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Factor V Leiden, prothrombin G20210A substitution and hormone therapy: indications for molecular screening testing[1)]

Faktor-V-Leiden, Prothrombin G20210A Substitution und Hormontherapie: Indikationen für molekulare Screening Tests

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Abstract

Venous thromboembolism is a well-known complication of oral contraception and hormonal replacement therapy. Inherited thrombophilia is viewed as an important determinant in modulating the effects of estrogens on thrombotic risk. An increasing number of kits for thrombophilic mutations [factor V Leiden, G20210A prothrombin and methylenetetrahydrofolate reductase (*MTHFR*) C677T genes] are becoming commercially available, and screening for inherited thrombotic risk is among the most requested genetic tests in molecular diagnostic laboratories. However, the question of routine genetic screening for thrombophilia before prescribing hormones is still a matter of debate. The purpose of this article is to discuss the usefulness and practical applications of thrombotic genetic testing to identify which women should be tested to improve both the safety and efficacy of individualized estrogen therapy.

Keywords: genetic testing; hormonal replacement therapy; inherited thrombophilia; oral contraception; thrombotic risk.

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Zusammenfassung

Venenthrombosen sind eine bekannte Komplikation bei oraler Kontrazeption und Hormonersatztherapie. Erbliche Thrombophilie wird als wichtiger Faktor gesehen, der Effekte von Östrogenen auf das Thromboserisiko beeinflusst. Die Zahl kommerziell vertriebener Testkits für Thrombophilie-Mutationen (Factor V Leiden, G20210A Prothrombin und MTHFR C677T Gene) steigt und das Screening auf erbliche Thrombose-Risikofaktoren gehört zu den am häufigsten angeforderten Leistungen in molekulardiagnostischen Labors. Die Frage nach der routinemäßigen Durchführung von Sceenings auf solche Risikofaktoren vor einer Verschreibung von Hormonpräparaten wird jedoch kontrovers diskutiert. In diesem Artikel werden der Wert und die praktischen Anwendungsmöglichkeiten von genetischen Tests auf Thrombose-Risikofaktoren diskutiert, um zu definieren, welche Patientinnen im Interesse der Erhöhung von Sicherheit und Effizienz einer individuellen Östrogentherapie getestet werden sollten.

Schlüsselwörter: erbliche Thrombophilie; genetische Tests; Hormonersatztherapie; orale Kontrazeption; Thromboserisiko.

Introduction

Exogenous hormones are used worldwide by women as oral contraceptives (OCs) and hormonal replacement therapy (HRT). Epidemiologic studies have shown a two- to six-fold increase in the relative risk of venous thromboembolism (VTE) with the use of either hormonal contraception or postmenopausal treatment [1]. Recently, it has been suggested that the risk/benefit ratio could be, in part, mediated by the genetic predisposition of women [2, 3]. In particular, genetic thrombophilia might be implicated in the risk of VTE in women who use exogenous hormones [2, 3]. The most common causes of genetic hypercoagulability known today are factor V Leiden, G20210A prothrombin polymorphisms, and the genetic variant C677T of the methylenetetrahydrofolate

reductase (*MTHFR*) gene (where the association appears to be much weaker) [4–6]. Indeed, diagnostic assays of VTE predisposition are among the most requested genetic tests in molecular diagnostic laboratories [7]. However, the question as to whether it is beneficial to screen women for genetic thrombophilia before prescribing hormones remains controversial. On the other hand, there is considerable debate over the new "predictive medicine" that could generate serious ethical, social and psychological consequences. Receiving genetic risk information may, therefore, be more harmful than positive by raising unnecessary anxieties and providing a real prospect of discrimination based on a person's genetic make-up [8, 9].

Furthermore, genetic tests are generally time-consuming and expensive, and they should not be used to "screen" the general population. However, testing for inherited thrombophilia could have a positive impact on the possibility of preventing VTE in high-risk women, as well as for careful clinical surveillance. Both physicians and women need to obtain accurate information concerning the appropriate use of genetic thrombophilia screening. The purpose of this article is to discuss the usefulness and the practical applications of thrombotic genetic testing to define which women should be tested to improve both the safety and efficacy of individualized estrogen therapy.

Hormone preparations and thrombotic risk

The first case of thrombosis associated with hormonal contraception occurred in 1961, when a nurse developed a pulmonary embolism shortly after starting a high-dose estrogen pill [10]. These early reports seemed to suggest that the thrombotic potential of OC use was related to its relatively high estrogen content of 50 μg or higher [11]. Subsequently, a number of European epidemiologic studies confirmed the association between OC and an increase in VTE, despite the lower overall estrogen content [12, 13]. The absolute risk of VTE is estimated at one event per 10,000 individuals per year among nonusers and 3–6 events per 10,000 per person-year among hormonal contraceptive users [12–14].

As with hormonal contraception, a number of studies have shown an increase in the risk of venous thrombosis after postmenopausal estrogen therapy [15]. The risk of VTE is 2.1- to 3.6-fold higher among users of oral or transdermal preparations [15]. Recently, the Women's Health Initiative (WHI) reported 34 events of VTE annually per 10,000 women treated with HRT vs. 16 VTE events per 10,000 women who were non-HRT users [16]. Again, the Heart and Estrogen/progestin Replacement Study [17] and the Estrogen Replacement and Atherosclerosis trial [18] found a 1.7% and 2.6% increase, respectively in VTE in women on HRT. Furthermore, HRT may also be associated with an early increase in the risk of coronary thrombotic events [19]. Interestingly, the pattern of early increased risk of arterial thrombosis mirrors the pattern of excess risk for VTE, which was also significantly increased during the first year [19]. Recently, it has been suggested that the benefit of HRT could have been obscured by an increased risk of cardiovascular disease in a subset of women genetically predisposed to a thrombotic complication [20]. In particular, genetic polymorphisms in genes regulating the coagulation and fibrinolytic cascade may contribute to estrogen-associated increase in risk for thrombotic events observed in the trials [2, 3]. During recent years, several studies have found an association between candidate genetic polymorphisms (fibrinogen, plasminogen activator, factor XIII, thrombin activatable fibrinolysis inhibitor, angiotensin-converting enzyme genes) and venous thrombosis [21–25]. In addition, the search for amino acid substitutions in factor V, other than that causing factor V Leiden, has identified some additional polymorphisms [26, 27]. However, the association of these genetic variants with thrombosis is still debated and definitive recommendations are not available as yet.

