

Education Case: Hereditary Thrombophilia With Double Heterozygous Factor V Leiden and Factor II c.*97G>A Mutations

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The following fictional case is intended as a learning tool within the Pathology Competencies for Medical Education (PCME), a set of national standards for teaching pathology. These are divided into three basic competencies: Disease Mechanisms and Processes, Organ System Pathology, and Diagnostic Medicine and Therapeutic Pathology. For additional information, and a full list of learning objectives for all three competencies, see <http://journals.sagepub.com/doi/10.1177/2374289521990788>.¹

Keywords

pathology competencies, organ system pathology, hematopathology, coagulation disorders, hemostasis, coagulation cascade, inherited thrombophilia, Factor V Leiden

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Primary Objective

HPCD2.6: Risk Factors for Thrombophilia. Give examples and discuss the pathophysiology of inherited versus acquired conditions that increase the risk of thrombophilia; *Competency 2: Organ System Pathology; Topic: Hematopathology—Platelets and Coagulation Disorders (HPCD); Learning Goal 2: Hemostasis*

Secondary Objective

Objective HPCD2.4: Proteases and the Coagulation Cascade. Describe how particular proteins that regulate the proteases to activate the clotting cascade either promote or inhibit coagulation; *Competency 2: Organ System Pathology Topic: Hematopathology—Platelets and Coagulation Disorders (HPCD) Learning Goal 2: Hemostasis*

Patient Presentation

A 32-year-old woman (G3P0) presents to her primary care provider for the evaluation of recurrent first trimester

miscarriages. She had 3 abortions in the last 4 years at 10, 9, and 12 weeks of gestation, respectively. Also, she has a history of lower limb deep vein thrombosis (DVT) at 27 years after a long flight. She has no history of pulmonary embolism, skin rash, or joint pain. The patient's menses are regular, occurring every 30 days and lasting for 5 days. She does not have any potential harmful environmental exposures. She has no known family history of chromosomal abnormalities. The previous pregnancies appeared to be progressing normally until the time of miscarriage.

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Diagnostic Findings, Part 1

Physical examination reveals an alert patient with no pain or distress. Vital signs include an oral temperature of 37.0 °C, heart rate 93 beats per minutes, respiratory rate 19 breaths/min, and blood pressure 133/86 mm Hg. Her cardiovascular and respiratory system are unremarkable. Abdominal examination reveals no tenderness or organomegaly. Pelvic examination and other systems are unremarkable.

Question/Discussion Points, Part 1

What Is the Definition of Recurrent Miscarriage?

Recurrent miscarriage is defined as 3 or more consecutive miscarriages. Miscarriage is defined as any spontaneous loss of a pregnancy from the time of conception until 24 weeks of gestation, which is the age of fetal viability.

What Are the Causes of Recurrent Early Trimester Miscarriage?

The etiology of recurrent miscarriage can be classified into genetic, anatomical, infectious, endocrine, immune, thrombophilic, or unexplained.² Genetic factors are the most common cause of early spontaneous miscarriage and include parental chromosomal rearrangements and fetal aneuploidy (having an abnormal number of chromosomes).³ Endocrine factors include poorly controlled diabetes mellitus and significant thyroid disease.⁴ Anatomical factors refer to congenital uterine malformations (such as septate uterus) and acquired uterine lesions (such as fibroids [leiomyomata]) that impede normal implantation by deforming the endometrial cavity.^{5,6} Immune factors are mainly represented by antiphospholipid syndrome, in which the body generates autoantibodies (mainly anticardiolipin antibodies) that induce placental injury by producing inflammatory and prothrombotic changes that affect endothelial and trophoblasts cells.⁷ Inherited thrombophilic disorders can cause recurrent fetal loss by inducing thrombosis of the uteroplacental circulation.³ Infective agents such as *Chlamydia*, *Listeria*, *Mycoplasma*, *Toxoplasma*, *Ureaplasma*, rubella, cytomegalovirus, and herpes virus have been linked to spontaneous miscarriage.⁸

What Are the Diagnostic Tests That Should Be Ordered for This Patient to Investigate the Etiology of Her Recurrent Miscarriage?

