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Venous thromboembolism laboratory testing (factor V Leiden and factor II c.*97G>A), 2025 revision: A technical standard of the American College of Medical Genetics and Genomics (ACMG)

Benjamin E. Kang[1], Shulin Zhang[2], Harry Lesmana[3,4], Jeffrey Dungan[5], Kara Reynolds[6], Saurav Guha[7], Hunter Best[8,9]; on behalf of the ACMG Laboratory Quality Assurance Committee[10,*]

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ABSTRACT

Venous thromboembolism (VTE) occurs when a blood clot forms in a vein. The etiology of VTE is multifactorial, including both environmental and genetic factors. Among the genetic factors, factor V Leiden and factor II c.*97G>A (formerly referred to as prothrombin 20210G>A) are the 2 most common genetic variants associated with VTE. Testing for these variants is one of the most common referrals in clinical genetics laboratories. Although the methodologies for testing these 2 variants are relatively straightforward, the clinical implementation can be complicated regarding test indications, risk assessment for occurrence, and recurrence of VTE and related genetic counseling. This document provides an overview of VTE, information about the variants and their influence on risk, considerations before initiating genetic testing, and the clinical and analytical sensitivity and specificity of the tests. Key information that should be included in the laboratory report is also provided. This document supersedes the Technical Standards and Guidelines for Venous Thromboembolism Laboratory Testing originally published in 2005 and revised in 2018. It is designed for genetic testing professionals familiar with the disease and the analysis methods.

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*Correspondence: ACMG. *Email address:* documents@acmg.net
Affiliations are at the end of the document.

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Introduction

This document updates the technical standards for Venous Thromboembolism Laboratory Testing (factor V Leiden [FVL] and factor II c.*97G>A), originally published in 2005[1] and revised in 2018.[2] The current revision includes updates to the population frequency of the FVL variant and factor II c.*97G>A based on the newly available population data, as well as updates to the databases of population frequency in the background section. It also revises the recommendation for testing of pregnant individuals and persons with recurrent adverse pregnant outcomes in the testing indications section and adds a section to discuss the clinical utility of methylenetetrahydrofolate reductase (*MTHFR*) testing for venous thromboembolism (VTE).

Background

Gene symbol/chromosome locus, OMIM entry and HGVS nomenclature

The *F5* (coagulation factor V) gene (HGNC:3542) is located on chromosome 1q24.2 and the OMIM number for the *F5* gene is 612309. The Human Genome Variation Society (HGVS) nomenclature of FVL is NC_000001.11:g.169549811C>T, NM_000130.5:c.1601G>A and NP_000121.2:p.Arg534Gln. This variant was previously designated as G1691A or Arg506Gln and is referred to as FVL. The *F2* (coagulation factor II, prothrombin) gene (HGNC:3535) is located on chromosome 11p11.2 and the OMIM number for the *F2* gene is 176930. The HGVS nomenclature of factor II c.*97G>A is NC_000011.10:g.46739505G>A, NM_000506.5:c.*97G>A. The genomic information of FVL and factor II c.*97G>A variants is summarized in Table 1. This variant was previously designated as G20210A or 20210G>A and is commonly referred to as factor II or prothrombin G20210A or 20210G>A.

Brief clinical description

Thrombosis is one of the most common causes of morbidity and mortality in the United States. The incidence of VTE is approximately 1 to 1.5 per 1000 person years, and an individual's absolute lifetime risk of VTE is approximately 11%.[3-5] The risk of VTE is age related. Before age 40, the risk is approximately 1 in 10,000 persons per year, and it increases to 1 in 100 persons per year after age 75.[6] Consequently, the economic burden is also significant; the clinical management of VTE costs the health care system an estimated $1.5 billion each year in the United States.[7,8] The recurrence risk is estimated to be approximately 20% within 5 years and 30% within 10 years after the first incidence.[9,10] Although the most frequent VTE event is deep vein thrombosis (DVT) in the legs, thrombosis can also occur in the veins of other sites, such as the upper extremities, pelvis, abdomen, cerebral venous sinuses, etc. Pulmonary embolism is the main life-threatening complication of DVT. It is estimated that one-third of VTE manifests as pulmonary embolism and two-thirds as other DVTs.[5] The etiology of VTE is multifactorial. Both environmental factors and genetic predispositions influence the hemostasis of the coagulation system. Environmental factors include smoking, male sex, older age, malignant neoplasm, prolonged immobilization, and surgery.[11] For individuals considering pregnancy and those at risk of breast cancer, additional risk factors include pregnancy, postpartum period, use of oral contraceptives (OCs), estrogen replacement therapy, tamoxifen, and raloxifene treatment.[12,13]

Although FVL and factor II c.*97G>A are the most common genetic predisposition factors, genetic defects in antithrombin III, protein C, protein S, or factor XIII also contribute to VTE.[14] A recent model which determined risks with thrombophilia factors determined that antithrombin III, protein C, and protein S have a more quantifiable association with VTE.[15] In addition, other single-nucleotide variants associated with VTE have also been identified.[16] Known genetic factors are present in about 25% of the screening population without a family history of VTE and up to 63% of familial cases.[17] Genetic predisposition factors often interact with various environmental factors to provoke thrombosis. However, approximately 50% of first-time VTE cases are apparently unprovoked.[18] Genetic counseling about genetic and nongenetic aspects of the risk is important.[19] Because of the coexistence of multiple risk factors for each individual, it is often challenging to integrate these risk factors to make a definitive prediction of occurrence or recurrence.

