THROMBOINCODE

Personalizing risk-assessment in **THROMBOSIS**

Ref. 1

The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII and affects cross-linked fibrin structure

Robert A. S. Ariëns, Helen Philippou, Chandrasekaran Nagaswami, John W. Weisel, David A. Lane, and Peter J. Grant

Factor XIII on activation by thrombin cross-links fibrin. A common polymorphism Val to Leu at position 34 in the FXIII A subunit is under investigation as a risk determinant of thrombosis. Because Val34Leu is close to the thrombin cleavage site, the hypothesis that it would alter the function of FXIII was tested. Analysis of FXIII subunit proteolysis by thrombin using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high-performance liquid chromatography showed that FXIII 34Leu was cleaved by thrombin more rapidly and by lower doses than 34Val. Mass spectrometry of isolated activation peptides confirmed the predicted single methyl group difference and demonstrated that the thrombin cleavage site is unaltered by Val34Leu. Kinetic analysis of activation peptide release demonstrated that the catalytic efficiency (k_{cat}/K_m) of thrombin was 0.5 for FXIII 34Leu and 0.2 (μmol/L)$^{-1}$ × sec^{-1} for 34Val. Presence of fibrin increased the catalytic efficiency to 4.8 and 2.2 (μmol/L)$^{-1}$ × sec^{-1}, respectively. Although the 34Leu peptide was released at a similar rate as fibrinopeptide A, the 34Val peptide was released more slowly than fibrinopeptide A but more quickly than fibrinopeptide B generation. Cross-linking of γ- and α-chains appeared earlier when fibrin was incubated with FXIII 34Leu than with 34Val. Fully activated 34Leu and 34Val FXIII showed similar cross-linking activity. Analysis of fibrin clots prepared using plasma from FXIII 34Leu subjects by turbidity and permeability measurements showed reduced fiber mass/length ratio and porosity compared to 34Val. The structural differences were confirmed by electron microscopy. These results demonstrate that Val34Leu accelerates activation of FXIII by thrombin and consequently affects the structure of the cross-linked fibrin clot. (Blood. 2000;96:988-995)

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Introduction

On cleavage of fibrinopeptides A and B by thrombin, fibrin spontaneously polymerizes into a network of multimeric strands, initially held together by noncovalent interaction. Blood coagulation factor XIII (FXIII) is activated by thrombin, and activated FXIII (FXIIIa) covalently cross-links the fibrin clot to increase resistance to chemical, mechanical, and proteolytic insults. FXIII is a tetrameric protransglutaminase consisting of 2 A subunits, which contain the active site, and 2 B subunits, which serve a carrier function for the A subunit in plasma.[1,2] FXIII is also found in platelets as an A-subunit dimer.[2] Platelets do not release FXIII on activation, but lysis of platelets entrapped in the blood clot may increase local concentrations of FXIII.[3]

FXIIIa catalyzes the introduction of γ-glutamyl-ϵ-lysine peptide bonds between fibrin γ- and α-chains. Other substrates of FXIIIa are α_2-antiplasmin,[4] von Willebrand factor,[5] thrombospondin,[6] and fibronectin.[7,8] Cross-linking of these substrates into the clot further contributes to the mechanical strength, viscosity, and resistance to proteolysis of fibrin. Activation of FXIII involves thrombin-induced cleavage of the peptide bond between Arg37 and Gly38 of the A subunit,[2,9] resulting in the release of an amino-terminal activation peptide. In a second step, calcium induces dissociation of the A-subunit dimer from the B subunit.[10,11] Both steps are essential for the activation of FXIII; the activation peptide release induces a conformational change in the A subunit and enables the dissociation of the subunits in the presence of calcium to unmask the catalytic site.

A common polymorphism with an allele frequency of around 25% has been identified in the FXIII A subunit (Val34Leu), 3 amino acids from the thrombin activation site.[12] Recent studies have reported that the prevalence of the Leu encoding allele is lower in patients with myocardial infarction,[13,14] deep vein thrombosis,[15,16] and cerebral infarction[17] when compared with matched control groups. These clinical studies suggest that this polymorphism may be a risk determinant of thrombosis in both the arterial and venous systems. Paradoxically, ex vivo and in vitro studies have suggested that possession of the Leu allele leads to increased cross-linking rates by FXIIIa.[18-20] The mechanisms by which this occurred remained unclear. In view of the close proximity of the Val34Leu polymorphism to the activation site, we tested the hypothesis that this polymorphism alters the activation rate of FXIII by thrombin and affects fibrin structure.

