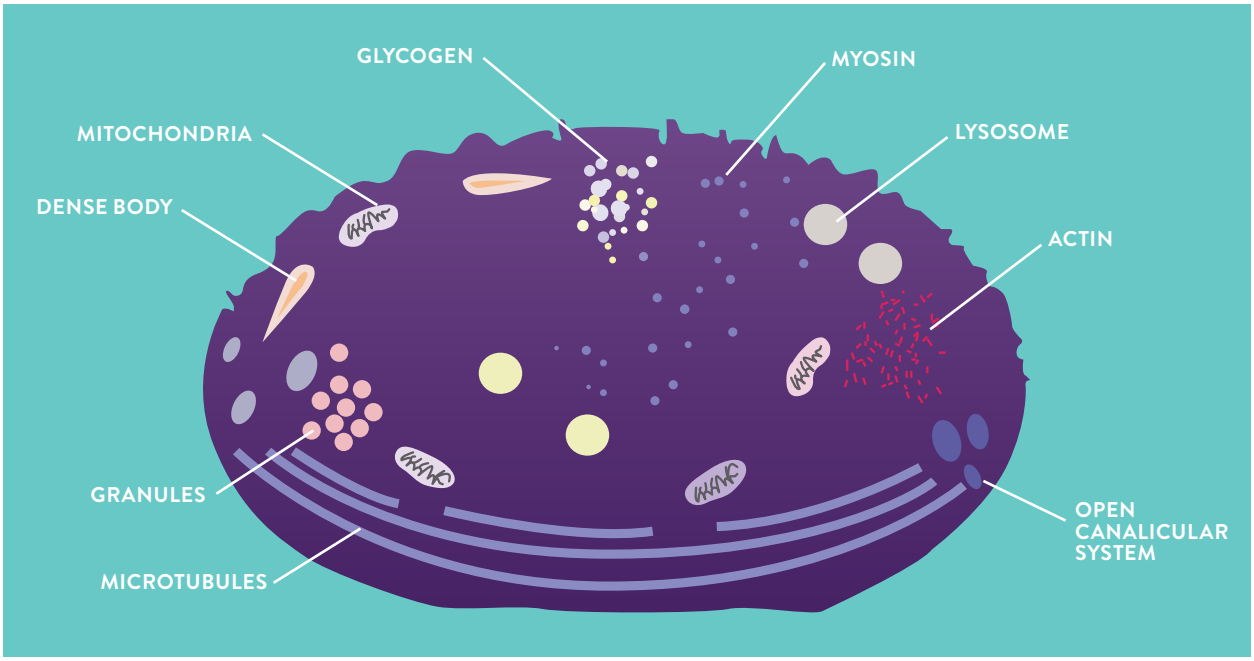


Figure 2

Located directly beneath the glycocalyx is the plasma membrane. This is a lipid bilayer that contains phospholipids, arachidonic acid (AA) and an enzyme called phospholipase A2. Phospholipase A2 reacts with the phospholipids located in the platelet membrane, leading to the release of AA. Arachidonic acid is then converted to the endoperoxides (PGG_2, PGH_2) by the enzyme cyclooxygenase.[2] Cyclooxygenase is a very important enzyme and is essential to the conversion of AA to the endoperoxides **(Figure 3)**. It is also an important target in antiplatelet therapy since it is inhibited by aspirin.

PGH_2 is then converted to thromboxane A_2 (TXA_2) by an enzyme called thromboxane synthase. The enzymes and substrates that lead to the conversion of AA to TXA_2 are found in the eicosanoid synthesis pathway. TXA_2 is released through the OCS into the circulation, where it binds to a specific receptor on inactive platelets in the circulation, leading to their activation. In addition to being a very potent platelet activator, TXA_2 also causes vasoconstriction, which slows down the flow of blood through the vessel, facilitating platelet adhesion. The structural zone of platelets is located adjacent to the plasma membrane and consists of circumferential bands of microtubules that form a cytoskeleton to maintain the discoid shape of the resting platelet.[2]

Located beneath the structural zone is the organelle zone, which contains alpha granules, dense granules, lysosomal granules, and mitochondria. The alpha granules (40–80/platelet) are the most numerous granules in platelets and secrete adhesive proteins, growth factors, and absorbed coagulation factors.[2]

The dense granules (4–8/platelet) secrete energy-related substances (ADP, ATP, GDP, GTP) that enhance platelet aggregation.[2] The membranous zone, called the dense tubular system (DTS), is located deep within the platelet and serves as a storage site for TXA_2 and calcium. It also weaves through the platelet cytoplasm to connect to the OCS, where constituents stored within the platelet are released to further enhance platelet aggregation.

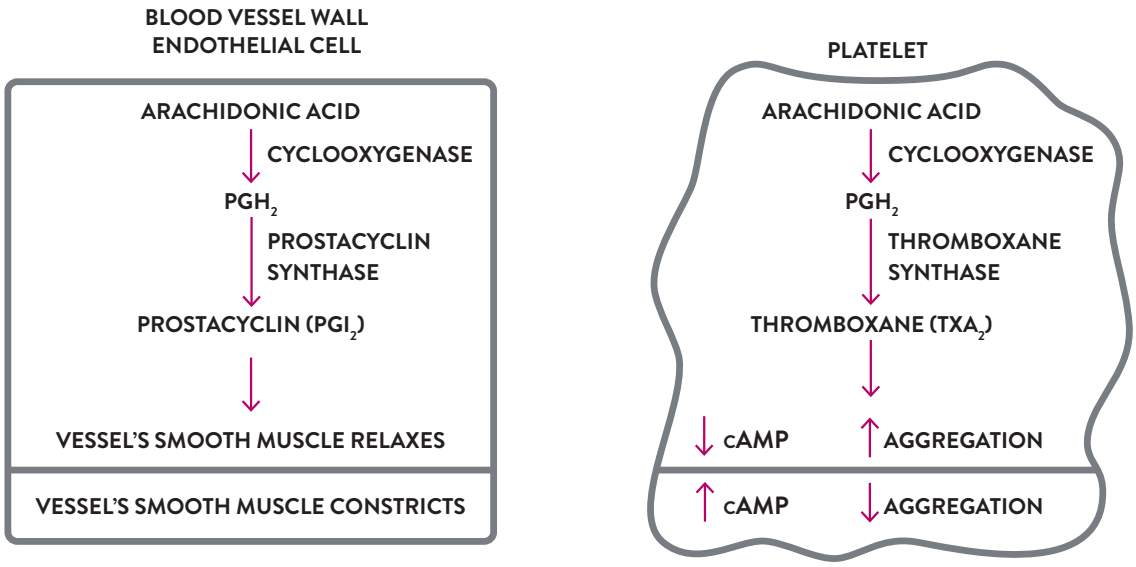


Figure 3

Platelet function can be divided into four stages: **(1)** adhesion, **(2)** shape change, **(3)** activation, and **(4)** secretion **(Table 2)**. Inactive, or resting, platelets circulate through the blood vessels, surveying the vessels for nicks and tears in the endothelium. When damage occurs to the blood vessel wall, platelets may bind directly to the exposed collagen microfibrils in veins and capillaries.[3] In areas of high-shear stress, such as arteries and arterioles, von Willebrand factor (vWF) is released from structures in endothelial cells called Weibel-Palade bodies. As vWF enters the lumen of the blood vessel, platelets bind to the released vWF via a specialized glycoprotein receptor, GPIb (part of the GPIb/V/IX receptor complex). Platelets are then pulled down to the exposed collagen microfibrils located in the subendothelial layer, forming a monolayer of adherent platelets **(Figure 4)**. Two additional platelet glycoprotein receptor complexes, GPIa/IIa ($\alpha_2\beta_1$ integrin) and GPVI, are also expressed on platelets and help to anchor the platelets to the exposed collagen layer.[4] GPVI is a major signaling receptor for collagen, contributing to collagen-induced activation of platelets.[5]