Arterial thrombosis is mainly caused by atherosclerosis. Stroke and coronary heart disease are the main manifestations of arterial thrombosis. Although arterial and venous thrombosis are traditionally viewed as distinct conditions with different pathophysiology and treatments, they share some common risk factors, such as aging, immobility, and obesity.[20-22]

Due in part to the high incidence of VTE, genetic testing for inherited thrombophilia is one of the most common tests in clinical genetics laboratories. FVL and factor II c.*97G>A are among the most commonly requested tests by clinicians. Testing for other inherited thrombophilias (antithrombin III, protein C, and protein S deficiency) is usually achieved by measuring protein function or antigen levels. Numerous variants have been reported in the antithrombin III (*SERPINC1*), protein C (*PROC*), and protein S (*PROS1*) genes. Overall, variants in the protein C, protein S, or antithrombin III genes account for approximately 5% to 10% of patients with thrombosis.[11,23-25]

Table 1 Genomic information of factor V Leiden and factor II c.*97G>A variants

Gene Information	Factor V Leiden	Factor II c.*97G>A
Gene name	Coagulation factor V (proaccelerin, labile factor)	Coagulation factor II (prothrombin)
Gene symbol	*F5*	*F2*
OMIM entry	612309	176930
Chromosomal location	1q24.2	11p11.2
Genomic coordinates	Chr.1:169,519,049 (GRCh37) Chr.1:169,549,811 (GRCh38)	Chr.11:46,761,055 (GRCh37) Chr.11:46,739,505 (GRCh38)
Nomenclature	NC_000001.11:g.169549811C>T NM_000130.5:c.1601G>A[a] NP_000121.2:p.Arg534Gln	NC_000011.10:g.46739505G>A NM_000506.5:c.*97G>A[b]
Clinical significance	Pathogenic, Risk factor	Pathogenic, Risk factor

[a]This variant was previously designated as G1691A or Arg506Gln and is referred to as factor V Leiden or FVL.
[b]This variant was previously designated as G20210A or 20210G>A and is commonly referred to as factor II or prothrombin G20210A or 20210G>A.

The pathophysiology of FVL and factor II c.*97G>A

In the normal coagulation system, activated protein C (APC) functions as a natural anticoagulant by inactivating coagulant factor Va and factor VIIIa in the presence of protein S. The initial APC cleavage at position Arg534 of factor V is required for the optimal exposure of factor V to subsequent cleavage. Subsequently, a rapid inactivation of factor V occurs by the APC cleavage at positions Arg334 and Arg707 (previously referred to as positions Arg306 and Arg679, respectively).[26,27] Alteration of the first APC cleavage site at Arg534 results in FVL that persists longer in the circulation compared with wild-type factor V, leading to more thrombin generation. FVL is found in 90% to 95% of all patients with APC resistance.[28,29]

Prothrombin (factor II) is a vitamin K-dependent protein. It is converted to thrombin in the presence of factor Va, factor Xa, calcium ions, and phospholipids. Thrombin has not only the function of catalyzing the conversion of fibrinogen to fibrin, the building block of a hemostatic plug, but it also activates platelets, factor V, factor VIII, and factor XIII.[30] The c.*97G>A variant is located in the 3′ untranslated region of the *F2* gene. This variant is associated with an elevated prothrombin level of 30% above normal in heterozygous individuals and 70% above normal in homozygous individuals.[31,32] The elevated prothrombin level is believed to play a key role in the pathogenesis of thrombosis.[31,33] Molecularly, the wild-type guanine at the cleavage site is the least efficient nucleotide to support 3′ end processing.[34] Factor II c.*97G>A variant up-regulates the 3′ end processing efficiency of the pre-mRNA, resulting in an increased pre-mRNA accumulation and elevated protein synthesis.[34,35] This variant is, therefore, a gain-of-function variant.

Mode of inheritance, population genetics, occurrence risk, and recurrent risk

Both FVL and factor II c.*97G>A exhibit a semidominant trait in that both heterozygotes and homozygotes are at an increased risk of VTE, with a greater risk in homozygotes, especially for FVL.

FVL

In the United States, FVL heterozygosity is present in 5.1%, 2.0%, and 1.2% of Europeans, Hispanics, and African Americans, respectively; the frequencies of homozygosity for the above populations are 65, 10, and 4 per 100,000 individuals correspondingly.[36-38] The population frequency of the FVL variant also varies among European countries. The population frequency of the FVL variant in Greece and Sweden is reported to be higher than Portugal and Italy (~7% vs 1.4%).[11] In the gnomAD database (gnomAD v4.1.0), the minor allele frequency (MAF) of the FVL variant ranges from almost 0% in the East Asian population to about 3.9% in the Middle Eastern population with an average global frequency of 2%.