A complete blood count is usually done to screen for anemia and other hematologic disorders. Sonohysterography or hysterosalpingography exclude uterine structural lesions. Fetal tissue and parental karyotyping are usually done to exclude structural chromosomal abnormalities (fetal aneuploidy or parental translocation). Histopathological examination of the aborted products of conception can also be helpful in excluding infections, molar pregnancy, or implantation site abnormality. Fetal karyotyping and examination of the aborted products of

conception require fetal (not parental) tissue, which requires them to be done at the time of miscarriage. Often, these 2 important investigations are not sent at the time of patient presentation due to the lack of clinical suspicion of an underlying abnormality. Laboratory evaluation for antiphospholipid syndrome includes anticardiolipin antibody, anti- β 2-glycoprotein, and lupus anticoagulant testing.

Diagnostic Findings, Part 2

The patient's complete blood count shows a white blood cell count of 5,500/ μ L with normal differential count, a hemoglobin level of 12.3 g/dL, and a platelet count of 300,000/ μ L. Workup for antiphospholipid syndrome is negative. Additional review of the patient's records reveals a family history significant for a mother with DVT at the age of 39 years, a sister with a mutation of factor II, and a maternal cousin with a mutation of factor V.

Question/Discussion Points, Part 2

Given This Patient's Family History, What Is the Primary Clinical Consideration?

The patient's family history of first- and second-degree relative with DVT and mutations of factor II and V raises the clinical concern of an underlying thrombophilic disorder.

What Is the Physiologic Role of the Coagulation Pathway?

The coagulation pathway (Figure 1) is a series of reactions that are carried out by many proteins and glycoproteins (known as coagulation factors) that act by cleaving/activating downstream proteins ultimately leading to fibrin formation and cross-linking, the end product of secondary hemostasis.⁹ In the coagulation cascade, each inactive enzyme precursor (zymogen) is activated to an active form that then catalyzes the next reaction in the cascade. Coagulation factors are generally indicated by Roman numerals (from I to XIII), followed by a lowercase a when active.

What Is the Definition and the Clinical Spectrum of Thrombophilia?

Thrombophilia can be defined as an increase tendency for arterial or venous thrombosis.¹⁰ Arterial thrombosis usually presents as myocardial infarction, cerebrovascular accidents, or peripheral arterial disease. Less frequently, it can involve renal, mesenteric, or retinal arteries.^{11,12} Venous thrombosis presents as venous thromboembolism (VTE). Deep venous thrombosis of the lower limbs is the most common form of VTE; however, thrombosis can occur in any part of the venous system (such as upper extremities, splanchnic veins, and cerebral sinuses). The incidence of VTE is approximately 1 to 1.5 per 1000 persons/year with an individual's absolute lifetime risk of 11%.¹³⁻¹⁵ Thrombophilic patients can also present with recurrent pregnancy loss, and rarely arterial thromboembolism.