Conversely, there is clinical evidence that indicates a strong association between increased risk of VTE and factor V Leiden or prothrombin G20210A substitution.

Thrombosis gene polymorphisms and risk with estrogen

Genetic risk factors for VTE have been known since 1965, when the first family with an identified hereditary tendency caused by antithrombin deficiency to thrombosis was reported [28]. However, genetic defects in the three principal anticoagulant proteins (antithrombin, protein C and protein S deficiencies) appear to be very rare and extremely heterogeneous. More recently, researchers have described new genetic abnormalities in the genes encoding factor V and prothrombin [29, 30]. These genetic disorders predispose to a hypercoagulable state and are well-conserved mutations. Moreover, a genetic polymorphism in the *MTHFR* gene has also been reported as a risk factor for deep venous thrombosis [4–6]. The relevance of these abnormalities lies in their high prevalence, which, contrary to deficiencies of natural anticoagulants, affects a large number of people (Table 1).

Factor V Leiden

Activated factor V (factor Va) is an essential cofactor for the conversion of prothrombin (factor II) to thrombin, and factor Va is inactivated by activated protein C (APC). APC cuts factor V into two parts. A common variation in the factor V gene (G1691A or factor V Leiden) is responsible for APC resistance. In fact, the resulting amino-acid substitution is located at one of the sites in factor V that is recognized, cleaved and inactivated by APC [29]. The lack of inactivation of factor V results in a predisposition to thrombosis, which is caused by the hypercoagulable condition. One copy of the mutated gene appears to be present in approximately 17–20% of patients with

Table 1 Prevalence of genetic defects for inherited thrombophilia.

Inherited defect	Prevalence in patients with thrombosis, %	Prevalence in the general population, %	Risk of venous thromboembolism, %
Antithrombin III	~1	0.02–0.04	~20–50
Protein C	~2–5	0.2–0.5	7–10
Protein S	~1–3	0.1–1	~2
Factor V Leiden	~20 (heterozygotes)	2–3 (heterozygotes)	~3–7 (heterozygotes) 50–100 (homozygotes)
Prothrombin G20210A	20	~2–3	~2–5 (heterozygotes) ~10 (homozygotes)
MTHFR C677T	15–20	5–15 (homozygotes)	2–3 (homozygotes)

venous thromboembolic events in the general Caucasian population [31–33]. Heterozygous carriers of factor V Leiden have a three- to seven-fold increased risk of VTE, while homozygous subjects have a 50- to 100-fold increased risk [6].

Several studies have reported that the risk of VTE associated with OC is 10- to 35-fold higher among factor V Leiden carriers in comparison with the risk among non-carriers and/or non-users [34–38]. The pooled odds ratio estimated by a recent meta-analysis showed a 15.62-fold increased risk [39].

It is also relevant to note that women homozygous for factor V manifested their first episode of VTE during OC use in 80% of cases [40].

This may be explained by the fact that estrogens induce an increase in acquired resistance to APC, leading to higher levels of activated factor V. Acquired APC resistance seems to be related to estrogen-associated increased levels of coagulation factors VII, VIII and decreased levels of physiological inhibitors, antithrombin and protein S [41]. Thus, mutation of factor V could further enhance plasma resistance to APC [42].

Like OCs, there are also data indicating a 13- to 16-fold increased risk of VTE during HRT among women with factor V Leiden [11, 43, 44]. Furthermore, women with factor V Leiden have a substantially increased risk of myocardial infarction or stroke on HRT compared to women without this mutation on HRT [45].

Prothrombin G20210A

A mutation in the prothrombin gene is the second most common cause of inherited thrombophilia. This mutation involves a single base-pair substitution (guanine → adenine) at nucleotide 20210 in the 3′-untranslated region of the prothrombin gene [30].

Carriers have higher prothrombin plasma levels, with increased risk of thrombosis [46]. Heterozygous carriers constitute approximately 2% of the general Caucasian population, and reach 20% in the population with recurrent or familial thrombotic events [47, 48]. Heterozygous subjects for the G20210A mutation carry an estimated two- to five-fold increased risk of venous thrombosis without other concomitant risk factors, and a more than additive synergistic risk of VTE when other thrombophilic risk factors, particularly factor V Leiden, are simultaneously present [47–49]. The risk of VTE is remarkably increased in women heterozygous for prothrombin G20210A polymorphism who are taking OC [37–39, 50–52]. For instance, a 150-fold increased risk of cerebral vein thrombosis has been reported in carriers of the prothrombin mutation on OC [52]. The overall increase in thrombotic risk for the combination of OC and G20210A mutation ranges from 16- to 59-fold [37–39, 50–52]. Few studies have investigated the relationship between prothrombin G20210A mutation and VTE in users of HRT.