FVL is present in approximately 20% of individuals with an initial episode of isolated DVT (19% heterozygous and 1% homozygous), 8.3% with isolated pulmonary embolism (8% heterozygous and 0.3% homozygous), and 16% with both DVT and pulmonary embolism (15% heterozygous and 1% homozygous).[39,40] The relative risk for VTE is approximately 6- to 8-fold for heterozygotes and 80-fold for homozygotes.[41,42] For individuals with FVL, a positive family history of VTE increases the risk of VTE 2.9-fold (95% CI, 1.5-5.7), and if a relative has VTE before the age of 50, the risk of VTE increases up to 5-fold (95% CI, 2.0-14.6). If there are multiple affected relatives, the risk could increase to 17-fold (95% CI, 2.2-143.1).[19,43]

Lifetime risk of VTE in heterozygotes is approximately 10% and close to 100% for FVL homozygotes (2.9 VTE events/1000 persons/year for heterozygotes and 15 VTE events/1000 persons/year for homozygotes).[44,45] Lifetime risks of VTE are higher when environmental risk factors, such as obesity and smoking, are also present.[44-47]

In terms of the recurrence risk, heterozygosity for FVL has at most a modest effect after the first VTE, with conflicting results between studies.[48] Some studies have demonstrated no increased recurrence risk for FVL heterozygotes.[49] However, homozygous FVL leads to a significant increase in recurrence. A systematic review reported odds

ratios of 1.56 and 2.65 for heterozygotes and homozygotes, respectively.[50]

*Factor II c.*97G>A*

The heterozygous factor II c.*97G>A variant is found in approximately 1% to 3% of European Americans, 1% of Hispanics, and 0.3% of African Americans in the United States.[19,36] The frequencies of homozygosity for factor II c.*97G>A are 12 per 100,000 and less than 1 per 100,000 individuals among Whites and Hispanics, respectively.[36] In the gnomAD database (gnomAD v4.1.0), the MAF of the factor II c.*97G>A variant ranges from almost 0% in the East Asian population to about 3.5% in the Ashkenazi Jewish population with an average global frequency of 1%.

Among symptomatic individuals, this variant is present in 6% of individuals with an initial episode of VTE.[38,51] In the absence of other acquired risk factors, the relative risk for venous thrombosis associated with the factor II c.*97G>A ranges from 1.9- to 11.5-fold; the majority of studies have shown a risk of 2- to 4-fold for heterozygotes.[51,52]

For individuals with the factor II c.*97G>A variant and a family history of VTE, the risk of VTE increases 3- to 4-fold.[40,43] The risk tends to be higher if the VTE occurred at a younger age or there are multiple affected family members.[40,43]

Homozygotes for factor II c.*97G>A are rare. The prevalence among the general population is 0.001% to 0.012% and 0.2% to 4% among individuals with VTE.[19] The annual risk of VTE in homozygotes has been reported to be 1.1%/year.[19] From a literature review of 49 cases, homozygous individuals display a striking phenotypic heterogeneity, ranging from asymptomatic individuals who were identified through family studies to individuals suffering from a fatal event in the neonatal period.[53]

The recurrence risk for VTE due to factor II c.*97G>A heterozygosity is at most moderate, with conflicting data and many studies showing no increased recurrence.[38,40] The recurrence risk of VTE for factor II c.*97G>A homozygotes is presumed to be higher than for heterozygotes, but this is not well defined because of the limited numbers of patients identified with this genotype.[40]

*FVL and factor II c.*97G>A double heterozygotes*

Because both FVL and factor II c.*97G>A are relatively common among White populations, individuals may harbor both variants. The estimated prevalence of double heterozygotes is 22 per 100,000.[36] Six to 12% of individuals who are heterozygous for the FVL with a VTE event also harbor the factor II c.*97G>A.[54] In the same meta-analysis consisting of 2310 White cases and 3204 controls, the odds ratio for VTE of double heterozygotes was 20.0 (95% CI, 11.1-36.1).[54]

Reports have demonstrated that patients who have had VTE and are heterozygous for both FVL and factor II c.*97G>A have a 3- to 9-fold increased risk for recurrent VTE, although 1 family study did not find an increased risk.[40] A systematic review showed a 5-fold increased risk.[50] A prospective study found an annual incidence of recurrent VTE of 12% per year in individuals heterozygous for both the FVL and factor II c.*97G>A vs 2.8% in those with neither variant.[55]