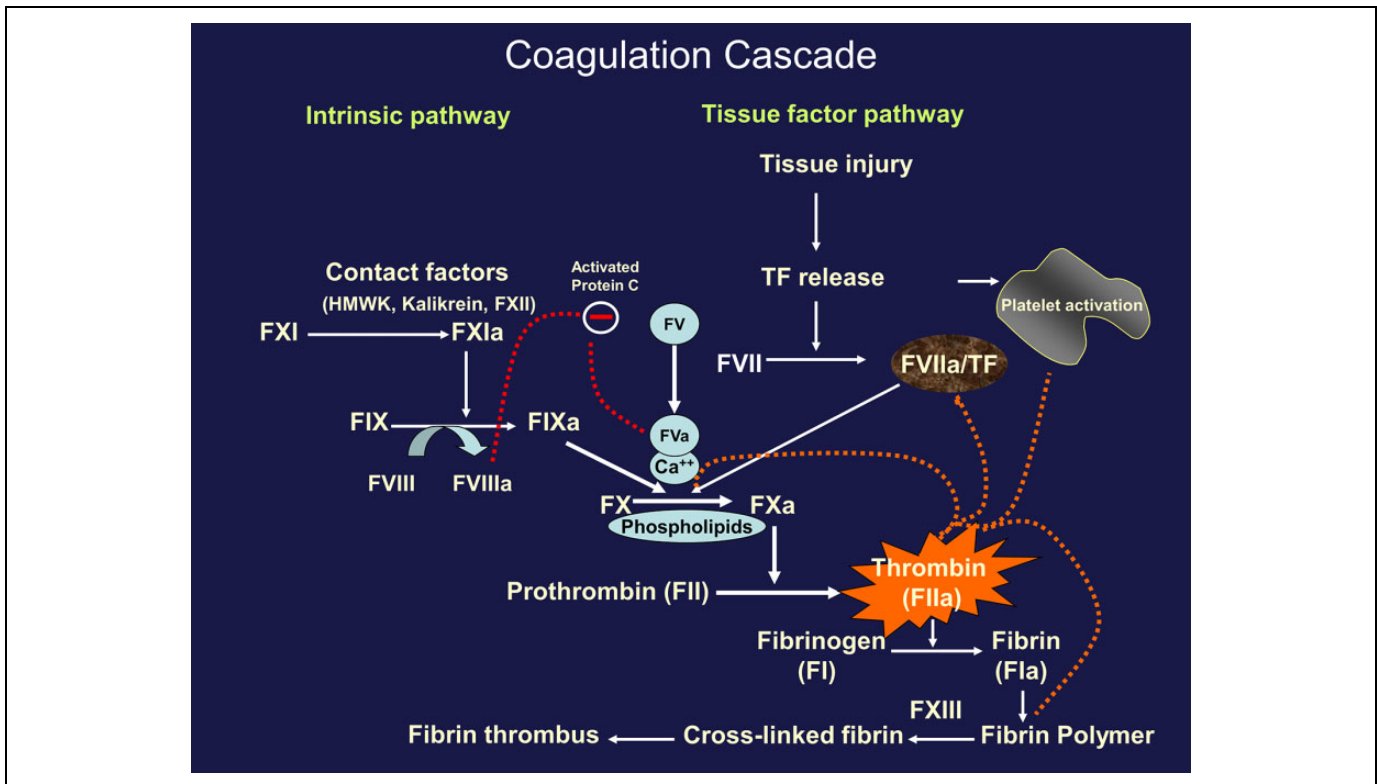


Figure 1. The 2 arms of the classic coagulation cascade, the tissue factor pathway (also known as the extrinsic pathway) and the contact activation pathway (also known as the intrinsic pathway), each consisting of different factors that both activate the common pathway (factor X, thrombin, and fibrin), leading to cross-linking of fibrin and formation of the hemostatic plug. It is worth mentioning that this classic cascade represents mostly in vitro events that is important for laboratory testing (PT, aPTT, and TT) and does not entirely represent the in vivo (physiological) coagulation cascade.

Pulmonary embolism is the main life-threatening complication of VTE.¹⁶

What Is the Etiology of Thrombophilia?

Thrombosis results from an imbalance between endogenous anti- and pro-thrombotic factors in the bloodstream.¹⁷ There are 3 factors, known as the Virchow's triad, that predispose individuals to thrombosis: vascular endothelial injury, blood stasis, and hypercoagulability.^{18,19} Endothelial damage includes atherosclerosis and mechanical injury that is caused by trauma, surgical manipulation, or catheter insertion. Blood stasis refers to impairment of the physiologic blood flow through the vascular (particularly venous) system. This can be caused by pregnancy or by prolonged immobility such as in people traveling long trips.^{18,19} Hypercoagulability is multifactorial and can be influenced by both hereditary and acquired factors. Hereditary thrombophilia is less common and is associated with certain mutations in proteins related to the coagulation cascade.^{17,20} Acquired hypercoagulability is much more common and can result from medications (eg, estrogen-containing contraceptives, hormone replacement therapies, tamoxifen, or raloxifene), malignancy, inflammatory state induced by pregnancy, trauma, surgery, infection and chronic

inflammatory conditions (eg, morbid obesity, autoimmune diseases, heavy smoking), antiphospholipid syndrome, and heparin-induced thrombocytopenia and thrombosis.^{17,21-24} Increased age is also an important contributor to thrombosis due to increased production of prothrombotic coagulation factors.^{20,25}

How Can Genetic Mutations Involving Different Factors in the Coagulation Cascade Have Different Physiologic Effects?

Mutations that affect the serum level, stability, or activity of individual clotting factors (eg, factor VIII, factor IX, factor XI, and von Willebrand factor) usually result in bleeding tendency. On the other hand, mutations involving inhibitors of coagulation (protein C, protein S, and antithrombin III) result in hypercoagulability (thrombophilia) with an increased risk of VTE.⁹

What Are the Most Common Genetic Mutations That Contribute to Thrombophilia?