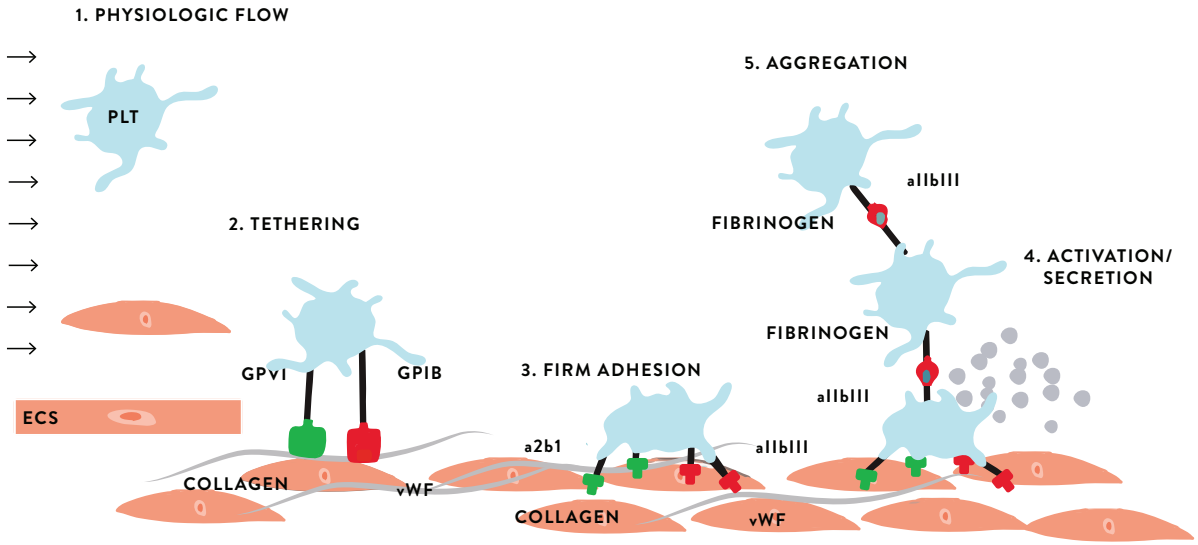
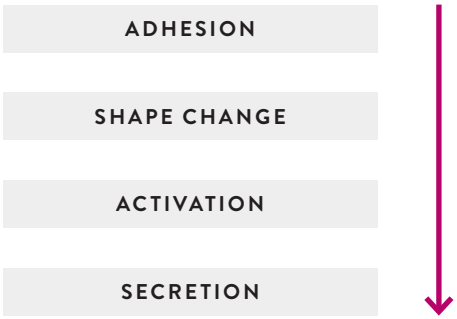


Figure 4: Physiologic Flow

When platelets stick to the damaged endothelial layer, the process is called platelet adhesion. The adherent platelets undergo a shape change and pseudopod formation, which increases the overall surface area of the platelets on the endothelial surface. Following adhesion and shape change, platelets are simultaneously activated and release their granular content. Activation involves the generation of TXA2 via the eicosanoid synthesis pathway. Activation leads to the expression of the glycoprotein IIb/IIIa (GPIIb/IIIa, $\alpha_{IIb}\beta_3$ integrin) receptors. The GPIIb/IIIa receptors expressed on adjacent platelets then bind to circulating fibrinogen in plasma, which serves as a "glue" for platelets to stick to each other **(Figure 4)**. This process is called platelet aggregation, which is driven by TXA2 and Ca^{++} stored in the DTS and released through the OCS to further enhance platelet activation.

Table 2: Platelet Function



Endothelial cells play a vital role in all phases of coagulation. They make up the innermost layer of all blood vessels and form a continuous single-cell layer as a barrier between the blood components located within the blood vessel and the interstitial compartments **(Figure 5)**.[6] Intact endothelial cells are covered with heparin sulfate proteoglycans, which contribute an anticoagulant effect by suppressing the activation of platelets and the coagulation cascade **(Figure 6a)**. Arachidonic acid, also found in the plasma membrane of ECs, is converted to PGG2 and PGH2 by cyclooxygenase, similar to what occurs in platelets. However, ECs contain prostacyclin synthase that converts the PGH2 into prostacyclin (PGI2), which is a very potent inhibitor of platelet aggregation and a vasodilator.[7] Nitric oxide (NO) is also released from ECs and causes vasodilation.

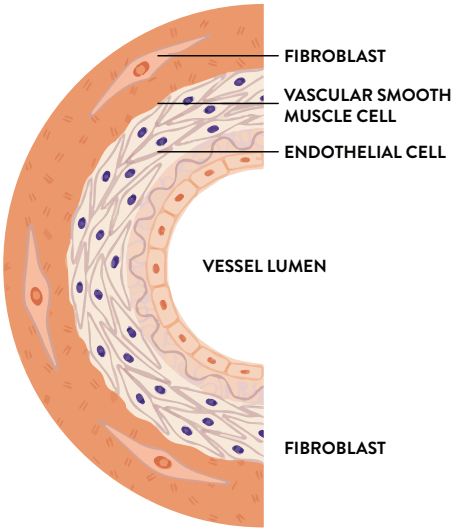


Figure 5

Endothelial cells also downregulate the coagulation cascade by converting thrombin from a procoagulant to an anticoagulant via the expression of thrombomodulin (TM) located on the endothelial cell surface. Thrombomodulin is a membrane-bound protein expressed on the surface of endothelial cells.[3] When thrombin binds to TM, it is no longer able

to feed back into the coagulation cascade to amplify more thrombin generation (procoagulant function). When thrombin binds to TM, the protein C pathway is activated, which downregulates the coagulation cascade (anticoagulant function) **(Figure 6b)**. In addition, ECs synthesize and release a protein, called tissue factor pathway inhibitor (TFPI), which rapidly inactivates the extrinsic pathway to downregulate thrombin generation.[7]

Procoagulant activities of ECs are initiated when the endothelial surface is disrupted, which results in TF expression in the lumen of the blood vessel. Tissue factor then activates the extrinsic pathway of the coagulation cascade. The damaged ECs also release vWF from the Weibel-Palade bodies, which mediates platelet adherence to the damaged endothelial surfaces. This contributes to platelet adhesion and activation through interaction of vWF with the GPIb platelet receptor. The vWF also protects FVIII in the circulation. Endothelial cells provide a surface for the coagulation reactions to occur. Endothelial cells also regulate fibrinolytic activity by releasing activators and inhibitors of plasminogen, which are discussed later.

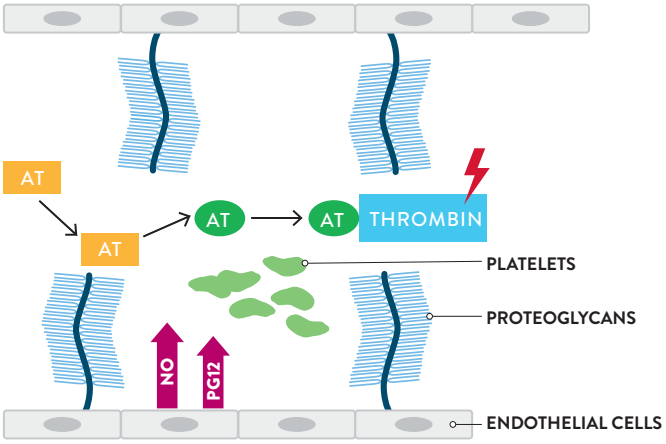


Figure 6a

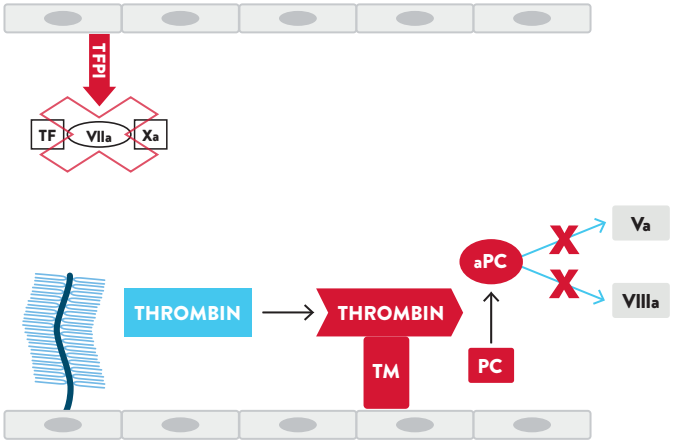


Figure 6b

QUIZ QUESTIONS

1. Primary hemostasis involves the interaction of _____ and _____

2. List four arbitrary zones of platelets.

   _____

   _____

   _____

   _____

3. What enzyme converts arachidonic acid to the endoperoxides (PGG2 and PGH2)?

   _____

4. List four functions of platelets in hemostasis.

   A _____

   B _____

   C _____

   D _____

5. Every blood vessel is surrounded by a single layer of _____

6. Which organelle in endothelial cells stores von Willebrand factor? _____

SECTION 3

SECONDARY HEMOSTASIS

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Name the coagulation factors.
2. Describe the sequential interaction of the coagulation factors.
3. Recognize the coagulation complexes that form.
4. Explain the function of the inhibitors of coagulation.