Variant spectrum

FVL accounts for 90% to 95% of cases with APC resistance.[56,57] Another variant in the factor V gene, called factor V R2 (rs1800595, NC_000001.11:g.169541110T>C, NM_000130.5:c.3980A>G, NP_000121.2:p.His1327Arg) also known as His1299Arg), has also been widely studied. The MAF of the R2 variant ranges from 0.9% in the African/African American population to about 8.5% in the Admixed American population with an average global frequency of 5% in the gnomAD database (gnomAD v4.1.0). It appears to confer a modest additional thrombotic risk when present in a compound heterozygous state with the FVL.[58] Compared with normal factor V, the factor V R2 variant has 73% of the APC cofactor activity.[59] In the homozygous state, the factor V R2 allele appears to cause a mild APC resistance.[60] With rare exceptions, it is usually in trans with the FVL and rarely found in FVL homozygotes.[58,61] The R2 variant alone is not associated with an increased risk of VTE.[62,63] However, it has been speculated that homozygous R2 can contribute significantly to APC resistance in the Japanese population because of a relatively high prevalence of homozygosity (1 in 350) and an extremely low presence of the FVL variant.[64]

Other variants in the factor V gene have also been described. Factor V Cambridge (rs118203906, NC_000001.11:g.169555299C>G, NM_000130.5:c.1001G>C p.Arg334Thr also known as Arg306Thr) and factor V Hong Kong (rs118203905, NC_000001.11:g.169555300T>C, NM_000130.5:c.1000A>G p.Arg334Gly also known as Arg306Gly) located in the APC cleavage site of the factor V gene were speculated to have functional implications.[65,66] By in vitro functional analysis, both factor V Cambridge and factor V Hong Kong variants showed a mild APC resistance with the APC response being in between that of the wild type and FVL variant.[67] In the gnomAD database (gnomAD v4.1.0), the MAF of factor V Hong Kong is reported in ranges from approximately 0.3% in the East Asian population to almost 0% in the European American and African/African American populations. Factor V Cambridge was found in ranges from approximately 0.2% of the Middle Eastern population to almost 0% of the European American and African/African American populations in the gnomAD database (gnomAD v4.1.0). Although anecdotal reports exist, studies do not support an association of these 2 variants with an increased risk of VTE at least in the Chinese and Mexican populations.[68-70] Large-scale studies of these variants and their risks related to thrombosis are still lacking.

Other rare variants such as factor V Liverpool (rs118203911, NC_000001.11:g.169552693A>G,

NM_000130.5:c.1160T>C p.Ile387Thr, also known as Ile359Thr) and factor V Nara (NC_000001.11:g.169523851A>G, NM_000130.5:c.5842T>C p.Trp1948Arg, also known as Trp1920Arg) have also been described in patients with VTE.[71,72] More and more rare alleles are expected to be discovered in the future because of the frequent use of the clinical exome sequencing (ES) and clinical genome sequencing (GS) in the clinical arena. However, it is not necessary to test routinely for these rare alleles for patients with VTE.

Regarding the factor II gene, the c.97*G>A variant accounts for the majority of reported alleles in patients with VTE. Other variants, such as prothrombin Yukuhashi (rs387907201, NC_000011.10:g.46739326G>T, NM_000506.5:c.1787G>T p.Arg596Leu), have been described.[73] These alleles do not have a frequency high enough to warrant routine clinical testing.

Methylenetetrahydrofolate reductase variants

The *MTHFR* gene encodes methylenetetrahydrofolate reductase which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The converted 5-methyltetrahydrofolate is crucial for the remethylation of homocysteine to methionine, affecting nucleic acids and protein synthesis. The 2 well-known polymorphic variants are c.665C>T p.Ala222Val and c.1286A>C p.Glu429Ala. These variants reduce enzyme activity and are prevalent in different ethnic groups.

It was previously hypothesized that reduced enzyme activity of MTHFR might lead to mild hyperhomocysteinemia, which, in turn, could increase the risk for VTE, coronary heart disease, and recurrent pregnancy loss. However, recent meta-analyses have shown no significant association between *MTHFR* variants and these conditions, questioning the clinical utility of such tests.[74,75]

Therefore, the American College of Medical Genetics and Genomics (ACMG) currently does not recommend routine *MTHFR* variant testing for thrombophilia, recurrent pregnancy loss, or other adverse perinatal events. In addition, *MTHFR* analysis should not be ordered as part of a routine evaluation for thrombophilia[76] or for reproductive carrier screening.[77] This position is also supported by the American College of Obstetricians and Gynecologists (ACOG) and the British Committee for Standards in Haematology and the British Society for Haematology.[78,79]

Testing indications

General indications

Testing for FVL and factor II c.97*G>A is recommended: (1) in patients with VTE when the results will influence treatment and clinical management decisions and (2) in patients and certain asymptomatic relatives to reduce the risk of provoked VTE through counseling about preventive measures in circumstances of elevated risk.[19,48,80] FVL and factor II c.*97G>A genotyping provides information on the recurrence risk of VTE and can inform decisions relevant to avoidable circumstantial risks, such as extended travel, contraceptive use, and approach to long term immobilization.[19] FVL and factor II c.97G>A homozygotes or double heterozygotes are defined as having high-risk thrombophilias, and the severity of the inherited thrombophilia (high or low risk) is a consideration for treatment decisions.[40,44,48,79,81-83] Testing is recommended for certain targeted populations/circumstances; it is not recommended indiscriminately for all patients with VTE or for the general population. Testing indications from different professional organizations vary. Some suggest that there is limited clinical utility of testing for inherited thrombophilias in a majority of patients with VTE.[38,79,84-86] However, it is acknowledged that this approach could miss the identification of homozygotes, for whom knowledge of this genotype would influence treatment or prevention.[79] Others recommend targeted testing of patients and relatives with increased risk.[47,49] This update incorporates several recommendations and suggestions from the American Society of Hematology.[15]