Materials and methods

Blood sampling and processing

Venous blood was obtained from individuals with the homozygous FXIII 34Val, homozygous 34Leu, and heterozygous genotypes after an overnight

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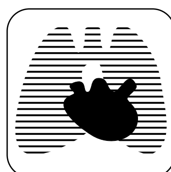

CHEST Supplement

ANTITHROMBOTIC THERAPY AND PREVENTION OF THROMBOSIS, 9TH ED: ACCP GUIDELINES

VTE, Thrombophilia, Antithrombotic Therapy, and Pregnancy

Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines

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Background: The use of anticoagulant therapy during pregnancy is challenging because of the potential for both fetal and maternal complications. This guideline focuses on the management of VTE and thrombophilia as well as the use of antithrombotic agents during pregnancy.

Methods: The methods of this guideline follow the Methodology for the Development of Antithrombotic Therapy and Prevention of Thrombosis Guidelines: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines in this supplement.

Results: We recommend low-molecular-weight heparin for the prevention and treatment of VTE in pregnant women instead of unfractionated heparin (Grade 1B). For pregnant women with acute VTE, we suggest that anticoagulants be continued for at least 6 weeks postpartum (for a minimum duration of therapy of 3 months) compared with shorter durations of treatment (Grade 2C). For women who fulfill the laboratory criteria for antiphospholipid antibody (APLA) syndrome and meet the clinical APLA criteria based on a history of three or more pregnancy losses, we recommend antepartum administration of prophylactic or intermediate-dose unfractionated heparin or prophylactic low-molecular-weight heparin combined with low-dose aspirin (75-100 mg/d) over no treatment (Grade 1B). For women with inherited thrombophilia and a history of pregnancy complications, we suggest not to use antithrombotic prophylaxis (Grade 2C). For women with two or more miscarriages but without APLA or thrombophilia, we recommend against antithrombotic prophylaxis (Grade 1B).

Conclusions: Most recommendations in this guideline are based on observational studies and extrapolation from other populations. There is an urgent need for appropriately designed studies in this population. ***CHEST 2012; 141(2)(Suppl):e691S–e736S***

Abbreviations: APLA = antiphospholipid antibody; aPPT = activated partial thromboplastin time; HIT = heparin-induced thrombocytopenia; INR = international normalized ratio; LMWH = low-molecular-weight heparin; NNT = number needed to treat; PE = pulmonary embolism; RR = risk ratio; UFH = unfractionated heparin

Summary of Recommendations

Note on Shaded Text: Throughout this guideline, shading is used within the summary of recommendations sections to indicate recommendations that are newly added or have been changed since the publication of Antithrombotic and Thrombolytic Therapy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Recommendations that remain unchanged are not shaded.

2.2.1. For pregnant patients, we recommend LMWH for the prevention and treatment of VTE, instead of UFH (Grade 1B)**.**

3.0.1. For women receiving anticoagulation for the treatment of VTE who become pregnant, we recommend LMWH over vitamin K antagonists during the first trimester (Grade 1A)**, in the second and third trimesters** (Grade 1B)**, and during late pregnancy when delivery is imminent** (Grade 1A)**.**

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3.0.2. For women requiring long-term vitamin K antagonists who are attempting pregnancy and are candidates for LMWH substitution, we suggest performing frequent pregnancy tests and substituting LMWH for vitamin K antagonists when pregnancy is achieved rather than switching to LMWH while attempting pregnancy (Grade 2C)**.**

Remarks: Women who place little value on avoiding the risks, inconvenience, and costs of LMWH therapy of uncertain duration while awaiting pregnancy and a high value on minimizing the risks of early miscarriage associated with vitamin K antagonist therapy are likely to choose LMWH while attempting pregnancy.