Factor V Leiden (FVL) and factor II c.*97G>A mutations are the most common genetic predisposition factors that contribute to thrombophilia. Other rare genetic defects such

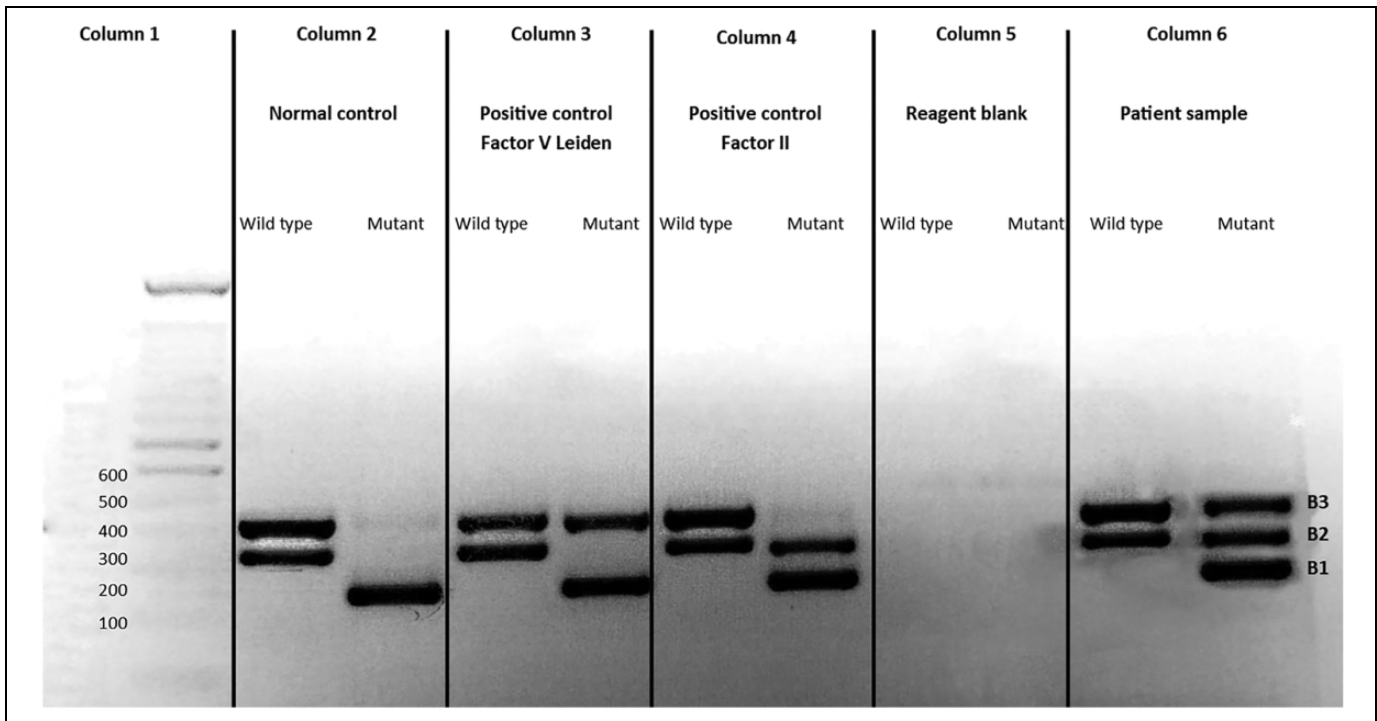


Figure 2. Each assay includes a size ladder (column 1) and 5 other columns (columns 2-6) in which different samples are added to reaction tubes. The size ladder represents the size of the migrating DNA fragments. Each one of the columns has 2 subcolumns in which the reactions occur: the wild type (wt) and the mutant types. In each column, the reagents are reacted with the 3 added primers generating different bands (B1, B2, and B3). The first band (B1) at 250 bp represents an amplification control primer. The second band (B2) at 340 bp represents the factor II mutation-specific DNA primer. The third band (B3) at 432 bp represents the factor V Leiden-specific DNA primer. The presence of migrating DNA fragments confirms the presence of the DNA sequence of interest. In column 2, a normal control (sample from healthy patient with no FVL or factor II mutations) is added to the reaction tube. Note the presence of 2 bands (B2 and B3) in the wt tube and the absence of those bands in the mutant reaction tube. The absence of both the factor V–specific (432 bp, B3) and the factor II–specific (340 bp, B2) bands in the wt reaction tube A, or the absence of the amplification control (250 bp, B1) in the mutant reaction, suggests either lack of template or polymerase chain reaction (PCR) inhibition and invalidates the run. In tubes 2 to 4, different controls are added as follows: normal control (column 2), heterozygous positive control samples for FVL (column 3), and heterozygous positive factor II (column 4). Column 5 include the test reagents with no added DNA sample, therefore, no bands are seen as there is no PCR reaction. Column 6 is the actual patient sample; note the presence of all 3 band (B1, B2, and B3) in the mutant reaction tube and absence of those bands in the wt tube.