Testing for FVL and factor II c.*97G>A is recommended in the following circumstances:

1. VTE at unusual sites (such as cerebral and splanchnic venous thrombosis)
2. for individuals with VTE provoked by pregnancy or postpartum
3. for individuals with VTE associated with the use of OCs
4. personal history of VTE with (a) a positive family history of thrombophilia diagnosed in a first- or second-degree relative or (b) 1 first-degree relative with VTE at a young age
5. individuals with low APC resistance activity

Testing is suggested in the following circumstances:

1. recurrent VTE
2. siblings of individuals known to be homozygous for FVL or factor II c.*97G>A
3. asymptomatic pregnant individual or individual contemplating pregnancy or estrogen use (OC or hormone replacement therapy) who has a first-degree relative with a history of VTE and is known to be homozygous FVL or is double heterozygotes of FVL and factor II c.97*G>A variant
4. asymptomatic pregnant individual or individual contemplating pregnancy with a previous nonestrogen-related VTE or VTE provoked by a minor risk factor because knowledge of the FVL and/or factor II c.*97G>A status may alter pregnancy-related thrombophylaxis

Routine testing is not generally recommended for patients with a personal or family history of arterial thrombotic disorders (such as coronary artery disease or ischemic stroke) because of a lack of evidence of the association

between inherited thrombophilias and arterial ischemic events.

Several clinical scenarios requiring special considerations

Testing of symptomatic vs asymptomatic individuals

Current genetic technologies have a high analytical sensitivity and specificity for the testing of FVL and factor II c.*97G>A. Currently, these tests are predominantly used for individuals with clinical symptoms of VTE. In a review of data from Europe, Australia, and the United States,[48] VTE accounts for 42% of the clinical referrals for testing. Other indications include arterial thrombosis (15%-23%), obstetric complication (13%-17%) and asymptomatic relatives (12%-16%). From a meta-analysis, the FVL genotype was shown to be predictive of the recurrence of VTE for the proband and of the occurrence for family members especially when FVL homozygosity was detected.[50] For factor II c.*97G>A, the predictive value is not conclusive with most studies demonstrating no increased recurrence risk for heterozygotes and not enough data for homozygotes.[44,48,50,80,81] Knowing the factor V or factor II genotype will neither alter the clinical management nor affect the decision for prophylaxis for many patients.[38,50] However, under certain circumstances, the knowledge of high-risk genotypes could influence clinical management.[48,80] Further investigation is needed to demonstrate whether testing of asymptomatic relatives to promote awareness of their risk of VTE would decrease the incidence of VTE; knowledge of their genotype might facilitate counseling on avoidable circumstantial situations.[19]

Asymptomatic family members may sometimes request genetic testing before being exposed to certain risk factors. It is generally not recommended to test asymptomatic minors as VTE rarely occurs before young adulthood even in the homozygous state.[44]

Prenatal testing and population screening

FVL and factor II c.*97G>A are relatively common among the general population and VTE can be fatal. However, universal prenatal testing and population screening are not indicated because of the low penetrance of these variants, later age of onset and lack of genotype-directed prophylaxis.

Testing of individuals with recurrent adverse pregnant outcomes

Pregnancy is associated with adaptive physiological changes resulting in an increased clotting risk and decreased anticoagulant activity. FVL and factor II c.*97G>A have been detected in approximately 40% and 17% of VTE cases, respectively, during pregnancy.[87-89] Risk stratification for pregnancy-associated VTE is reviewed by Rodger.[83] ACOG and the Society for Maternal-Fetal Medicine recommend that every pregnant individual requires an assessment for VTE risk.[90,91] The first and second most significant individual risk factors for VTE in pregnancy are (1) prior thrombosis and (2) known thrombophilia. The risk of VTE in heterozygous patients without a personal history or affected first-degree relative(s) is only minimally increased compared with the general population.[82,88,89] A comprehensive investigation of a patient's personal and family history of thrombosis and individualized risk assessment is recommended before the initiation of genetic testing.[19,82,92] However, it is often challenging to collect a thorough personal and family history in the current clinical settings.[92] Screening for an FVL variant and factor II c.*97G>A variant is not recommended for individuals with a history of fetal loss or adverse pregnancy outcomes without a personal history of VTE or a family history of thrombosis due to insufficient clinical evidence.[93] For individuals with a known genotype, some professional organizations recommend prophylactic treatment to prevent VTE for homozygotes and double heterozygotes.[82]