3.0.3. For pregnant women, we suggest limiting the use of fondaparinux and parenteral direct thrombin inhibitors to those with severe allergic reactions to heparin (eg, HIT) who cannot receive danaparoid (Grade 2C)**.**

3.0.4. For pregnant women, we recommend avoiding the use of oral direct thrombin (eg, dabigatran) and anti-Xa (eg, rivaroxaban, apixaban) inhibitors (Grade 1C)**.**

4.0.1. For lactating women using warfarin, acenocoumarol, or UFH who wish to breast-feed, we recommend continuing the use of warfarin, acenocoumarol, or UFH (Grade 1A)**.**

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4.0.2. For lactating women using LMWH, danaparoid, or r-hirudin who wish to breast-feed, we recommend continuing the use of LMWH, danaparoid, or r-hirudin (Grade 1B)**.**

4.0.3. For breast-feeding women, we suggest alternative anticoagulants rather than fondaparinux (Grade 2C)**.**

4.0.4. For breast-feeding women, we recommend alternative anticoagulants rather than oral direct thrombin (eg, dabigatran) and factor Xa inhibitors (eg, rivaroxaban, apixaban) (Grade 1C)**.**

4.0.5. For lactating women using low-dose aspirin for vascular indications who wish to breast-feed, we suggest continuing this medication (Grade 2C)**.**

5.1.1. For women undergoing assisted reproduction, we recommend against the use of routine thrombosis prophylaxis (Grade 1B)**.**

5.1.2. For women undergoing assisted reproduction who develop severe ovarian hyperstimulation syndrome, we suggest thrombosis prophylaxis (prophylactic LMWH) for 3 months postresolution of clinical ovarian hyperstimulation syndrome rather than no prophylaxis (Grade 2C)**.**

Remarks: Women who are averse to taking medication for very small benefit and those who consider self-injecting a considerable burden will be disinclined to use LMWH for extended thrombosis prophylaxis. Given that the absolute benefit decreases as time from the hyperstimulation event increases, such women will be very disinclined to continue prophylaxis throughout the entire resultant pregnancy.

6.2.1. For women undergoing cesarean section without additional thrombosis risk factors, we recommend against the use of thrombosis prophylaxis other than early mobilization (Grade 1B)**.**

6.2.2. For women at increased risk of VTE after cesarean section because of the presence of one major or at least two minor risk factors, we suggest pharmacologic thromboprophylaxis (prophylactic LMWH) or mechanical prophylaxis (elastic stockings or intermittent pneumatic compression) in those with contraindications to anticoagulants while in hospital following delivery rather than no prophylaxis (Grade 2B)**.**

Remarks: The reduced bleeding risk with mechanical prophylaxis should be weighed against the inconvenience of elastic stockings and intermittent pneumatic compression.

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6.2.3. For women undergoing cesarean section who are considered to be at very high risk for VTE and who have multiple additional risk factors for thromboembolism that persist in the puerperium, we suggest that prophylactic LMWH be combined with elastic stockings and/or intermittent pneumatic compression over LMWH alone (Grade 2C).

6.2.4. For selected high-risk patients in whom significant risk factors persist following delivery, we suggest extended prophylaxis (up to 6 weeks after delivery) following discharge from the hospital (Grade 2C).

7.1.1. For pregnant women with acute VTE, we recommend therapy with adjusted-dose subcutaneous LMWH over adjusted-dose UFH (Grade 1B).

7.1.2. For pregnant women with acute VTE, we recommend LMWH over vitamin K antagonist treatment antenatally (Grade 1A).

7.1.3. For pregnant women with acute VTE, we suggest that anticoagulants should be continued for at least 6 weeks postpartum (for a minimum total duration of therapy of 3 months) in comparison with shorter durations of treatment (Grade 2C).

7.1.4. For pregnant women receiving adjusted-dose LMWH therapy and where delivery is planned, we recommend discontinuation of LMWH at least 24 h prior to induction of labor or cesarean section (or expected time of neuraxial anesthesia) rather than continuing LMWH up until the time of delivery (Grade 1B).

8.2.1. For all pregnant women with prior VTE, we suggest postpartum prophylaxis for 6 weeks with prophylactic- or intermediate-dose LMWH or vitamin K antagonists targeted at INR 2.0 to 3.0 rather than no prophylaxis (Grade 2B).