Secondary hemostasis involves activation and interaction of the clotting factors **(Table 3)**. Clotting factors consist of substrates, cofactors, and enzymes. Most are synthesized in the liver. There are approximately 16 procoagulants in the coagulation cascade. Eight of these are enzymes that circulate as inactive precursors, called zymogens. The remaining procoagulants are cofactors that bind to and stabilize their respective enzymes.[3] The names of the coagulation factors and their locations in the coagulation cascade are provided in **Table 3**.

Table 3

Factor	Name	Pathway
I	Fibrinogen	Common
II	Prothrombin	Common
III	Tissue factor	Extrinsic
V	Labile factor	Common
VII	Proconvertin (Stabile factor)	Extrinsic
VIII	Antihemophilic factor	Intrinsic
IX	Plasma thromboplastin component (PTC)	Intrinsic
X	Stuart-Prower factor	Common

Factor	Name	Pathway
XI	Plasma thromboplastin antecedent (PTA)	Intrinsic
XII	Hageman factor	Intrinsic
XIII	Fibrin-stabilizing factor	Common?
PK	Prekallikrein (Fletcher)	Intrinsic
HMWK	High-molecular-weight kininogen (Fitzgerald)	Intrinsic
IV	Ionized calcium	
VI	Not assigned	

The coagulation cascade involves a series of amplification reactions whereby a zymogen is activated by an enzyme into an active serine protease **(Figure 7)**. The newly activated serine protease then activates the next zymogen in the pathway, creating a cascade of reactions. These reactions have also been described as a waterfall effect.

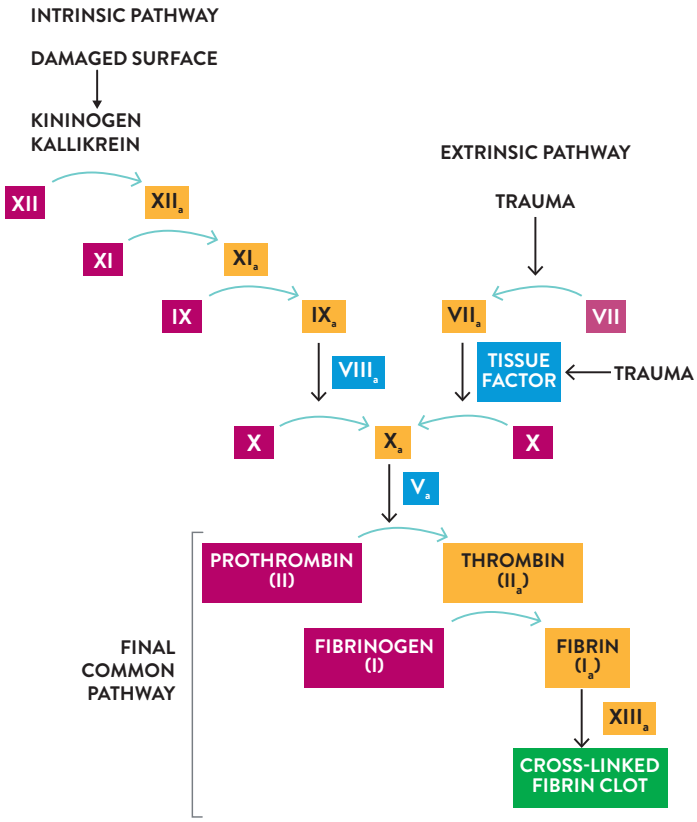


Figure 7

Activation of the coagulation cascade leads to the generation of thrombin and fibrin. Thrombin is a key player in the coagulation cascade, where it has numerous functions, both procoagulant and anticoagulant. As a procoagulant, thrombin feeds back to the intrinsic and common pathways to amplify its own generation, which leads to the formation of a fibrin clot. As an anticoagulant, thrombin activates protein C, which downregulates factors Va and VIIIa, leading to an inhibition of thrombin generation. Thrombin activates platelets, which also contribute to platelet plug formation. Additionally, thrombin regulates fibrinolysis through the production of thrombin-activatable fibrinolysis inhibitor (TAFI), which inhibits fibrinolysis. When TAFI levels are elevated, there is an increased risk for thrombosis. Decreased levels are associated with an increased risk for bleeding.

The clotting factors are divided into the **(1)** contact group, **(2)** prothrombin group, and **(3)** fibrinogen group. The contact group consists of prekallikrein (PK), also know as Fletcher factor; high-molecular-weight kininogen (HMWK), also known as Fitzgerald factor; FXII; and FXI. A qualitative/quantitative defect in PK, HMWK, or FXII results in a prolonged APTT, withan increased risk of thrombosis rather than bleeding. These three factors may play a greater role in activating the complement system, the kallikrein-kinin system, and the fibrinolytic system, rather than the coagulation system. FXII functions as a link between all four of these systems.

The prothrombin group consists of FII, FVII, FIX, and FX. These are also vitamin K-dependent factors (VKDF) and contain 10–12 glutamic acid units near their terminal ends.[3] In the presence of vitamin K, they undergo a post-translational modification, whereby γ-carboxylation of glutamic residues occurs, creating a negatively charged protein. The negative charge allows the VKDFs to bind to the phospholipid surface of platelets, using Ca^{2+} as a bridge **(Figure 8)**. Vitamin K is an essential cofactor required for the modification.

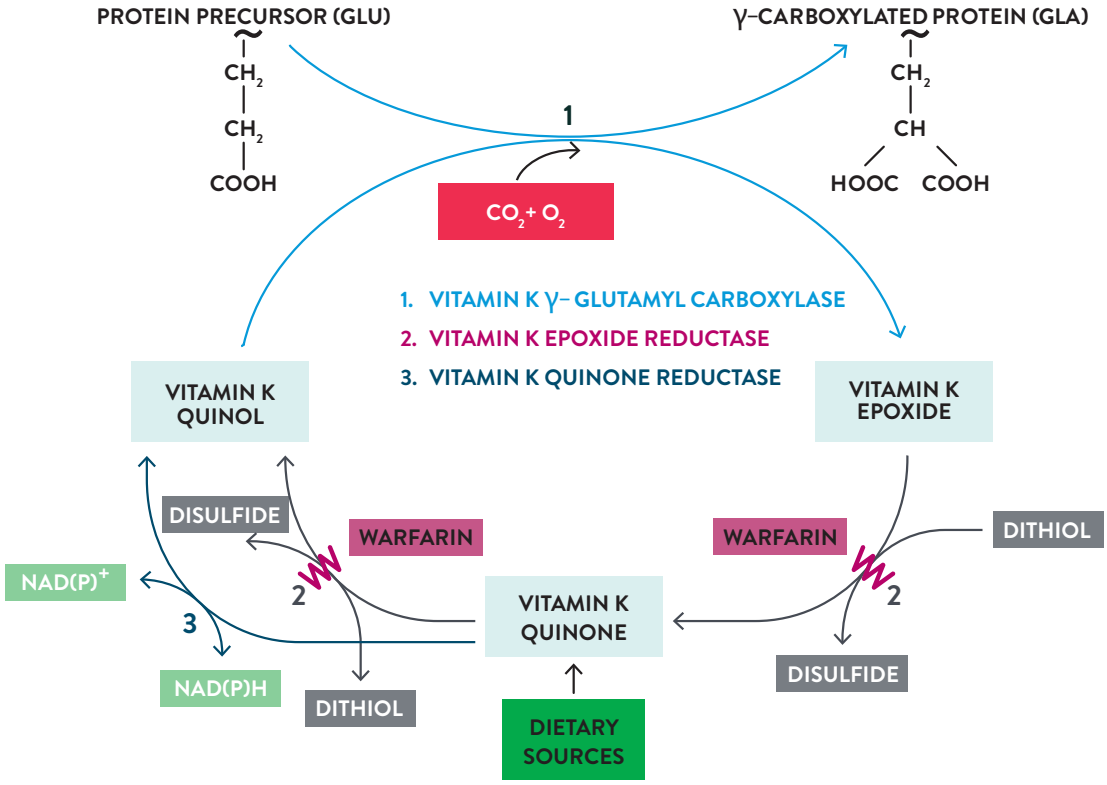


Figure 8

The fibrinogen group consists of FI, FV, FVIII, and FXIII. These factors are acted upon by thrombin and are consumed during the clotting process. All of the clotting factors in the coagulation cascade are serine proteases, except for FXIII, which is a transglutaminase. Serine proteases contain a reactive serine residue in their active centers and act by hydrolyzing peptide bonds.