Patients who have experienced utero-placental thrombosis-related adverse pregnancies, such as fetal loss, preeclampsia, fetal growth restriction, and placental abruption, are often referred for genetic testing.[48] However, the relationship between inherited thrombophilia and utero-placental thrombosis or preeclampsia is unclear.[48,94,95] Routine genetic testing for these conditions is currently not recommended by ACOG.[94]

Patients considering taking estrogen-containing OCs or hormone replacement therapy

It is well known that OCs pose an additional risk of thrombosis among individuals harboring the FVL and/or factor II c.*97G>A.[96] In a meta-analysis, OC users showed an odds ratio of 1.8 (95% CI, 1.20-2.71) compared with nonusers among FVL heterozygotes in which the odds ratio is 1.63 (95% CI, 1.01-2.65) for factor II c.*97G>A heterozygotes.[97] Nevertheless, the absolute risk in these individuals is less than 1%, the threshold at which prophylaxis is considered.[83] Although ACOG recommends a consideration of alternative contraceptive options, screening all patients for inherited thrombophilias before initiating contraception is not recommended.[86,98,99]

Hormone replacement therapy (HRT) is associated with a 2- to 4-fold increased risk of VTE in users compared with nonusers.[44] Individuals on HRT with FVL have an odds ratio of 13.16 (95% CI, 4.28-40.27) for VTE compared with those without this variant.[100] Retrospective studies suggest that transdermal HRT is not as prothrombotic as oral HRT.[101-103] Genetic screening of prospective HRT users has not been proven to be beneficial.[102] A family and personal history of thrombosis should be carefully evaluated for all patients before initiating HRT, and a positive history may warrant thrombophilia screening.

For asymptomatic relatives of patients with FVL or factor II c*97G>A considering OCs or HRT, there are no published data regarding whether genetic testing would benefit or change the clinical management of OC or HRT use.[38]

*FVL and factor II c.*97G>A variants as secondary findings*

Clinical ES or GS is now frequently used as a diagnostic tool for pediatric and adult patients. Because of the relatively high population frequency of FVL and factor II c*97G>A, it is not surprising that these variants are often identified as secondary findings during ES or GS testing. There is currently no consensus on whether to report these variants. Individual laboratories may have different policies. Factor V and factor II are not among the list of genes proposed by ACMG in which secondary finding of pathogenic variants are recommended to be reported.[104-107] Although these 2 variants increase the risk of VTE, which can have fatal outcomes, such as pulmonary embolism, it is important to be aware that the penetrance of these variants is rather low. If a laboratory decides to report these variants as secondary findings, genetic counseling should be recommended. Additionally, the ES/GS consent should indicate specific genes that the laboratory includes in the secondary findings, and it should be obtained from the patients or guardians before testing.

Informed consent

Obtaining informed consent is generally not mandatory for FVL and factor II c.*97G>A testing unless required by state-specific laws/regulations for genetic testing. However, individuals should be aware that any genetic test could possibly have implications for insurability or have other social and psychological implications. Other family members may be at an increased risk of VTE if the proband tests positive. Genetic counseling should be available when necessary. As for all other genetic tests, testing laboratories are encouraged to have mechanisms to collect pretest clinical information that includes the patient's date of birth, racial/ethnic background, indication for testing, and specific family history. When testing indications are found to be inappropriate by the clinical laboratory, testing laboratories are encouraged to communicate with the referring physician to recommend test cancellation.

Clinical validity, clinical utility, clinical sensitivity, and specificity

Clinical validity is defined as the test's ability to accurately and reliably identify or predict the disorder or phenotype of interest. Several meta-analyses support the clinical validity of FVL (either heterozygote or homozygote) to predict the recurrence of VTE in the proband and VTE occurrence in family members.[38,50] For factor II c.*97G>A, there is only limited evidence regarding the predictive value for the recurrence risk of VTE in probands, and it is inconclusive whether this variant could predict VTE in family members.[50] FVL and factor II c.*97G>A double heterozygotes seem to be predictive for the occurrence of VTE among family members, but there is insufficient information to draw a firm conclusion. However, the risk of VTE in family members with this genotype is likely to be at least as high as for FVL alone.[38,50]

Clinical utility is defined as whether the clinical test results could change the patient's clinical management. There is no consensus regarding the role of genotype for determining the treatment regimen for VTE. Current antithrombotic recommendations from professional organizations largely do not focus on genotype for most VTE patients.[88,108] For many patients, the clinical utility of genetic testing for VTE is not high.[50] However, for certain circumstances, such as pregnant individuals with previous VTE and a positive family history, the clinical utility has been acknowledged.[48,79,80,88]