as antithrombin, protein C, protein S, or factor XIII can also be contributing factors.^{16,26} Factor V Leiden is a pathogenic variant protein that results from a point mutation in the factor V gene. The c.1601G>A DNA sequence change leads to protein sequence change p.Arg534Gln or R534Q. This variant was previously designated as c.1691G>A, p.Arg506Gln, or R506Q.¹⁶ Prothrombin c.*97G>A mutation is the second most common mutation associated with inherited thrombophilia after FVL. The factor II mutation is designated c.*97G>A at the coding DNA level or r.1997G>A at the RNA level. Historically it was reported as 20210G>A.

Diagnostic Findings, Part 3

Factor II and factor V mutation testing (Figure 2) shows a heterozygous pattern for both the FVL mutation and the c.*97G>A mutation of the factor II (prothrombin) gene.

Question/Discussion Points, Part 3

What Are the Functional Roles of Factor II and Factor V in the Coagulation Cascade?

Activated factor V is an essential cofactor to factor Xa in converting prothrombin (factor II) to thrombin (activated factor II) which in turn catalyzes the crosslinking of fibrin polymers required for the formation of a hemostatic plug (Figure 1).⁹ Thrombin also has an essential role in activating other coagulation factors that both augment and inhibit the formation of more thrombin, such as activated protein C (APC).⁹

How Does Factor V Leiden Lead to Thrombophilia?

Normally, APC, in the presence of protein S, functions as a natural anticoagulant by cleaving/inactivating activated factor Va and factor VIIIa. Activated protein C cleaves factor V initially at position p.Arg534 followed by 2 other cleavage sites (p.Arg334 and p.Arg707).^{27,28} The FVL inhibits APC cleavage

Table 1. Risk of Venous Thrombosis Compared With Healthy Individuals Not Having Factor II or Factor V Variants.

Mutation	Fold increase in risk of venous thrombosis compared with individuals without this variant
FVL heterozygote	4- to 8-fold increase ^{39,40}
FVL homozygote	80-fold increase ⁴¹
Factor II c.*97G>A heterozygote	2- to 4-fold increase ⁴¹
Factor II c.*97G>A homozygote	Inconclusive due to the relatively few number of individuals with this genotype ⁴²
FVL and factor II c.*97G>A double heterozygote	20-fold increase ⁴³

Abbreviation: FVL, Factor V Leiden.

by eliminating the first, and the most essential, cleavage site (p.Arg534). This results in reduction of inactivation of factor V and longer persistence in circulation, leading to delayed attenuation, and more generation of thrombin, resulting in a hypercoagulable state (thrombophilia). Factor V Leiden accounts for at least 90% to ~95% of cases with APC resistance.²⁹⁻³²

How Does Factor II Mutation c.*97G>A Lead to Thrombophilia?

In the normal coagulation cascade, thrombin catalyzes the conversion of fibrinogen to fibrin, which is the building block of the hemostatic plug. Thrombin also activates factor V, factor VIII, factor XIII, and platelets. The c.*97G>A is a gain-of-function mutation variant that occurs in the 3' untranslated region of the factor II gene. The substitution of guanine to adenine upregulates processing efficiency of the 3' end of the precursor messenger RNA (pre-mRNA). This results in an increased accumulation and stabilization of the pre-mRNA, leading to more protein generation and a higher plasma protein level.^{33,34} Prothrombin is elevated by 30% in heterozygous individuals and 70% in homozygous individuals.^{35,36} An elevated prothrombin level is believed to be the underlying mechanism leading to increased risk of thrombosis.^{35,37}

What Is the Mode of Inheritance for Factor V Leiden and Factor II c.*97G>A Mutations?