Recently, the Estrogen and Thromboembolism Risk (ESTHER) study confirmed associations between increased VTE risk in postmenopausal women and current use of oral estrogen or the presence of either factor V Leiden or prothrombin G20210A mutations [53]. In women who both carry a prothrombotic mutation and use oral estrogen, VTE risk is 25-fold increased compared with non-users without mutation. However, women using transdermal estrogen and carrying a prothrombotic mutation have a VTE risk close to that of non-users with a prothrombotic mutation (four-fold increased VTE risk compared to controls).

In addition, several studies have also found an association between the presence of prothrombin G20210A polymorphism and the risk of myocardial infarction or stroke in women on HRT who were also carriers of the allele A compared with the wild-type genotype [54, 55].

Thermolabile MTHFR

Elevated levels of homocysteine (Hcy) have been recognized as an independent risk factor for venous thrombosis [56–58]. Women with hyperhomocysteinemia during OC use had a 20-fold higher risk of cerebral vein thrombosis compared with women without this risk factor [59]. Nutritional deficiencies of essential cofactors or enzyme substrates in Hcy metabolism, including cobalamin (vitamin B_{12}), folate, and pyridoxine (vitamin B_6), are the most common causes of hyperhomocysteinemia [60]. Genetic defects in genes encoding enzymes and cofactors involved in Hcy metabolism may result in elevated plasma Hcy concentrations. In the last decade, several studies have been conducted to elucidate the Hcy-modifying impact of these polymorphisms [61].

Table 2 Risk of venous thromboembolism (VTE) associated with oral contraception (OC) and hormonal replacement therapy (HRT) in women with thrombophilic defects.

Thrombophilic defect	Relative risk, odds ratio	
	OC-associated VTE	HRT-associated VTE
Factor V Leiden	10–35	13–16
Prothrombin G20210A	16–59	3–10
Factor V and prothrombin (double heterozygote)	17–86	NA
MTHFR C677T	NA	NA

NA, not available.

However, the results do not allow firm conclusions to be drawn. In contrast, the 677C>T mutation in the *MTHFR* gene is a well-established genetic cause of mild hyperhomocysteinemia [61, 62].

The *MTHFR* mutation is very common and is distributed in a heterogeneous manner; the homozygous form is more common in North America and Europe than among African Americans [63]. For instance, the homozygous state has been reported in up to 23% of the Italian population [64]. Although the presence of two *T* alleles appears to be a significant independent risk factor for hyperhomocysteinemia, the association between this homozygosity and clinical outcomes of thromboembolism is still debated [65–70]. Indeed, the Copenhagen City Heart Study demonstrated that while ischemic cardiovascular disease or thromboembolism cases had increased Hcy, *MTHFR* C677T homozygotes with genetically elevated Hcy levels did not have increased disease risk [71]. However, a recent meta-analysis of prospective and retrospective studies demonstrated a modest association between Hcy and venous thrombosis, and the 677TT genotype was associated with a 20% increased risk of venous thrombosis compared with the 677CC genotype, providing some support for causality [72].

At present, there is little information on the thrombotic risk of both OC and HRT in women with *MTHFR* C677T polymorphism. Sidney et al. reported that this polymorphism did not increase the risk of VTE in users of low-estrogen OC formulations [73]. However, women with *MTHFR* 677T variants did not show decreased Hcy in response to HRT as demonstrated for the *CC* genotype, suggesting that they may gain reduced cardiovascular benefits from HRT [74].

Finally, a recent investigation also reported a two-fold increase in myocardial infarction in young women with the *TT* genotype who had low folate levels compared with the *CC* genotype [75].

Combined defects

The risk of VTE can further increase in the presence of combined defects. For instance, carriers of both C677T *MTHFR* and factor V Leiden or the G20210A prothrombin mutation have an increased risk of VTE [48]. As regards OC, some evidence indicates that women carrying both factor V Leiden and the prothrombin G20210A mutation or other inherited thrombophilic conditions (antithrombin, protein C, protein S deficiencies) show a further increase in the risk of thrombotic events, particularly during the first year of use [50, 76]. In particular, women on OC who are double heterozygous for factor V Leiden and the prothrombin G20210A mutation have a 17- to 86-fold increase for thrombotic risk [50, 51]. In summary, the data available indicate that women with thrombotic mutations might be at high risk for an estrogen-associated VTE during hormonal contraception and HRT (Table 2).

Candidates for screening

Venous thromboembolic disease is now recognized as multicausal, resulting from gene-gene and gene-environment interactions [77]. Genetic testing for the identification of heritable thrombophilia has become common

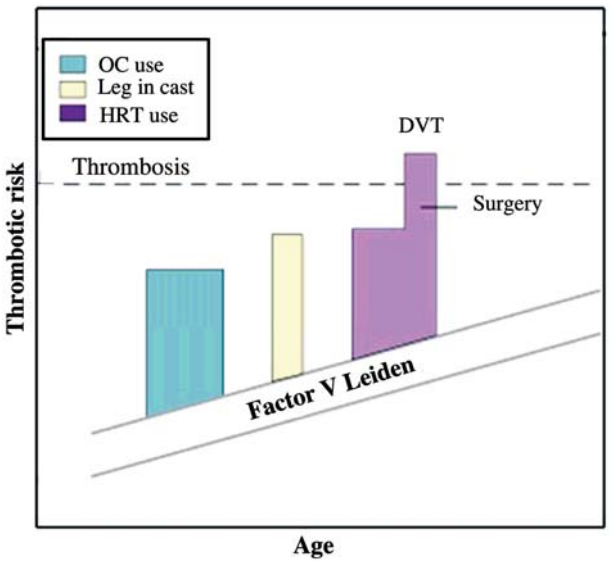

Figure 1 Graphic representation of individual thrombotic risk over time. An underlying factor V Leiden mutation provides a theoretically constant increased risk. The thrombotic risk increases with age and acquired risk factors. This cumulative risk may increase to the threshold for thrombosis and result in deep venous thrombosis [modified from Konkle et al. [77]]

practice. Knowledge of a thrombotic mutation may allow for appropriate prophylaxis on the occurrence of circumstantial risk factors, such as surgery, immobilization or estrogen therapies (Figure 1).