The coagulation factors are referred to using Roman numerals. The letter "a" placed after the Roman numeral indicates that the zymogen has been activated. The coagulation cascade consists of three pathways: **(1)** intrinsic, **(2)** extrinsic, and **(3)** common. Activation of any one of the pathways ultimately leads to thrombin generation. The coagulation cascade is generally described as a Y-shaped model, with the intrinsic and extrinsic pathways converging onto the final common pathway **(Figure 7).**

The extrinsic pathway is activated following vascular injury when tissue factor (TF) "extrinsic" to the blood vessel is introduced into the lumen of the blood vessel. TF is a membrane-bound glycoprotein found on the surface of almost all cells, except endothelial cells.[7,8] It rapidly binds to circulating FVII, forming a complex called the TF:VIIa complex. The TF:VIIa binds to FX (located in the common pathway) and converts FX to FXa. This leads to activation of the common pathway via the extrinsic pathway and a rapid burst of thrombin generation. The TF:VIIa complex also converts FIX (located in the intrinsic pathway) to FIXa, and FIXa converts FX to FXa. As a result, the intrinsic pathway is also able to activate the common pathway. Therefore, all three pathways are linked together at FX.

The intrinsic pathway is activated when FXII interacts with a negatively charged surface, such as collagen found in the subendothelial layer of blood vessels. FXIIa circulates in plasma bound to HMWK and converts PK to kallikrein. FXII is activated when it contacts a negatively charged surface, such as collagen in a damaged blood vessel. It converts FXI to FXIa. FXIa converts FIX to FIXa. FIXa in the presence of FVIIIa forms a complex called the "intrinsic" tenase complex and converts FX to FXa on the platelet surface **(Figure 9)**. FVIIIa functions as a cofactor in this reaction and is activated by the initial burst of thrombin from activation of the extrinsic pathway.

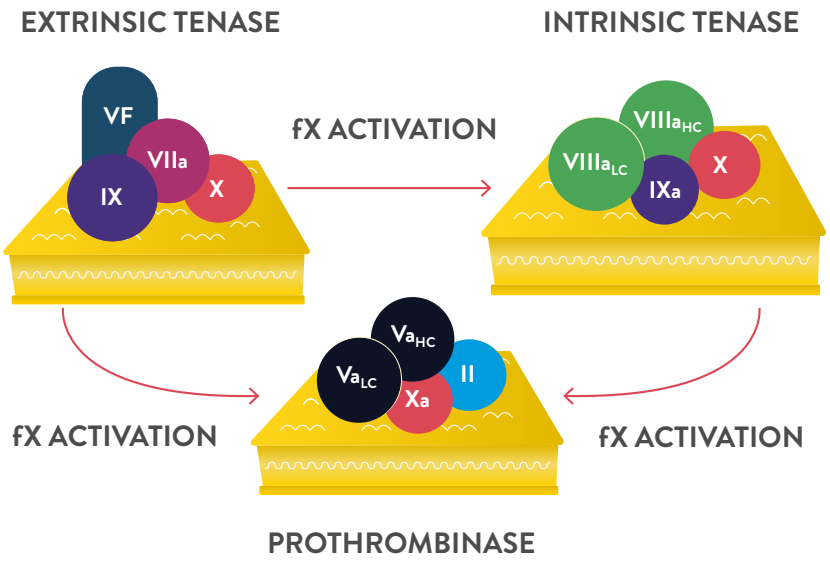


Figure 9

The common pathway begins with the conversion of FX to FXa via the extrinsic tenase complex (TF:FVIIa) or the intrinsic tenase complex (FIXa:VIIIa) on the platelet surface. FXa in the presence of FVa converts prothrombin (FII) to thrombin (FIIa). Thrombin is initially generated by the extrinsic pathway and, via an amplification loop, activates FXI in the intrinsic pathway **(Figure 10)**. When a sufficient amount of thrombin is generated via this amplification loop, it converts fibrinogen into soluble fibrin monomers.

FVa functions as a cofactor in this reaction. The soluble fibrin monomers are cross-linked together to become insoluble fibrin monomers via the action of FXIIIa. FXIII, a transglutaminase, is converted to FXIIIa by thrombin. Thrombin also participates in an amplification reaction to generate more thrombin by directly converting FXI to XIa, FVIII to FVIIIa, and FV to FVa. These three reactions ensure the continued generation of thrombin during fibrin clot formation. Thrombin has also been shown to activate platelets and fibrinolysis and induces the release of TAFI.

Once thrombin generation begins, it eventually needs to be turned off. This is achieved by a group of proteins that are natural inhibitors of coagulation: **(1)** tissue factor pathway inhibitor (TFPI), **(2)** antithrombin (AT), and **(3)** activated protein C (aPC) **(Figure 11)**. The extrinsic pathway is downregulated when endothelial cells release TFPI. TFPI first binds to FXa and forms a complex to shut off FXa. The TF:Xa complex then binds to FVIIa to shut off FVIIa.[3] Thus, TFPI rapidly shuts off thrombin generated from the extrinsic side of the coagulation cascade. Even though thrombin generation diminishes quickly, small amounts of residual thrombin are able to amplify continued thrombin generation via the intrinsic pathway.

Antithrombin is a serine-protease inhibitor produced in the liver that preferentially inhibits thrombin and FXa **(Figure 11)**. It also inhibits the other serine proteases in the coagulation cascade. Its activity is greatly enhanced by heparin or heparin-like molecules expressed on the endothelium.[8] The aPC forms when thrombin binds to the thrombomodulin on the endothelial surface and thrombin is converted from a procoagulant to an anticoagulant molecule. The thrombin:thrombomodulin complex converts protein C (PC) into aPC. Protein S (PS) serves as a cofactor for this reaction. Activated protein C inhibits FVa and FVIIIa located in the common and intrinsic pathways, respectively, resulting in the inactivation of thrombin generated by the intrinsic pathway of the coagulation cascade **(Figure 11)**.

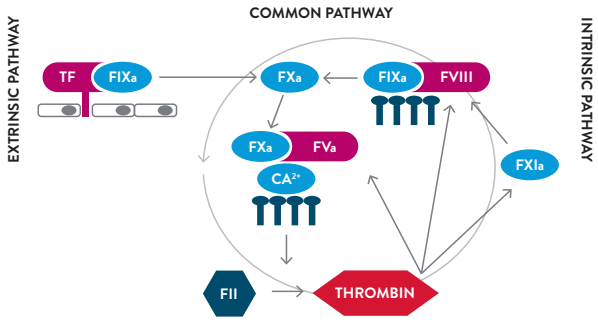


Figure 10

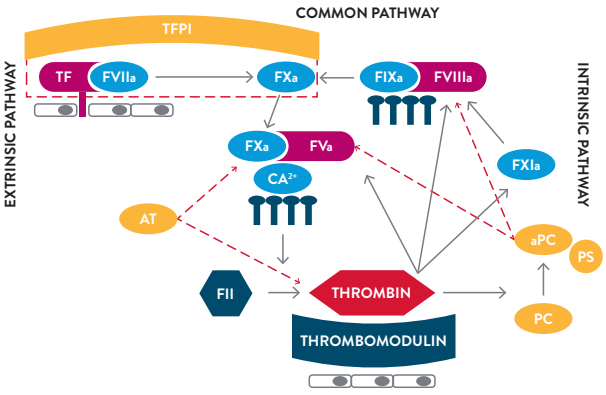


Figure 11

QUIZ QUESTIONS

1. Clotting factors consist of _____, and _____ .

2. List the three groups of clotting factors.

   A _____

   B _____

   C _____

3. Which enzyme is a key player in the coagulation cascade, with multiple functions?

   _____

4. List the vitamin K-dependent factors.

   A _____

   B _____

   C _____

   D _____

5. Name the three pathways of the coagulation cascade.

   A _____

   B _____

   C _____

6. Name three naturally occurring inhibitors of the coagulation.

   A _____

   B _____

   C _____

SECTION 4

FIBRINOLYSIS

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Define fibrinolysis.
2. Identify key players in the fibrinolytic process.
3. Describe the mechanism of action of the enhancers and inhibitors of fibrinolysis.