Both mutations exhibit a codominant trait in that both heterozygotes and homozygotes are at an increased risk of VTE, with a greater risk in homozygotes, especially for factor V Leiden.¹⁶

How Can Mutations in Factor II and Factor V Be Tested?

Many laboratory-developed tests and US Food and Drug Administration–approved testing platforms are now being used by clinical laboratories.¹⁶ Detection of these variants can be done using many methods such as polymerase chain reaction (PCR)–restriction fragment length polymorphism, allele-specific PCR, melting curve analysis, flap endonuclease + Fluorescence Resonance Energy Transfer, Taqman real-time PCR, and fluorescent probe-based allelic discrimination.¹⁶ Sanger sequencing is conventionally used as the “gold standard” for small nucleotide changes, and it may be useful for clinical laboratories to use this technique to establish the controls during the validation stage of the assays. Routine use

of Sanger sequencing is not necessary and not common due to cost, turnaround time, as well as limited capacity for multiplexing.¹⁶

What Is Polymerase Chain Reaction With Amplification-Refractory Mutation System?

Polymerase chain reaction–Amplification-refractory mutation system (ARMS) is a simple method for detecting any mutation involving single base changes or small deletions. Amplification-refractory mutation system is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. After DNA extraction from the patient's specimen, amplification using 2 allele-specific or amplification refractory mutation system reactions is used to detect both wild and the FVL mutation and the factor II mutation (c.*97 G>A): one with primers to the normal sequences and one with primers to the variant sequences. Another set of primers specific for the F9 gene are added to confirm the addition of the patient sample. Polymerase chain reaction products are electrophoresed through 2% agarose, and the ethidium stained gel is visualized and photographed (Figure 2). Following an ARMS reaction, the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele. This provides simultaneous detection of factor V and factor II mutations and permits the identification of normal, heterozygous, or homozygous variant genotypes.³⁸

How Much Does the Risk of VTE Increase in Patients With Factor V Leiden and Factor II Mutations?

The increased risk of VTE (Table 1³⁹⁻⁴³) depends on the type of mutant variant (FVL vs. factor II c.*97G>A) and the zygosity status (homozygous vs. heterozygous). When these mutations are present in women taking oral contraceptives, the risk for VTE and particularly cerebrovascular embolism are dramatically elevated (35- to 150-fold, depending upon the mutation and zygosity).¹⁶

Which Patients Should Be Tested for Mutations in Factor II and Factor V?

Population-based screening in otherwise healthy individuals is not recommended. Testing for these mutations is appropriate

for persons at the time of first unprovoked VTE (particularly if younger than 50 years), patients with recurrent VTE or VTE at an unusual site (sites other than upper or lower limbs), patients with history of VTE in more than one family member or in a first-degree relative at a young age, and finally in patients with low APC resistance activity.¹⁶ Our patient fits the criteria for testing because she has a personal history of VTE at a young age (27 years old) as well as a first-degree relative with VTE at a young age (mother with VTE at age 39).

How Should This Patient Be Managed?

In addition to her recurrent first trimester miscarriages, this patient has a previous episode of lower limb DVT that was provoked by a long flight. As she is not pregnant at this time and her previous DVT was provoked by a transient risk factor, she does not require long-term anticoagulation therapy.⁴⁴ However, if she becomes pregnant, low-molecular-weight heparin should be started and continued throughout pregnancy and for 6 weeks postpartum. Anticoagulation would decrease her risk of developing VTE during pregnancy, however, it is still unclear if anticoagulation will prevent fetal loss in patients with recurrent miscarriages. She should also maintain a healthy lifestyle (not smoking, maintaining physical activity) and avoid estrogen-containing contraceptives.

Teaching Points

- Recurrent miscarriage is defined as 3 or more consecutive miscarriages of less than 24 weeks of gestation.
- The etiology of recurrent miscarriage can be genetic, anatomical, infectious, endocrine, immune, thrombophilic, or unexplained.
- Thrombophilia is a multifactorial condition that manifests clinically as venous or arterial thromboembolism. Pulmonary embolism is the main life-threatening complications of thrombophilia.
- Thrombophilia can be hereditary or acquired. Acquired thrombophilia is more common.
- Acquired thrombophilia has a poorly understood etiology with many associated risk factors: smoking, older age, presence of malignant neoplasms, and history of a period of prolonged immobilization. Additional risks for women include pregnancy, use of estrogen containing contraceptives or replacement therapy, and tamoxifen and raloxifene treatments.
- Hereditary thrombophilia is associated with certain mutations in proteins related to the coagulation cascade such as Factor V, prothrombin, protein C, protein S, and antithrombin-III.
- The coagulation pathway is a cascade of reactions that are carried out by coagulation factors that act by activating downstream proteins, ultimately leading to formation of the fibrin necessary for clot formation.
- Factor V Leiden and factor II c.*97G>A mutations are the most common genetic factors predisposing

individuals to thrombophilia. Other mutations are present but are rare, therefore these 2 variants are the only variants tested routinely by clinical laboratories.