However, the question as to whether it is beneficial to screen women for thrombotic mutations before prescribing estrogen treatment remains controversial. In fact, the American College of Medical Genetics stated that practices regarding testing for factor V Leiden vary considerably, and no consensus has emerged [78]. Although the presence of thrombotic mutations, in particular factor V Leiden mutation, remarkably increases the risk during OC therapy [13], the absolute risk is believed to be modest (2 extra VTEs per 10,000 OC users per year). However, because of the higher baseline incidence of VTE in older postmenopausal women, the absolute risk is >20-fold greater than in younger OC users [79]. In fact, it is important to remember that age is an independent cardiovascular risk factor that favors thrombotic events. Thus, despite the lower estrogen dose and the use of natural estrogen and progestogen, the excess number of thrombotic events attributable to HRT is much higher (40 extra VTEs per 10,000 OC users per year) [17, 18, 79]. Selective screening based on prior personal or family history has been recommended [80, 81]. In contrast, indiscriminate thrombophilia screening could be inappropriate and unjustified. On the one hand, there is considerable debate over the psychological and social problems of genetic testing in asymptomatic individuals [6, 80, 81]. On the other hand, selective thrombophilia screening in women with a personal and/or family history of VTE could itself be considered a contraindication for OC, regardless of any thrombophilic defect [82]. However, a recent analysis showed that selective history-based screening was more cost-effective in all four different patient groups considered: women prior to prescribing OC or HRT, women at the onset of pregnancy, and patients prior to major orthopedic surgery, compared with no screening [83].

Thrombophilia also increases the risk of pregnancy-associated VTE, as well as the risk of poor pregnancy outcome associated with uteroplacental thrombosis [79, 84–86]. However, thrombophilic defects are prevalent and most carriers remain asymptomatic. General screening of all women before pregnancy is not justifiable. Until more specific clinical guidelines are available, genetic testing is recommended in high-risk subgroups of women (Table 3). Specifically, any woman with a personal or family history of VTE or arterial thrombosis who is contemplating starting OC, HRT or pregnancy should be screened for possible hereditary thrombophilia.

Furthermore, potential candidates for genetic screening are women with recurrent complications of pregnancy other than venous thromboembolism before starting exogenous hormone therapy. In these situations, genetic testing might offer important opportunities for patient management, such as appropriate secondary prophylactic antithrombotic therapy or avoidance of additional risk factors. Moreover, molecular analysis may also help to evaluate the need for primary prophylaxis in asymptomatic family members exposed to high-risk acquired situations.

Table 3 Candidate women for genetic screening for prothrombotic mutation before oral contraception, hormonal replacement therapy, and pregnancy.

- All women with previous episodes of VTE or arterial thrombosis
- Asymptomatic women who are first-degree relatives of patients with inherited VTE
- Women with recurrent pregnancy loss or unexplained intrauterine fetal growth retardation or stillbirth

Diagnostic tool for thrombophilia screening

Selected blood tests can be useful in the diagnosis of venous thromboembolism [87]. Deficiencies of antithrombin, protein C, and protein S can be caused by many different genetic mutations. For this reason, molecular tests for protein C, protein S and antithrombin III are technically very complex and rarely performed in clinical settings. More useful and largely applied are the immunological or functional tests of coagulation, which allow detection of the defects in the three principal anticoagulant proteins. APC resistance can be assessed in plasma with activated partial thromboplastin time (APTT)-based methods with and without APC, as originally described by Dahlback et al. [88]. Another possibility is offered by APTT-based methods in which test plasma is prediluted with factor V-deficient plasma [89]. The APTT-based test gives an estimation of the anticoagulant function in vivo and provides information on the thrombotic risk associated with inherited and acquired APC resistance. Indeed, exogenous estrogen often produces acquired resistance to APC; consequently, this serologic test can be abnormal even if the genotype is normal. Therefore, in women on exogenous estrogen or pregnant (physiologic hyperestrogenemia), only a polymerase chain reaction (PCR) test is appropriate for factor V Leiden determination, since it is not affected by exogenous estrogen. In addition, testing for the factor V Leiden mutation has to be performed to confirm positive and borderline cases by clotting assay.

There is no sensitive and specific test for the prothrombin gene mutation, which requires a cDNA-PCR test. Hcy measurement is recommended, while PCR analysis for the C677T MTHFR polymorphism can be of limited usefulness.

Until recently, the cost of DNA assays for gene polymorphism made screening impractical. However, these costs are decreasing and genetic screening could be a cost-effective strategy before use of OC or HRT. Indeed, new DNA-based thrombotic risk kits have become widely