After a clot forms, the fibrinolytic system is activated to enzymatically degrade insoluble fibrin. As a result, fibrinolysis prevents the blood vessel from becoming completely occluded after clot formation. It also aids in tissue repair and the removal of clots in areas where they are not supposed to be. There are six key components in fibrinolysis: **(1)** plasminogen, **(2)** plasmin, **(3)** tissue plasminogen activator (tPA), **(4)** plasminogen activator inhibitor (PAI-1), **(5)** alpha-2 antiplasmin (α2AP), and (6) thrombin-activatable fibrinolysis inhibitor (TAFI).[3,5] Plasminogen is produced in the liver and circulates as a precursor molecule that is converted into plasmin (a serine protease) by the enzyme tPA. tPA is produced and secreted by endothelial cells as thrombin is produced and a fibrin clot forms.[8] Once the clot is formed, plasmin binds to fibrin monomers and cleaves them into fibrin degradation products (FDP), which consist of fragments X, Y, D and E **(Figure 12)**.[9]

After repeated rounds of degradation, the end product is a DD-dimer fragment (DD). Plasmin degrades fibrinogen, as well, also producing fragments X, Y, D and E. However, the D-dimer product that results from plasmin degradation of fibrin is specific for fibrin. Factors V and VIII are also degraded by plasmin.

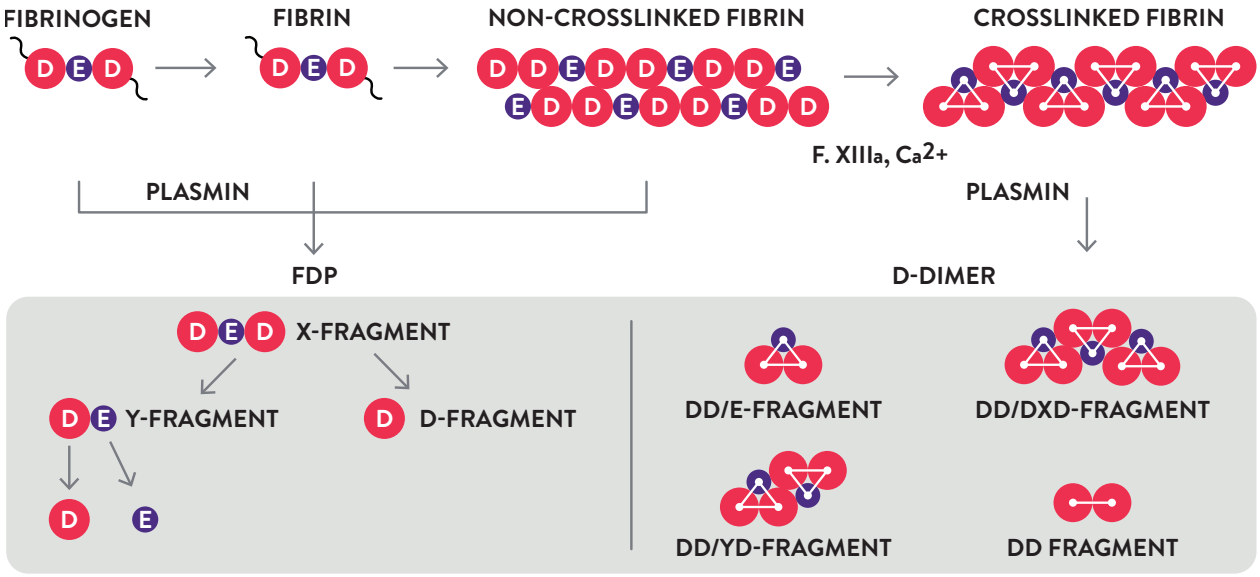


Figure 12

The regulation of plasmin has to be tightly controlled in order to prevent severe bleeding. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) are endothelial-derived regulators of fibrinolytic activity **(Figure 13)**. tPA increases fibrinolysis by upregulating the conversion of plasminogen to plasmin. Both plasminogen and tPA bind to the fibrin clot in order for plasminogen to be converted to plasmin. PAI-1 downregulates fibrinolysis by directly inhibiting the action of tPA. Alpha-2 antiplasmin is a protein produced in the liver that is capable of binding to and inhibiting free-floating plasmin that escapes from the fibrin clot. Thrombin-activatable fibrinolysis inhibitor (TAFI), produced as a result of thrombin generation, inhibits fibrinolysis by preventing tPA and plasmin from binding to the fibrin clot.[3] TAFI is activated when thrombin binds to TM on the damaged endothelium.

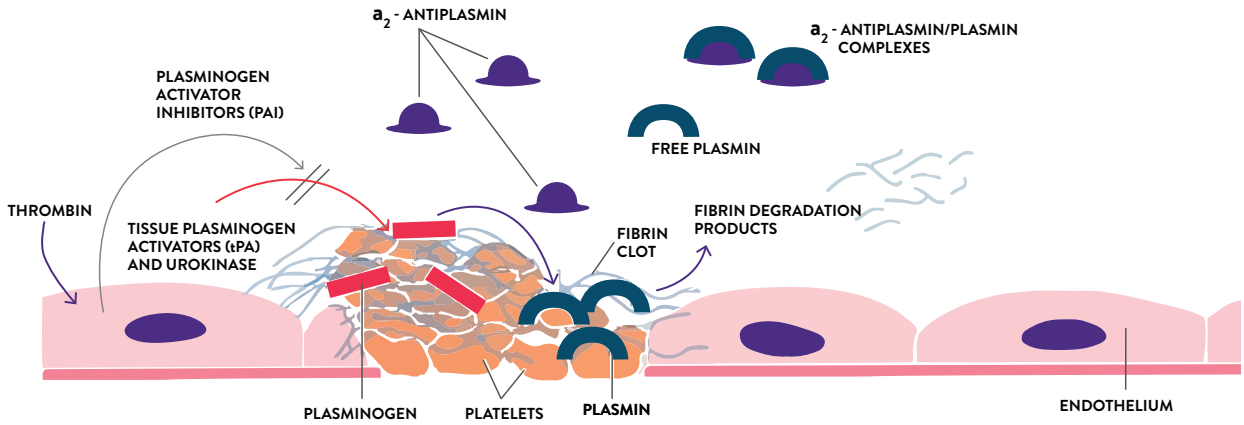
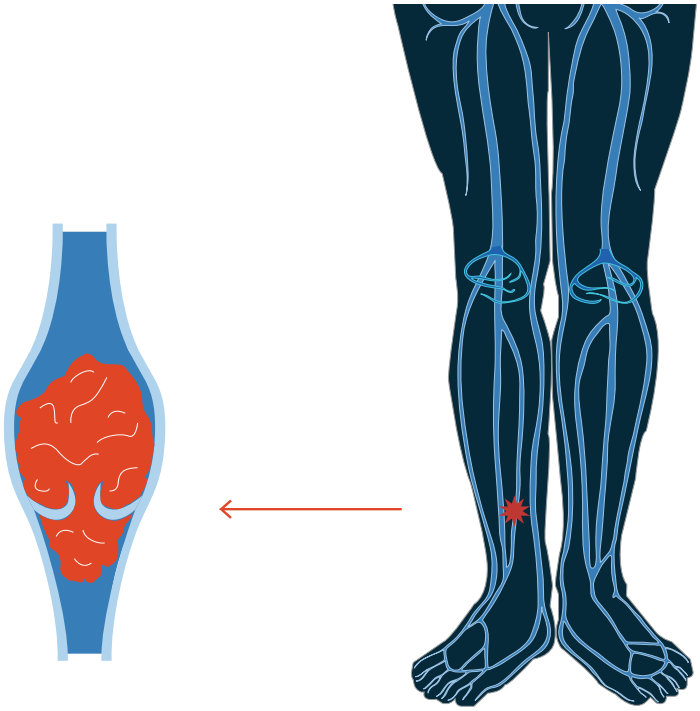


Figure 13



Deep vein thrombosis (DVT):
Clot forming in the deep veins of the leg

QUIZ QUESTIONS

1. Which system is responsible for cleaving unwanted fibrin after thrombin generation?

_____

2. Name six key players in fibrinolysis.

   A _____

   B _____

   C _____

   D _____

   E _____

   F _____

3. The end product that represents the action of plasmin on the fibrin clot is _____

SECTION 5

ROUTINE COAGULATION ASSAYS

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Name routine assays used in coagulation screening.
2. Associate assays with pathways of the coagulation cascade.
3. Discuss reagents used to perform coagulation assays.
4. Define the INR and its use.
5. Correlate abnormal findings in coagulation screening tests to specific factor deficiencies.
6. Identify and associate pre- and post analytical variables in coagulation testing.

Routine screening assays performed in the coagulation laboratory include prothrombin time (PT), including the INR (PT/INR); activated partial thromboplastin time (APTT); fibrinogen assay(FIB); thrombin time (TT); and D-dimer (DD) **(Table 4)**. When the results of these assays are outside of the normal reference interval, they may be suggestive of either a bleeding or a clotting disorder and require further investigation involving more specialized coagulation testing.