- Activated protein C normally cleaves activated factor V, leading to its inhibition. Factor V Leiden eliminates the APC cleavage site, therefore abolishing its inhibitory effect, which increases the risk of thrombosis.
- Factor II c.*97G>A mutation upregulates the processing efficiency of the pre-mRNA, which results in a higher plasma protein level, increasing the risk of thrombosis.
- The increased risk of thrombosis depends on the type and the zygosity of the pathogenic variant. From individuals who have a pathogenic variant, women taking oral contraceptives have a dramatically elevated risk (up 150-fold increase, depending upon the mutation and zygosity). Treatment with anticoagulation depends on the individual risk, risk factors, and previous episodes of thromboembolism.


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References

1. Knollmann-Ritschel BEC, Regula DP, Borowitz MJ, Conran R, Prystowsky MB. Pathology competencies for medical education and educational cases. *Acad Pathol.* 2017;4. doi:10.1177/2374289517715040
2. Rai R, Regan L. Recurrent miscarriage. *Lancet.* 2006;368:601-611.
3. Garrido-Gimenez C, Alijotas-Reig J. Recurrent miscarriage: causes, evaluation and management. *Postgrad Med J.* 2015;91:151-162.
4. Arredondo F, Noble LS. Endocrinology of recurrent pregnancy loss. *Semin Reprod Med.* 2006;24:33-39.
5. Casini ML, Rossi F, Agostini R, Unfer V. Effects of the position of fibroids on fertility. *Gynecol Endocrinol.* 2006;22:106-109.
6. Sugiura-Ogasawara M, Ozaki Y, Katano K, Suzumori N, Mizutani E. Uterine anomaly and recurrent pregnancy loss. *Semin Reprod Med.* 2011;29:514-521.
7. Coulam CB, Faulk WP, McIntyre JA. Immunotherapy for recurrent spontaneous abortion and its analogies to treatment for cancer. *Am J Reprod Immunol.* 1991;25:114-119.
8. Nigro G, Mazzocco M, Mattia E, Di Renzo GC, Carta G, Anceschi MM. Role of the infections in recurrent spontaneous abortion. *J Matern Fetal Neonatal Med.* 2011;24:983-989.
9. Dahlback B. Blood coagulation. *Lancet.* 2000;355:1627-1632.
10. Moll S. Thrombophilia: clinical-practical aspects. *J Thromb Thrombolysis.* 2015;39:367-378.