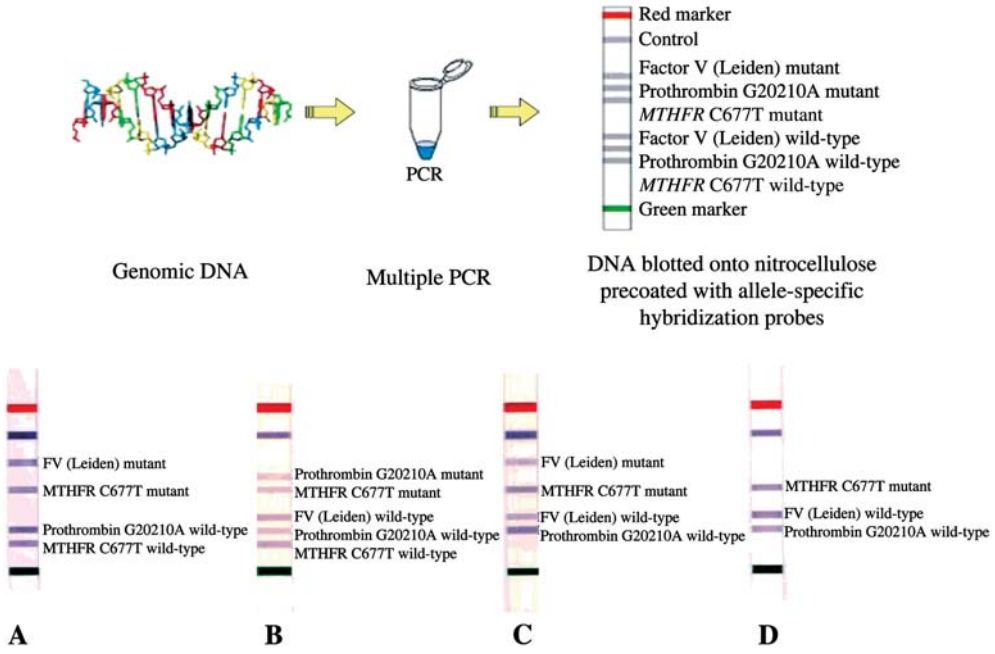

Figure 2 Examples of molecular screening for factor V Leiden, prothrombin G20210A and *MTHFR* C677T mutations in candidate women using a typical commercial kit. (A) Patient homozygous for factor V Leiden and heterozygous for *MTHFR* 677T variant. (B) Patient heterozygous for prothrombin 20210A and *MTHFR* 677T mutation. (C) Patient heterozygous for factor V Leiden and homozygous for *MTHFR* 677T variant. (D) Patient homozygous for *MTHFR* 677T variant.

used, since they allow simultaneous detection of thrombotic mutations.

These kits involve simple and fast genotyping methods based on PCR technology. In particular, the multiplex allele-specific amplification (ASA-PCR) technology represents a valid alternative to standard protocols such as restriction fragment length polymorphism-PCR or sequencing, especially when simultaneous determination of multiple genetic mutations is required (Figure 2). However, several additional commercial assays are available. Using a commercial thrombotic risk kit, it is possible to obtain all three genetic results within 5–8 h.

Conclusions

Inherited thrombophilia is viewed as an important determinant in modulating the safety and efficacy of hormone therapy. Genetic screening may provide a new tool to improve both the safety and efficacy of individualized estrogen therapy. Factor V Leiden and prothrombin mutations increase the risk of VTE during OC use and HRT. Current evidence indicates that screening for thrombophilic defects might be advisable in women with a personal or family history of VTE. Testing for inherited thrombophilic disorders, to be used in decision-making about HRT use, would be premature in unselected patients. Therefore, it is crucial for physicians to develop specific knowledge for appropriate practical use of genetic tests, as well as for avoiding anxiety among women.

References

1. Gomes MP, Deitcher SR. Risk of venous thromboembolic disease associated with hormonal contraceptives and hormone replacement therapy: a clinical review. Arch Intern Med 2004;164:1965–76.
2. Herrington DM, Klein KP. Invited review: pharmacogenetics of estrogen replacement therapy. J Appl Physiol 2001; 91:2776–84.
3. Braunstein JB, Kershner DW, Bray P, Gerstenblith G, Schulman SP, Post WS, et al. Interaction of hemostatic genetics with hormone therapy: new insights to explain arterial thrombosis in postmenopausal women. Chest 2002;121:906–20.
4. Kottke-Marchant K. Genetic polymorphisms associated with venous and arterial thrombosis: an overview. Arch Pathol Lab Med 2002;126:295–304.
5. Endler G, Mannhalter C. Polymorphisms in coagulation factor genes and their impact on arterial and venous thrombosis. Clin Chim Acta 2003;330:31–55.
6. Federici C, Gianetti J, Andreassi MG. Genomic medicine and thrombotic risk: who, when, how and why? Int J Cardiol 2006;106:3–9.
7. Schifreen RS, Storts DR, Buller AM. The challenge of using SNPs in the understanding and treatment of disease. Biotechniques 2002;18:20–1.
8. Fleming DA. Ethical considerations of genetic testing. J Clin Ethics 2002;13:316–23.