The PT/INR, APTT, FIB, and TT are clot-based assays.[10] Clot-based assays are very good screening tests that are based on thrombin generation and that evaluate level and functionality of the clotting factors in the coagulation cascade. If any of the clotting factors are decreased, absent, or nonfunctional, an abnormal test result is obtained in the screening assay. Clot-based assays are subject to a number of pre-analytical variables that may prolong or shorten the result **(Table 5)**. The DD assay is a latex-immunoassay and is not affected by the preanalytical variables listed in Table 5.

Table 4: Routine Coagulation Assays

Routine coagulation assays
Prothrombin time
APTT
Fibrinogen
Thrombin time
D-dimer

Table 5: Preanalytical Variables in Coagulation Testing

Error	Test and effect
Short draw (<2.7 mL)	PT/APTT – falsely prolonged; may influence fibrinogen and D-dimer
Serum or clotted sample	PT/APTT/ fibrinogen– no clot formation
Hemolysis	PT/APTT – falsely shortened
Refrigerated before centrifugation	PT – falsely shortened
Excessive/vigorous mixing	PT/APTT – falsely shortened
Heparin contamination from a line draw	APTT – falsely prolonged
Lipemia	PT/APTT – may not get a result using photo-optical methodology
Prolonged storage	APTT – perform testing within 4 hours (within 1 hour if heparinized) at RT PT – perform testing within 24 hours at RT
Excessive stasis during venous blood draw	PT/APTT - prolonged
Capillary blood draw: risk for tissue-fluid admixture	PT/APTT - shortened
Stressed patient before or at blood draw	APTT - shortened; F VIII - increased

PROTHROMBIN TIME

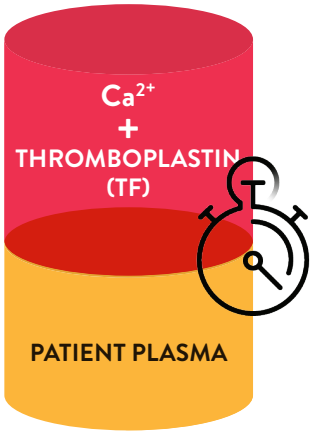


Figure 14

The PT was described in 1935 by Quick and was developed to measure prothrombin.[10] The PT measures the time it takes for a clot to form when a patient's plasma is combined with a thromboplastin reagent and calcium **(Figure 14).** Thromboplastin contains a source of phospholipids and tissue factor. The PT is prolonged when there are deficiencies in the extrinsic and/or common pathways of the coagulation cascade or in the presence of natural or therapeutic anticoagulants. The PT is most sensitive to deficiencies in FVII and moderately sensitive to deficiencies in FV and FX.[3] It also detects deficiencies in three of the vitamin K- dependent factors (VKDF): FII, FVII, and FX. The PT also is used to monitor patients on oral anticoagulant therapy (OAC), such as warfarin (Coumadin®).

Since many laboratories often use different thromboplastin reagents from different manufacturers that have different sensitivities to decreases in the VKDFs, the use of the PT assay to monitor OAC has been modified to include the international normalized ratio (INR). The INR is a ratio that compares the laboratory's thromboplastin reagent to the World Health Organization international reference thromboplastin reagent, which has an ISI of 1.0 **(Box 1)**.[3,9] An ISI near 1.0 is more responsive to decreases in the VKDFs. The INR is a calculation derived by dividing the patient's PT value by the geometric mean (GM) of the PT of a sufficient number of normal donor plasmas, raised to the power of the ISI. The GM is preferred to the arithmetic mean for determining the INR.

The ISI, or international sensitivity index, is a value that is provided by the reagent/analyzer manufacturer and is a measure of the sensitivity of a particular thromboplastin reagent to a warfarin-induced decrease in the VKDFs. The INR should not be considered a substitute for the PT and is intended to be used only in patients who are on OAC since it is standardized in relation to the universal standard for patients on stabilized warfarin therapy. An elevated PT is often seen in patients with disseminated intravascular coagulation (DIC), liver disease, and vitamin K deficiency.[3] The PT is not prolonged in FXIII deficiency.

In 1959, Owren described a modification of the PT, called thrombotest. This PT reagent contains fibrinogen and FV and is, therefore, only sensitive to vitamin K- dependent factors. The thrombotest assay can be done not only in plasma but also in a low volume of whole blood, which may be an advantage in certain conditions.

$$\text{INR} = \left(\frac{\text{PATIENT PT (SEC)}}{\text{GM PT (SEC)}} \right)^{\text{ISI}}$$

Box 1

ACTIVATED PARTIAL THROMBOPLASTIN TIME

The APTT was described in 1953 by Langdell, Wagner and Brinkhaus and modified in 1961 by Proctor and Rappaport. The APTT measures the time it takes for a clot to form when a patient's plasma is combined with a partial thromboplastin reagent, which consists of phospholipids and an activator; after incubating for a few minutes to complete the activation, calcium is added for starting the clotting reaction **(Figure 15)**. The partial thromboplastin does not contain tissue factor phospholipids or tissue factor. Activators, such as celite, ellagic acid, kaolin and silica, provide a negatively charged surface to activate the contact factors in the absence of tissue factor.[9] The APTT is prolonged when there are deficiencies in the intrinsic and/or common pathway. The APTT was developed to identify deficiencies in the intrinsic pathway (e.g., hemophilias); however, it is also commonly used to monitor patients on heparin. The APTT is also prolonged in the presence of specific factor inhibitors, global inhibitors that interfere with thrombin generation, and lupus anticoagulants. The APTT is not prolonged with deficiencies of FVII or FXIII.

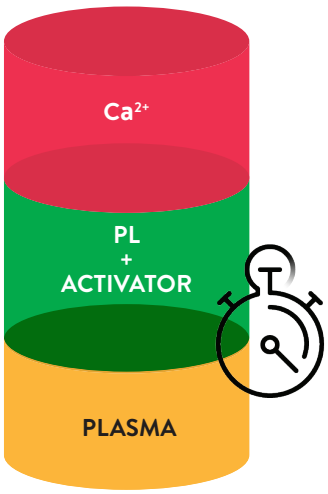


Figure 15

FIBRINOGEN ASSAY

The fibrinogen test, first described by von Clauss, is a functional assay used to measure the functional level of fibrinogen, and it is the recommended method for measuring fibrinogen.[3] The test involves adding a high concentration of bovine or human thrombin reagent to diluted (1:10) patient plasma and then recording the time to clot formation. The clotting time from the patient is compared with a standard curve that is constructed by testing known concentrations of fibrinogen with the thrombin time. The thrombin time, reported in seconds, is converted to mg/dL (or g/L) of fibrinogen **(Figure 16)**. The von Clauss technique detects both quantitative and qualitative deficiencies in fibrinogen. There are other methods for determining fibrinogen concentration that include immunologic, gravimetric, turbidimetric, and PT-derived fibrinogen. These other methods are often normal in dysfibrinogenemia (qualitative defects), where the fibrinogen molecule is abnormal.

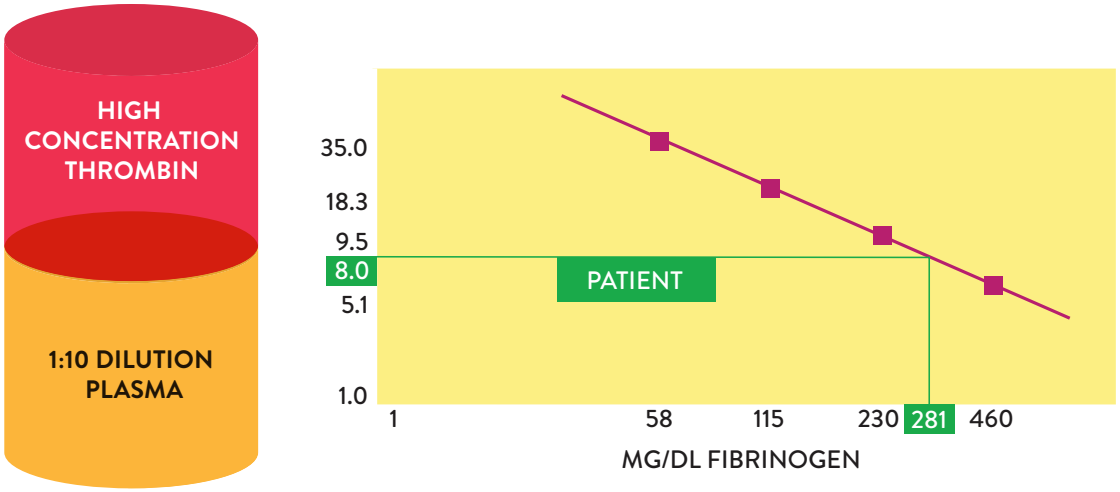


Figure 16

THROMBIN TIME

The thrombin time involves adding a low concentration of thrombin reagent (bovine or human) to undiluted patient plasma and then measuring the time to clot formation, which is reported in seconds. The TT is prolonged in hypofibrinogenemia, dysfibrinogenemia, severe liver disease, and heparin therapy. The TT is very sensitive to levels of heparin. The reptilase time is very similar to the thrombin time.