11. Lowe GD. Arterial disease and venous thrombosis: are they related, and if so, what should we do about it? *J Thromb Haemost.* 2006;4:1882-1885.
12. Insull W Jr. The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. *Am J Med.* 2009;122:S3-s14.
13. Silverstein MD, Heit JA, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ III. Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study. *Arch Intern Med.* 1998;158:585-593.
14. White RH. The epidemiology of venous thromboembolism. *Circulation.* 2003;107:14-8.
15. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. *J Thromb Haemost.* 2007;5:692-699.
16. Zhang S, Taylor AK, Huang X, et al. Venous thromboembolism laboratory testing (factor V Leiden and factor II c.*97G>A), 2018 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG). *Genet Med.* 2018;20:1489-1498.
17. Armstrong EM, Bellone JM, Hornsby LB, Treadway S, Phillippe HM. Acquired thrombophilia. *J Pharm Pract.* 2014;27:234-242.
18. Lurie JM, Png CYM, Subramaniam S, et al. Virchow's triad in "silent" deep vein thrombosis. *J Vasc Surg Venous Lymphat Disord.* 2019;7:640-645.
19. Stone J, Hangge P, Albadawi H, et al. Deep vein thrombosis: pathogenesis, diagnosis, and medical management. *Cardiovasc Diagn Ther.* 2017;7(suppl 3):S276-s284.
20. Lowe GD. Venous and arterial thrombosis: epidemiology and risk factors at various ages. *Maturitas.* 2004;47:259-263.
21. Bauduer F, Lacombe D. Factor V Leiden, prothrombin 20210A, methylenetetrahydrofolate reductase 677 T, and population genetics. *Mol Genet Metab.* 2005;86:91-99.
22. Jick H, Derby LE, Myers MW, Vasilakis C, Newton KM. Risk of hospital admission for idiopathic venous thromboembolism among users of postmenopausal oestrogens. *Lancet.* 1996;348:981-983.
23. Varas-Lorenzo C, Garcia-Rodriguez LA, Cattaruzzi C, Troncon MG, Agostinis L, Perez-Gutthann S. Hormone replacement therapy and the risk of hospitalization for venous thromboembolism: a population-based study in southern Europe. *Am J Epidemiol.* 1998;147:387-390.
24. Heit JA, Spencer FA, White RH. The epidemiology of venous thromboembolism. *J Thromb Thrombolysis.* 2016;41:3-14.
25. Favalaro EJ, Franchini M, Lippi G. Aging hemostasis: changes to laboratory markers of hemostasis as we age—a narrative review. *Semin Thromb Hemost.* 2014;40:621-633.
26. Lijfering WM, Rosendaal FR, Cannegieter SC. Risk factors for venous thrombosis—current understanding from an epidemiological point of view. *Br J Haematol.* 2010;149:824-833.
27. Heeb MJ, Kojima Y, Greengard JS, Griffin JH. Activated protein C resistance: molecular mechanisms based on studies using purified Gln506-factor V. *Blood.* 1995;85:3405-3411.
28. Kalafatis M, Bertina RM, Rand MD, Mann KG. Characterization of the molecular defect in factor VR506Q. *J Biol Chem.* 1995;270:4053-4057.
29. de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. *Blood.* 1999;93:1271-1276.
30. Rodeghiero F, Tosetto A. Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. *Ann Intern Med.* 1999;130:643-650.
31. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature.* 1994;369:64-67.
32. Voorberg J, Roelse J, Koopman R, et al. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. *Lancet.* 1994;343:1535-1536.
33. Gehring NH, Frede U, Neu-Yilik G, et al. Increased efficiency of mRNA 3' end formation: a new genetic mechanism contributing to hereditary thrombophilia. *Nat Genet.* 2001;28:389-392.
34. Danckwardt S, Gehring NH, Neu-Yilik G, et al. The prothrombin 3'end formation signal reveals a unique architecture that is sensitive to thrombophilic gain-of-function mutations. *Blood.* 2004;104:428-435.
35. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood.* 1996;88:3698-3703.
36. Soria JM, Almasy L, Souto JC, et al. Linkage analysis demonstrates that the prothrombin G20210A mutation jointly influences plasma prothrombin levels and risk of thrombosis. *Blood.* 2000;95:2780-2785.
37. Makris M, Preston FE, Beauchamp NJ, et al. Co-inheritance of the 20210A allele of the prothrombin gene increases the risk of thrombosis in subjects with familial thrombophilia. *Thromb Haemost.* 1997;78:1426-1429.
38. Little S. Amplification-refractory mutation system (ARMS) analysis of point mutations. *Curr Protoc Hum Genet.* 2001;Chapter 9:Unit 9.8.
39. Vandenbroucke JP, Koster T, Briet E, Reitsma PH, Bertina RM, Rosendaal FR. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation. *Lancet.* 1994;344:1453-1457.
40. Middeldorp S, Henkens CM, Koopman MM, et al. The incidence of venous thromboembolism in family members of patients with factor V Leiden mutation and venous thrombosis. *Ann Intern Med.* 1998;128:15-20.
41. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood.* 1995;85:1504-1508.
42. Reich LM, Bower M, Key NS. Role of the geneticist in testing and counseling for inherited thrombophilia. *Genet Med.* 2003;5:133-143.
43. Tosetto A, Rodeghiero F, Martinelli I, et al. Additional genetic risk factors for venous thromboembolism in carriers of the factor V Leiden mutation. *Br J Haematol.* 1998;103:871-876.
44. Ortel TL, Neumann I, Ageno W, et al. American Society of Hematology 2020 guidelines for management of venous thromboembolism: treatment of deep vein thrombosis and pulmonary embolism. *Blood Adv.* 2020;4:4693-4738.