9. Bates BR, Templeton A, Achter PJ, Harris TM, Condit CM. What does "a gene for heart disease" mean? A focus group study of public understandings of genetic risk factors. Am J Med Genet 2003;119:156–61.
10. Jordan WM. Pulmonary embolism. Lancet 1961;2:1146–7.
11. Rosendaal FR, Helmerhorst FM, Vandenbroucke JP. Female hormones and thrombosis. Arterioscler Thromb Vasc Biol 2002;22:201–10.
12. Jick H, Jick SS, Gurewich V, Myers MW, Vasilakis C. Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. Lancet 1995; 346:1589–93.
13. Vandenbroucke JP, Rosing J, Bloemenkamp KW, Middeldorp S, Helmerhorst FM, Bouma BN, et al. Oral contraceptives and the risk of venous thrombosis. N Engl J Med 2001;344:1527–35.
14. Jick H, Kaye JA, Vasilakis-Scaramozza C, Jick SS. Risk of venous thromboembolism among users of third generation oral contraceptives compared with users of oral contraceptives with levonorgestrel before and after 1995: cohort and case-control analysis. Br Med J 2000;321:1190–5.
15. Rosendaal FR, Helmerhorst FM, Vandenbroucke JP. Oral contraceptives, hormone replacement therapy and thrombosis. Thromb Haemost 2001;86:112–23.
16. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. J Am Med Assoc 2002;288: 321–33.
17. Grady D, Wenger NK, Herrington D, Khan S, Furberg C, Hunninghake D, et al. Postmenopausal hormone therapy increases risk for venous thromboembolic disease. The Heart and Estrogen/progestin Replacement Study. Ann Intern Med 2000;132:689–96.
18. Herrington DM, Vittinghoff E, Howard TD, Major DA, Owen J, Reboussin DM, et al. Factor V Leiden, hormone replacement therapy, and risk of venous thromboembolic events in women with coronary disease. Arterioscler Thromb Vasc Biol 2002;22:1012–7.
19. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. J Am Med Assoc 1998;280:605–13.
20. Herrington DM. The HERS trial results: paradigms lost? Heart and Estrogen/progestin Replacement Study. Ann Intern Med 1999;131:463–6.
21. Renner W, Cichocki L, Forjanics A, Koppel H, Gasser R, Pilger E. G-455A polymorphism of the fibrinogen beta gene and deep vein thrombosis. Eur J Clin Invest 2002; 32:755–8.
22. Sartori MT, Danesin C, Saggiorato G, Tormene D, Simioni P, Spiezia L, et al. The PAI-1 gene 4G/5G polymorphism and deep vein thrombosis in patients with inherited thrombophilia. Clin Appl Thromb Hemost 2003;9:299–307.
23. Fatini C, Gensini F, Sticchi E, Battaglini B, Prisco D, Fedi S, et al. ACE DD genotype: an independent predisposition factor to venous thromboembolism. Eur J Clin Invest 2003;33:642–7.
24. Zidane M, de Visser MC, ten Wolde M, Vos HL, de Monye W, Bertina RM, et al. Frequency of the TAFI –438 G/A and factor XIIIA Val34Leu polymorphisms in patients with objectively proven pulmonary embolism. Thromb Haemost 2003;90:439–45.
25. Komanasin N, Catto AJ, Futers TS, van Hylckama Vlieg A, Rosendaal FR, Ariens RA. A novel polymorphism in the factor XIII B-subunit (His95Arg): relationship to sub-unit dissociation and venous thrombosis. J Thromb Haemost 2005;3:2487–96.
26. van Wijk R, Nieuwenhuis K, van den Berg M, Huizinga EG, van der Meijden BB, Kraaijenhagen RJ, et al. Five novel mutations in the gene for human blood coagulation factor V associated with type I factor V deficiency. Blood 2001;98:358–67.
27. Mingozzi F, Legnani C, Lunghi B, Scanavini D, Castoldi E, Palareti G, et al. A FV multiallelic marker detects genetic components of APC resistance contributing to venous thromboembolism in FV Leiden carriers. Thromb Haemost 2003;89:983–9.
28. Egeberg O. Inherited antithrombin deficiency causing thrombophilia. Thromb Diath Haemorrh 1965;13:516–30.
29. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994;369:64–7.
30. 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;88:3698–703.
31. Rees DC. World distribution of factor V Leiden. Lancet 1995;346:1133–4.
32. Tripodi A, Mannucci PM. Laboratory investigation of thrombophilia. Clin Chem 2001;47:1597–606.
33. 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; 85:1504–8.
34. 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;344:1453–7.
35. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Buller HR, Vandenbroucke JP. Enhancement by factor V Leiden mutation of risk of deep-vein thrombosis associated with oral contraceptives containing a third-generation progestagen. Lancet 1995;346:1593–6.
36. Andersen BS, Olsen J, Nielsen GL, Steffensen FH, Sorensen HT, Baech J, et al. Third generation oral contraceptives and heritable thrombophilia as risk factors of non-fatal venous thromboembolism. Thromb Haemost 1998;79:28–31.
37. Martinelli I, Cattaneo M, Taioli E, De Stefano V, Chiusolo P, Mannucci PM. Interaction between the G20210A mutation of the prothrombin gene and oral contraceptive use in deep vein thrombosis. Arterioscler Thromb Vasc Biol 1999;19:700–3.
38. Spannagl M, Heinemann LA, Schramm W. Are factor V Leiden carriers who use oral contraceptives at extreme risk for venous thromboembolism? Eur J Contracept Reprod Health Care 2000;5:105–12.
39. Wu O, Robertson L, Langhorne P, Twaddle S, Lowe GD, Clark P, et al. Oral contraceptives, hormone replacement therapy, thrombophilias and risk of venous thromboembolism: a systematic review. The Thrombosis: Risk and Economic Assessment of Thrombophilia Screening (TREATS) Study. Thromb Haemost 2005;94:17–25.