The assay uses the venom from the Bothrops atrox snake to catalyze the conversion of fibrinogen to fibrin rather than thrombin. Reptilase cleaves fibrinopeptide A from the fibrinogen molecule, whereas thrombin cleaves both fibrinopeptides A and B. The reptilase time is prolonged in hypofibrinogenemia, dysfibrinogenemia, and severe liver disease; however, it is insensitive to heparin and, therefore, normal in patients on heparin therapy. The reptilase is most often used in conjunction with the thrombin time.

D-DIMER ASSAY

The DD assay measures specific degradation products of fibrin that result from the action of plasmin on cross-linked fibrin **(Figures 17, 18)**. DD is formed when fibrin monomers are cross-linked by FXIIIa, and upon plasmin digestion, the cross-linked fibrin monomers are cleaved between the D-E-D domains, releasing the DD fragments. The DD assay uses a monoclonal antibody (attached to microlatex beads) that is specific for epitopes on the D-dimer that comes from cross-linked fibrin. The coated microlatex beads agglutinate when patient plasma containing D-dimer is added. DD is often elevated in patients with inflammation and can aid in the diagnosis of venous thromboembolism (VTE). When used in combination with additional pretest clinical probability factors, the DD has a very high negative predictive value and can be used for the exclusion of deep vein thrombosis and pulmonary embolism. Therefore, a positive DD is not specific for VTE, and a negative DD is highly unlikely for VTE.

Another assay that historically has been used to detect the presence of FDPs is the fibrin degradation product (FDP) assay. This is a semi-quantitative assay that measures circulating levels of fibrinogen degradation products, cross-linked and non-cross-linked fibrin degradation products produced by the action of plasmin. Since the DD assay is specific for the breakdown of fibrin, many institutions no longer perform the FDP assay.

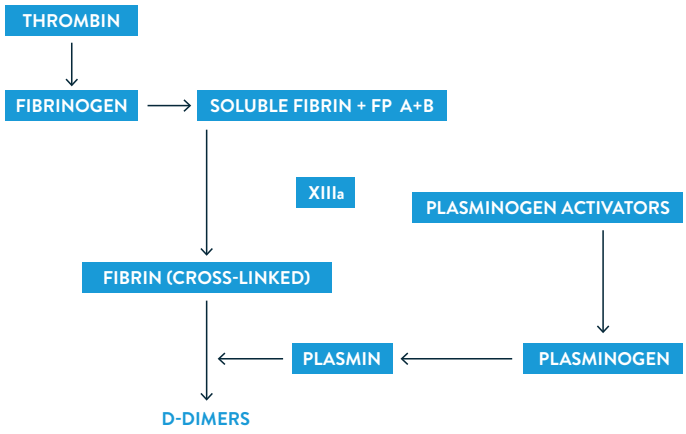


Figure 17. D-Dimer

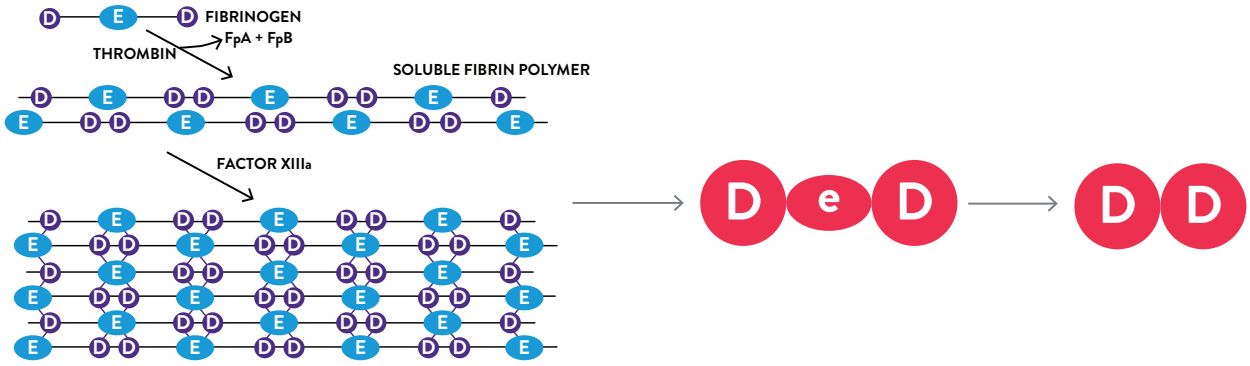

FIBRINOGEN
FpA + FpB
THROMBIN
SOLUBLE FIBRIN POLYMER
FACTOR XIIIa
D
E
D
e
D
D
D

Figure 18

QUIZ QUESTIONS

1. Which coagulation assay measures a decrease in the coagulation factors found in the extrinsic pathway?

---

2. Which coagulation assay measures a decrease in the coagulation factors found in the intrinsic pathway?

---

3. Both the PT and APTT are prolonged in FXIII deficiency. True or false?

---

4. Which of the following assays is sensitive to heparin therapy: thrombin time or reptilase time?

---

5. Which coagulation assay can be used to rule out deep vein thrombosis when used with other pretest clinical probability factors?

GLOSSARY, APPENDIX, AND REFERENCES

GLOSSARY

Activated partial thromboplastin time: Clot-based screening test that measures deficiencies of the intrinsic and common pathways. The test is also used to monitor unfractionated heparin therapy.

Activated protein C: Inhibitor of the coagulation pathway that specifically downregulates FVa and FVIIIa.

Adhesion: Property of platelets binding to the damaged endothelial surface.

Aggregation: Property of platelets binding to each other to form a platelet plug.

Alpha granules: Platelet granules that store and release a variety of hemostatic proteins.

Alpha-2 antiplasmin: Serine protease inhibitor that downregulates free-floating plasmin.

Arachidonic acid: Essential fatty acid found in the plasma membrane. It is a precursor of thromboxane, which promotes platelet aggregation.

Coagulation: Ability of blood to form a solid clot. Often used synonymously with hemostasis.

Coumadin®: A vitamin K antagonist used for the prevention of pathologic clots in patients, also known as warfarin.

Cyclooxygenase: Enzyme that converts arachidonic acid to the endoperoxides PGG2 and PGH2, also a target for aspirin therapy.

Dense granules: Platelet organelles that secrete energy substances, such as ADP and ATP.

Dense tubular system: Platelet organelle that stores calcium and thromboxane A2.

Disseminated intravascular coagulation: Excessive, uncontrolled activation of thrombin, leading to consumption of the coagulation factors, platelets and fibrinolytic proteins.

Dysfibrinogenemia: Structurally abnormal fibrinogen molecule.

Ecchymoses: Small purple or red lesions on the skin measuring larger than 3 mm in diameter. They represent bleeding into the skin from capillaries and small blood vessels.

Endoperoxides: Intermediate compounds that lead to formation of the prostaglandins.

Endothelium: Cell layer that lines the surface of all blood vessels.

Enzymes: Molecules that act as catalysts to speed up reactions.

Fibrin degradation products: Breakdown fragments resulting from the action of plasmin on fibrin.

Fibrinogen: Plasma glycoprotein produced in the liver that is a precursor of fibrin.

Fibrinolysis: Normal process whereby the fibrin clot is broken into fragments by the enzyme plasmin.

Geometric mean: Special type of average derived by multiplying numbers together and taking the nth root of the product. It is used to calculate the international normalized ratio (INR).

Glycocalyx: Amorphous, spongy exterior layer covering the plasma membrane of platelets.

Glycolipids: Conjugated lipids containing one or more covalently linked carbohydrate residues.

Glycoproteins: Proteins covalently linked to a carbohydrate residue.

Hemophilia: Group of hereditary bleeding disorders caused by a defective/absent FVIII or FIX protein.

Hemostasis: Process whereby blood is maintained in a fluid state. Often used synonymously with coagulation.

Heparin: Highly sulfated mucopolysaccharide used as an anticoagulant to treat pathologic clots.