The clinical sensitivity of FVL or factor II c.*97G>A can be defined as the proportion of individuals who have had (or will have) VTE and are variant positive. Overall, the clinical sensitivity of FVL for isolated VTE is between 20% to 50%.[39,109] It is 16% for individuals with both DVT and pulmonary embolism and for those with isolated pulmonary embolism.[39] Age is a strong risk factor for thrombosis. The risk for VTE in heterozygotes of FVL increased with age at a rate significantly greater than that in wild type.[110] The clinical sensitivity was found to be approximately 29.5% in a study of 380 individuals with at least 1 thromboembolic event.[111] FVL has been found in 20% to 46% of patients with VTE during pregnancy.[112,113] The clinical sensitivity of the factor II c.*97G>A variant for an initial episode of VTE is about 6%.[51]

Clinical specificity can be defined as the proportion of individuals who do not have or will not develop VTE and do not have a variant. The false-positive rate is 1 minus the clinical specificity. The low penetrance of these 2 variants is the main reason for less than 100% clinical specificity. Analytical error is possible, but this is likely to be a much smaller factor in cases of clinical false-positive test results. The clinical specificity for FVL has not been firmly established but can be no lower than 95% (this assumes that all 5% of the population with a variant is clinical false positive). Similarly, the clinical specificity for the factor II c.*97G>A test is likely to be no lower than 98% (if all 2% of heterozygotes are clinical false positives). Given the low penetrance of these variants (ie, most individuals with a pathogenic variant will not develop VTE), the estimation of clinical specificity of these 2 variants are reasonably reliable.

The penetrance of the FVL variant is considered low for heterozygotes. The cumulative incidence of VTE at age 60 is about 6.5% for heterozygotes, and the lifetime risk for heterozygotes is estimated to be approximately 10%.[44,81] Penetrance for homozygotes has been estimated at 15% to 20% from population screening studies.[45,81,114] Another study showed an incidence of 15 VTE/1000 person years for homozygotes.[45] The risk of VTE is expected to be higher in FVL-positive asymptomatic individuals identified from thrombophilic families than those who were identified from population screening. It is difficult to estimate the absolute

penetrance for these variants alone because it is very common that additional risk factors also coexist.

Technical performance

Assay considerations

Because both FVL and factor II c.*97G>A variants are single-nucleotide substitutions, any assay that is amenable to detecting single-nucleotide changes can be used for clinical testing. Currently, laboratory developed tests, research use only reagents, and Food and Drug Administration-approved testing platforms are all being used by clinical laboratories. Individual laboratories may choose assays based on their sample volume, laboratory workflow, and number of employees, etc. Assays can be designed based on polymerase chain reaction (PCR), restriction-fragment-length polymorphism, allele-specific PCR, flap endonuclease plus Förster resonance energy transfer, melting curve analysis, Taqman real-time PCR, or fluorescent probe-based allelic discrimination, etc. Details of the underlying chemistry, quality control, and advantage/disadvantage of individual assays can be found in the general technical standards published by the ACMG. 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 because of cost, turnaround time, and limited capacity for multiplexing. Each laboratory is responsible for the in-house validation/verification required by regulatory agencies, such as the Clinical Laboratory Improvement Amendments and the College of American Pathologists. Participation in proficiency testing or sample exchange with other clinical laboratories is recommended to ensure the assay quality.

Positive controls

Positive controls can be obtained from the National Institute of General Medical Sciences Human Genetic Cell Repository (http://catalog.coriell.org) or other resources. Genomic DNA from patients identified as heterozygous or homozygous and confirmed by alternative methodologies, other laboratories, or Sanger sequencing can also be used as the assay control if consent is obtained from these patients.

Sample preparation

Most assays are amenable to the use of genomic DNA prepared from blood or other tissue sources using a variety of extraction protocols. Platforms integrating DNA extraction and PCR steps are also available.

Analytical sensitivity and specificity

The analytical sensitivity of an assay is defined as the proportion of biological samples with a known variant that is correctly classified as having a positive test result. The analytical specificity is the proportion of biological samples without a specific variant that is correctly classified as having a negative test result.

Segal et al[50] carried out a meta-analysis to investigate the analytical sensitivity and specificity of these 2 variants. This analysis included 43 individual studies with more than 11,000 subjects collectively for the genotyping of FVL and factor II c.*97G>A using different platforms.[50] The majority of the studies used PCR-restriction-fragment-length polymorphism as the reference standard. The concordance rate between the various platforms and the reference standard ranged between 98% to 100%, indicating that the analytical sensitivity and specificity are not lower than 98%.[115] In the clinical setting, the analytical sensitivity and specificity of assays testing these 2 variants is also very high. A collection of data from ACMG/College of American Pathologists external proficiency testing between 1999 and 2003, the National External Quality Assessment Schemes from the United Kingdom and Europe between 1999 and 2002, and the Royal College of Pathologists of Australasia between 1998 and 2003 demonstrated a 98.8% and 99.3% analytical sensitivity and specificity for FVL and 98.3% and 99.6% for factor II c.*97G>A.[116] Hertzberg et al[117] reported the result of a 5-year external quality assurance program in Australia. Among 3799 responders, the rate of successfully identifying specific genetic alterations were 98.13% and 98.84% for FVL and factor II c.*97G>A, respectively.