40. Rintelen C, Mannhalter C, Ireland H, Lane DA, Knobl P, Lechner K, et al. Oral contraceptives enhance the risk of clinical manifestation of venous thrombosis at a young age in females homozygous for factor V Leiden. Br J Haematol 1996;93:487–90.
41. Lippi G, Manzato F, Brocco G, Franchini M, Guidi G. Prothrombotic effects and clinical implications of third-generation oral contraceptives use. Blood Coagul Fibrinolysis 2002;13:69–72.
42. Rosing J, Tans G, Nicolaes GA, Thomassen MC, van Oerle R, van der Ploeg PM, et al. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. Br J Haematol 1997;97:233–8.
43. Rosendaal FR, Vessey M, Rumley A, Daly E, Woodward M, Helmerhorst FM, et al. Hormonal replacement therapy, prothrombotic mutations and the risk of venous thrombosis. Br J Haematol 2002;116:851–4.
44. Lowe G, Woodward M, Vessey M, Rumley A, Gough P, Daly E. Thrombotic variables and risk of idiopathic venous thromboembolism in women aged 45–64 years. Relationships to hormone replacement therapy. Thromb Haemost 2000;83:530–5.
45. Glueck CJ, Wang P, Fontaine RN, Tracy T, Sieve-Smith L, Lang JE. Effect of exogenous estrogen on atherothrombotic vascular disease risk related to the presence or absence of the factor V Leiden mutation (resistance to activated protein C). Am J Cardiol 1999;84:549–54.
46. Franco RF, Trip MD, ten Cate H, van den Ende A, Prins MH, Kastelein JJ, et al. The 20210 G→A mutation in the 3′-untranslated region of the prothrombin gene and the risk for arterial thrombotic disease. Br J Haematol 1999; 104:50–4.
47. De Stefano V, Martinelli I, Mannucci PM, Paciaroni K, Chiusolo P, Casorelli I, et al. The risk of recurrent deep venous thrombosis among heterozygous carriers of both factor V Leiden and the G20210A prothrombin mutation. N Engl J Med 1999;341:801–6.
48. De Stefano V, Zappacosta B, Persichilli S, Rossi E, Casorelli I, Paciaroni K, et al. Prevalence of mild hyperhomocysteinaemia and association with thrombophilic genotypes (factor V Leiden and prothrombin G20210A) in Italian patients with venous thromboembolic disease. Br J Haematol 1999;106:564–8.
49. Martinelli I, Bucciarelli P, Margaglione M, De Stefano V, Castaman G, Mannucci PM. The risk of venous thromboembolism in family members with mutations in the genes of factor V or prothrombin or both. Br J Haematol 2000;111:1223–9.
50. Emmerich J, Rosendaal FR, Cattaneo M, Margaglione M, De Stefano V, Cumming T, et al. Combined effect of factor V Leiden and prothrombin 20210A on the risk of venous thromboembolism – pooled analysis of 8 case-control studies including 2310 cases and 3204 controls. Study Group for Pooled-Analysis in Venous Thromboembolism. Thromb Haemost 2001;86:809–16 (Erratum in: Thromb Haemost 2001;86:1598).
51. Legnani C, Palareti G, Guazzaloca G, Cosmi B, Lunghi B, Bernardi F, et al. Venous thromboembolism in young women; role of thrombophilic mutations and oral contraceptive use. Eur Heart J 2002;23:984–90.
52. Martinelli I, Sacchi E, Landi G, Taioli E, Duca F, Mannucci PM. High risk of cerebral-vein thrombosis in carriers of a prothrombin-gene mutation and in users of oral contraceptives. N Engl J Med 1998;338:1793–7.
53. Straczek C, Oger E, Beau Yon de Jonage-Canonico M, Plu-Bureau G, Conard J, Meyer G, et al. Estrogen and Thromboembolism Risk (ESTHER) Study Group. Prothrombotic mutations, hormone therapy, and venous thromboembolism among postmenopausal women: impact of the route of estrogen administration. Circulation 2005;112:3495–500.
54. Glueck CJ, Wang P, Fontaine RN, Tracy T, Sieve-Smith L, Lang JE. The effect of exogenous estrogen on atherothrombotic vascular disease risk relates to the presence or absence of the 20210 G/A prothrombin gene mutation: a cross-sectional study of 230 hyperlipidemic women. Circulation 2000;102:II278–9.
55. Psaty BM, Smith NL, Lemaitre RN, Vos HL, Heckbert SR, LaCroix AZ, et al. Hormone replacement therapy, prothrombotic mutations, and the risk of incident nonfatal myocardial infarction in postmenopausal women. J Am Med Assoc 2001;285:906–13.
56. Fermo I, D'Angelo SV, Paroni R, Mazzola G, Calori G, D'Angelo A. Prevalence of moderate hyperhomocysteinemia in patients with early-onset venous and arterial occlusive disease. Ann Intern Med 1995;123:747–53.
57. den Heijer M, Koster T, Blom HJ, Bos GM, Briet E, Reitsma PH, et al. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. N Engl J Med 1996;334:759–62.
58. den Heijer M, Rosendaal FR, Blom HJ, Gerrits WB, Bos GM. Hyperhomocysteinemia and venous thrombosis: a meta-analysis. Thromb Haemost 1998;80:874–7.
59. Martinelli I, Battaglioli T, Pedotti P, Cattaneo M, Mannucci PM. Hyperhomocysteinemia in cerebral vein thrombosis. Blood 2003;102:1363–6.
60. Haynes WG. Hyperhomocysteinemia, vascular function and atherosclerosis: effects of vitamins. Cardiovasc Drugs Ther 2002;16:391–9.
61. Gellekink H, den Heijer M, Heil SG, Blom HJ. Genetic determinants of plasma total homocysteine. Semin Vasc Med 2005;5:98–109.
62. Kang SS, Zhou J, Wong PW, Kowlisyn J, Strokosch G. Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet 1988;48:536–45.
63. Franco RF, Araujo AG, Guerreiro JF, Elion J, Zago MA. Analysis of the 677 C→T mutation of the methylenetetrahydrofolate reductase gene in different ethnic groups. Thromb Haemost 1998;79:119–21.
64. Key NS, McGlennen RC. Hyperhomocyst(e)inemia and thrombophilia. Arch Pathol Lab Med 2002;126:1367–75.
65. Kluijtmans LA, Den Heijer M, Reitsma PH, Heil SG, Blom HJ, Rosendaal FR. Thermolabile methylenetetrahydrofolate reductase and factor V Leiden in the risk of deep-vein thrombosis. Thromb Haemost 1998;79:254–8.
66. Domagala TB, Adamek L, Nizankowska E, Sanak M, Szczeklik A. Mutations C677T and A1298C of the 5,10-methylenetetrahydrofolate reductase gene and fasting plasma homocysteine levels are not associated with the increased risk of venous thromboembolic disease. Blood Coagul Fibrinolysis 2002;13:423–31.
67. Zalavras ChG, Giotopoulou S, Dokou E, Mitsis M, Ioannou HV, Tzolou A, et al. Lack of association between the C677T mutation in the 5,10-methylenetetrahydrofolate reductase gene and venous thromboembolism in Northwestern Greece. Int Angiol 2002;21:268–71.
68. Morelli VM, Lourenco DM, D'Almeida V, Franco RF, Miranda F, Zago MA, et al. Hyperhomocysteinemia increases the risk of venous thrombosis independent of the C677T mutation of the methylenetetrahydrofolate reductase gene