GLOSSARY (CONTINUED)

High-molecular-weight kininogen: Contact factor protein in the intrinsic pathway of the coagulation cascade that plays a role in coagulation, fibrinolysis, and inflammation.

Hypofibrinogenemia: Decreased amount of fibrinogen.

Inhibitor: Protein antibody that interferes with the action of a coagulation factor complex.

International normalized ratio: Calculated ratio of a patient's prothrombin time result to the geometric mean of prothrombin time reference interval. It is also referred to as the INR and is used instead of the prothrombin time in patients taking coumarin drugs.

International sensitivity index: Measure of the sensitivity of the prothrombin time reagent to a warfarin-induced decrease of the vitamin K-dependent factors, also known as the ISI.

Lysosomal granules: Membrane-bound sacs in the cell that contain hydrolytic enzymes responsible for ingesting bacteria.

Mitochondria: Oval or round structures in cells that provide energy in the form of ATP.

Nitric oxide: Substance released by endothelial cells that relaxes blood vessels.

Petechiae: Small purple or red lesions on the skin measuring less than 3 mm in diameter. They represent bleeding into the skin from capillaries and small blood vessels.

Phospholipase A2: An enzyme found in the plasma membrane that converts phosphatidyl-inositol to arachidonic acid.

Plasmin: Enzyme derived from plasminogen that cleaves fibrin into fibrin degradation products, or FDPs.

Plasminogen: Inactive precursor of plasmin.

Plasminogen activator inhibitor: Protein inhibitor of tissue plasminogen activator (tPA).

Prekallikrein: Contact factor protein found in the intrinsic pathway of the coagulation cascade. Prekallikrein plays a role in coagulation, fibrinolysis and inflammation.

Primary hemostasis: First phase of coagulation that involves the interaction of platelets with the damaged endothelial layer to form the primary platelet plug.

Procoagulant: Protein-clotting factor that leads to the formation of a fibrin clot via the generation of thrombin.

Prostacyclin: Protein found in endothelial cells that is a very potent inhibitor of platelet aggregation and a very potent vessel dilator.

Prostacyclin synthase: Enzyme found in endothelial cells that converts the endoperoxides (PGG2, PGH2) to prostacyclin.

Prostaglandins: Family of unsaturated 20-carbon fatty acids that are cleaved from membrane phospholipids.

Prothrombin time: Test used to measure the activity of FII, FV, FVII, and FX in the coagulation cascade; also known as the PT. The INR is derived from the PT.

Secondary hemostasis: Series of reactions involving the coagulation clotting factors that results in the formation of a stable, insoluble fibrin clot.

Serine protease: Proteolytic enzyme that contains serine in its active site. Serine proteases result from the activation of a zymogen to an active protein.

Soluble fibrin monomer: Protein structure that results from the action of thrombin on fibrinogen. Soluble fibrin monomers form the basis for a fibrin clot.

GLOSSARY (CONTINUED)

Substrate: Substance that is acted upon by an enzyme.

Thrombin: Serine protease that converts fibrinogen to fibrin. Thrombin also activates FV, FVIII, FXI, FXIII, and platelets.

Thrombin-activatable fibrinolysis inhibitor: Serine protease inhibitor of fibrinolysis generated by thrombin.

Thrombin time: Test that measures the conversion of fibrinogen to fibrin.

Thrombomodulin: Cell surface receptor protein located on endothelial cells that converts thrombin from an anticoagulant to a procoagulant.

Thromboxane A2: Very potent platelet activator and vessel constrictor.

Tissue factor: Membrane-bound glycoprotein that is expressed on most cells. When expressed in the lumen of the blood vessel, it activates FVII to initiate the extrinsic pathway of the coagulation cascade.

Tissue factor pathway inhibitor: Naturally occurring inhibitor of tissue factor.

Tissue plasminogen activator: Serine protease found on endothelial cells that converts plasminogen to plasmin.

Vasoconstriction: Process of narrowing the blood vessel to decrease blood flow.

Vasodilation: Process of widening the blood vessel to increase blood flow.

Venous thromboembolism: Syndrome that includes deep vein thrombosis (DVT) and pulmonary embolism (PE) that results from a combination of hereditary and acquired risk factors for thrombosis.

Von Willebrand factor: Large multimeric protein that plays a role in platelet adhesion and protecting FVIII in the circulation.

Warfarin: Vitamin K antagonist used for the prevention of pathologic clots in patients, also known as Coumadin®.

Weibel-Palade body: Organelle found in endothelial cells where von Willebrand factor is stored.

Zymogen: Inactive precursor protein that is converted to an active form by an enzyme.

APPENDIX A: ANTICOAGULANT AGENTS

WARFARIN

Warfarin is an oral vitamin K antagonist that interferes with the action of the vitamin K-dependent factors (II, VII, IX, X) resulting in decreased thrombin generation. Indications include treatment of arterial and venous thrombosis, ischemic stroke in nonvalvular atrial fibrillation, and patients with mechanical heart valves. The anticoagulant activity of warfarin is monitored using the INR. The therapeutic range for most indications is an INR of 2-3, and for some patients with a mechanical heart valve, the INR may be slightly higher.

HEPARIN

Heparin is a heterogeneous mixture of glycosaminoglycans and consists of unfractionated heparin (UFH) and low molecular weight heparin (LMWH). The functional unit of the heparin consists of a unique pentasaccharide sequence that binds to antithrombin and inhibits thrombin, Xa, and the other serine proteases. Both types of heparin are indicated in patients with arterial and venous thrombosis and prevention of secondary thrombosis in patients with a prior pathologic clot. UFH ranges in size from 3,000–30,000 daltons and can be monitored with the APTT assay or a chromogenic anti-Xa assay. The accepted therapeutic range for the APTT in patients on UFH is generally 1.5–2.5 times normal. When using the chromogenic anti-Xa assay, the therapeutic range is 0.3–0.7 anti-Xa units/mL. LMWH is derived from UFH and is about 1/3 the size of UFH. As a result, it has a great anti-Xa inhibitory activity. In addition, it has a greater bioavailability (> 80%). LMWH can only be monitored using a chromogenic anti-Xa assay. The therapeutic range for LMWH is 0.5–1.1 anti-Xa units/mL for twice-daily dosing and 1–2 anti-Xa units/mL for once-daily dosing.

DIRECT ORAL ANTICOAGULANTS

The direct oral anticoagulant (DOACs), also know as target-specific oral anticoagulants (TSOACs) are a new class of oral anticoagulants that target a single-specific enzyme in the coagulation cascade rather than multiple enzymes. They consist of dabigatran, a specific inhibitor of thrombin (IIa), and rivaroxaban, apixaban, and edoxaban, specific inhibitors of factor Xa. All of these drugs were designed to replace warfarin, and have been reported to be either non-inferior or superior to warfarin in the prevention of nonvalvular atrial fibrillation and improved bleeding risks compared to warfarin. The DOACs were designed to not require monitoring in patients. However, there are a number of situations where monitoring may be necessary. All of these agents may prolong the PT/INR and/or APTT depending on the particular manufacturer's reagent used by the laboratory.

APPENDIX B: QUIZ ANSWERS

SECTION 1 OVERVIEW OF COAGULATION AND FIBRINOLYSIS

1. Coagulation refers to the ability of the blood to form a solid clot following vascular injury.
2. Blood vessels, platelets, coagulation factors

SECTION 2 PRIMARY HEMOSTASIS

1. Blood vessels (endothelium), platelets
2. Peripheral zone, structural zone, organelle zone, membranous zone
3. Cyclooxygenase
4. Adhesion, shape change, secretion, aggregation
5. Endothelial cells
6. Weibel-Palade body

SECTION 3 SECONDARY HEMOSTASIS

1. Enzymes, cofactors, substrates
2. Thrombin
3. Contact group, prothrombin group, fibrinogen group
4. FII, FVII, FIX, FX
5. Intrinsic pathway, extrinsic pathway, common pathway
6. Antithrombin, activated protein C (aPC), tissue factor pathway inhibitor (TFPI)

SECTION 4 FIBRINOLYSIS

1. Fibrinolytic
2. Plasminogen, plasmin, tissue plasminogen activator (tPA), alpha-2 antiplasmin, plasminogen activator inhibitor-1 (PAI-1), thrombin-activatable fibrinolysis inhibitor (TAFI)
3. D-dimer

SECTION 5 ROUTINE COAGULATION ASSAYS

1. PT
2. APTT
3. False
4. Thrombin time
5. D-dimer

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CORELABORATORY.ABBOTT/HEMATOLOGY

Abbott