We can conclude that the analytical sensitivity and specificity are excellent for both variants regardless of the testing platforms. Commercial kits and new methodologies for detecting the FVL and factor II c.*97G>A are being introduced into the market frequently. It is the responsibility of the laboratory director and/or medical director to evaluate and validate any new methodology before the implementation of clinical testing. If a proper clinical quality assurance protocol is instituted, the majority of the testing platforms will yield consistent genotyping results.

Laboratory result interpretations

Each laboratory may develop their own reporting format with content pertaining to the requirements of federal, state, and other regulatory agencies. Information regarding genotype, related risk for thrombosis, and potential clinical implications are integral components in a clinical genetic report. A recommendation for genetic counseling may also be included. Reports may be tailored for the specific clinical indications, if available, especially when the testing indication is not VTE (eg, recurrent pregnancy losses, planning to use OCs, testing of asymptomatic individuals because of family history). Reports should clearly state that a positive result can only suggest an elevated risk but cannot definitively predict the occurrence or recurrence of a VTE in a specific individual.

Normal results

Venous thrombosis is a relatively common disorder in the general population. Genetic causes can only be identified in about 25% of White patients without a family history.[17] The genetic causes in other ethnic groups are largely unknown. Health care providers need to be aware that negative genetic testing results are unlikely to significantly reduce the recurrence risk derived from clinical and family history. Patients' clinical management and implementation of a healthier lifestyle toward preventing recurrent VTE should not be altered because of a negative genetic testing result.

FVL heterozygote

Individuals heterozygous for FVL have an approximately 4- to 7- or 8-fold increased risk of venous thrombosis compared with individuals without this variant.[96,118]

FVL homozygote

Individuals homozygous for FVL have an approximately 80-fold increased risk of venous thrombosis compared with individuals without this variant.[42]

Factor II c.*97G>A heterozygote

Individuals heterozygous for factor II c.*97G>A have an approximately 2- to 4-fold increased risk of venous thrombosis compared with individuals without this variant.[42]

Factor II c.*97G>A homozygote

The associated risk of the homozygous c.*97G>A genotype and VTE is not conclusive because of the relatively few number of individuals with this genotype.[119] However, it is presumed to be higher than the risk for the heterozygous c.97*G>A genotype.[40] The risk of VTE is estimated to be 1.1% per person per year for homozygotes.[19]

FVL and factor II c.*97G>A double heterozygote

Between 1.4% to 10% of symptomatic heterozygotes of FVL also harbor the factor II c.*97G>A.[12,36,54] Individuals harboring both FVL and factor II c.*97G>A have about a 20-fold increased risk of VTE compared with individuals without either variant (about 4-fold compared with individuals carrying FVL alone).[120] The pooled odds ratio for recurrence of VTE in a proband is 4.81 (95% CI, 0.50-46.3) compared with normal controls.[50]

Alternative testing methods

APC resistance can be diagnosed by a functional coagulation assay that measures the ability of activated protein C to inactivate factor Va. A positive APC resistance may indicate an FVL variant; however, depending upon the assay used, it may be affected by variants or conditions other than FVL. Testing of prothrombin levels is not a functional alternative assay for factor II c.*97G>A genetic testing.[40]

Conclusion

FVL and factor II c.*97G>A are the 2 most common genetic variants associated with VTE. Depending on the heterozygous, homozygous, or double heterozygous status of FVL and factor II c.*97G>A, the risk of VTE varies. However, there is insufficient clinical evidence to support assessment of *MTHFR* variants or measurement of fasting homocysteine levels in the evaluation of a thrombophilic etiology for VTE. It is critical to perform FVL and factor II c.*97G>A testing only for appropriate individuals with a personal or family history of VTE, not for routine general population screening. Additionally, various testing methodologies, including Taqman real-time PCR and next-generation sequencing, can be used to detect FVL and factor II c.*97G>A variants. Therefore, these technical standards serve as a guide for clinical FVL and factor II c.*97 G>A variants testing that should be applied to any methodology currently used.

Conflict of Interest

All workgroup members receive salary for providing clinical services that may be relevant to the content of this document in either the laboratory or patient care setting at their listed affiliations. The following workgroup members have additional conflicts of interest: Benjamin E. Kang and Jeffrey Dungan (Natera [stock]),

Affiliations

[1]Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN; [2]Department of Pathology and Laboratory Medicine, University of Kentucky, Lexington, KY; [3]Department of Medical Genetics and Genomics, Cleveland Clinic Lerner College of Medicine, Cleveland, OH; [4]Case Western Reserve University, Cleveland, OH; [5]Division of Clinical Genetics, Department of Obstetrics and Gynecology, Northwestern University Feinberg School of Medicine, Chicago, IL; [6]Fetal Diagnostic and Treatment Center, Department of Obstetrics and Gynecology, Michigan Medicine, Ann Arbor, MI; [7]New York Genome Center, New York, NY; [8]Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT; [9]ARUP Laboratories, Salt Lake City, UT; [10]American College of Medical Genetics and Genomics, Bethesda, MD

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