in selected Brazilian patients. Blood Coagul Fibrinolysis 2002;13:271–5.
69. Ray JG, Langman LJ, Vermeulen MJ, Evrovski J, Yeo EL, Cole DE. Genetics University of Toronto Thrombophilia Study in Women (GUTTSI): genetic and other risk factors for venous thromboembolism in women. Curr Control Trials Cardiovasc Med 2001;2:141–9.
70. Varela ML, Adamczuk YP, Forastiero RR, Martinuzzo ME, Cerrato GS, Pombo G, et al. Major and potential prothrombotic genotypes in a cohort of patients with venous thromboembolism. Thromb Res 2001;104:317–24.
71. Frederiksen J, Juul K, Grande P, Jensen GB, Schroeder TV, Tybjaerg-Hansen A, et al. Methylenetetrahydrofolate reductase polymorphism (C677T), hyperhomocysteinemia, and risk of ischemic cardiovascular disease and venous thromboembolism: prospective and case-control studies from the Copenhagen City Heart Study. Blood 2004;104: 3046–51.
72. Den Heijer M, Lewington S, Clarke R. Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiological studies. J Thromb Haemost 2005;3:292–9.
73. Sidney S, Petitti DB, Soff GA, Cundiff DL, Tolan KK, Quesenberry CP Jr. Venous thromboembolic disease in users of low-estrogen combined estrogen-progestin oral contraceptives. Contraception 2004;70:3–10.
74. Brown CA, McKinney KQ, Young KB, Norton HJ. The C677T methylenetetrahydrofolate reductase polymorphism influences the homocysteine-lowering effect of hormone replacement therapy. Mol Genet Metab 1999;67: 43–8.
75. Tanis BC, Blom HJ, Bloemenkamp DG, van den Bosch MA, Algra A, van der Graaf Y, et al. Folate, homocysteine levels, methylenetetrahydrofolate reductase (MTHFR) 677C→T variant, and the risk of myocardial infarction in young women: effect of female hormones on homocysteine levels. J Thromb Haemost 2004;2:35–41.
76. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Vandenbroucke JP. Higher risk of venous thrombosis during early use of oral contraceptives in women with inherited clotting defects. Arch Intern Med 2000;160:49–52.
77. Konkle BA, Schafer AI. Hemostasis, thrombosis, fibrinolysis, and cardiovascular disease. In: Zipes D, Libby P, Bonow R, Branuwald E, editors. Braunwald's heart disease, 7th ed. Philadelphia, PA: Elsevier-Saunders, 2005: 2067–92.
78. Grody WW, Griffin JH, Taylor AK, Korf BR, Heit JA, ACMG Factor V Leiden Working Group. American College of Medical Genetics consensus statement on factor V Leiden mutation testing. Genet Med 2001;3:139–48.
79. Kujovich JL. Hormones and pregnancy: thromboembolic risks for women. Br J Haematol 2004;126:443–54.
80. Mannucci PM. Laboratory detection of inherited thrombophilia: a historical perspective. Semin Thromb Hemost 2005;31:5–10.
81. Hertzberg MS. Genetic testing for thrombophilia mutations. Semin Thromb Hemost 2005;31:33–8.
82. Cosmi B, Coccheri S. Thrombophilia in young women candidate to the pill: reasons for and against screening. Pathophysiol Haemost Thromb 2002;32:315–7.
83. Wu O, Robertson L, Twaddle S, Lowe G, Clark P, Walker I, et al. The Thrombosis: Risk and Economic Assessment of Thrombophilia Screening (TREATS) Study. Screening for thrombophilia in high-risk situations: a meta-analysis and cost-effectiveness analysis. Br J Haematol 2005;131:80–90.
84. Martinelli I, De Stefano V, Taioli E, Paciaroni K, Rossi E, Mannucci PM. Inherited thrombophilia and first venous thromboembolism during pregnancy and puerperium. Thromb Haemost 2002;87:791–5.
85. Kupferminc MJ, Eldor A, Steinman N, Many A, Bar-Am A, Jaffa A, et al. Increased frequency of genetic thrombophilia in women with complications of pregnancy. N Engl J Med 1999;340:9–13.
86. Blumenfeld Z. Thrombophilia-associated pregnancy wastage. Fertil Steril 1999;72:765–74.
87. Caprini JA, Glase CJ, Anderson CB, Hathaway K. Laboratory markers in the diagnosis of venous thromboembolism. Circulation 2004;109:I4–8.
88. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C. Prediction of a cofactor for activated protein C. Proc Natl Acad Sci USA 1993;90:1004–8.
89. Tripodi A, Mannucci PM. Laboratory investigation of thrombophilia. Clin Chem 2001;47:1597–606.