8.2.2. For pregnant women at low risk of recurrent VTE (single episode of VTE associated with a transient risk factor not related to pregnancy or use of estrogen), we suggest clinical vigilance antepartum rather than antepartum prophylaxis (Grade 2C).

8.2.3. For pregnant women at moderate to high risk of recurrent VTE (single unprovoked VTE, pregnancy- or estrogen-related VTE, or multiple prior unprovoked VTE not receiving long-term anticoagulation), we suggest antepartum prophylaxis with prophylactic- or intermediate-dose LMWH rather than clinical vigilance or routine care (Grade 2C).

8.2.4. For pregnant women receiving long-term vitamin K antagonists, we suggest adjusted-dose LMWH or 75% of a therapeutic dose of LMWH throughout pregnancy followed by resumption of long-term anticoagulants postpartum, rather than prophylactic-dose LMWH (Grade 2C).

9.2.1. For pregnant women with no prior history of VTE who are known to be homozygous for factor V Leiden or the prothrombin 20210A mutation and have a positive family history for VTE, we suggest antepartum prophylaxis with prophylactic- or intermediate-dose LMWH and postpartum prophylaxis for 6 weeks with prophylactic- or intermediate-dose LMWH or vitamin K antagonists targeted at INR 2.0 to 3.0 rather than no prophylaxis (Grade 2B).

9.2.2. For pregnant women with all other thrombophilias and no prior VTE who have a positive family history for VTE, we suggest antepartum clinical vigilance and postpartum prophylaxis with prophylactic- or intermediate-dose LMWH or, in women who are not protein C or S deficient, vitamin K antagonists targeted at INR 2.0 to 3.0 rather than routine care (Grade 2C).

9.2.3. For pregnant women with no prior history of VTE who are known to be homozygous for factor V Leiden or the prothrombin 20210A mutation and who do not have a positive family history for VTE, we suggest antepartum clinical vigilance and postpartum prophylaxis for 6 weeks with prophylactic- or intermediate-dose LMWH or vitamin K antagonists targeted at INR 2.0 to 3.0 rather than routine care (Grade 2B).

9.2.4. For pregnant women with all other thrombophilias and no prior VTE who do not have a positive family history for VTE, we suggest antepartum and postpartum clinical vigilance rather than pharmacologic prophylaxis (Grade 2C).

10.2.1. For women with recurrent early pregnancy loss (three or more miscarriages before 10 weeks of gestation), we recommend screening for APLAs (Grade 1B).

10.2.2. For women with a history of pregnancy complications, we suggest not to screen for inherited thrombophilia (Grade 2C).

10.2.3. For women who fulfill the laboratory criteria for APLA syndrome and meet the clinical

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APLA criteria based on a history of three or more pregnancy losses, we recommend antepartum administration of prophylactic- or intermediate-dose UFH or prophylactic LMWH combined with low-dose aspirin, 75 to 100 mg/d, over no treatment (Grade 1B)**.**

10.2.4. For women with inherited thrombophilia and a history of pregnancy complications, we suggest not to use antithrombotic prophylaxis (Grade 2C)**.**

11.1.1. For women considered at risk for preeclampsia, we recommend low-dose aspirin throughout pregnancy, starting from the second trimester, over no treatment (Grade 1B)**.**

11.2.1. For women with two or more miscarriages but without APLA or thrombophilia, we recommend against antithrombotic prophylaxis (Grade 1B)**.**

12.1.1. For pregnant women with mechanical heart valves, we recommend one of the following anticoagulant regimens in preference to no anticoagulation (all Grade 1A)**:**

(a) Adjusted-dose bid LMWH throughout pregnancy. We suggest that doses be adjusted to achieve the manufacturer's peak anti-Xa LMWH 4 h postsubcutaneous-injection or

(b) Adjusted-dose UFH throughout pregnancy administered subcutaneously every 12 h in doses adjusted to keep the mid-interval aPTT at least twice control or attain an anti-Xa heparin level of 0.35 to 0.70 units/mL or

(c) UFH or LMWH (as above) until the 13th week, with substitution by vitamin K antagonists until close to delivery when UFH or LMWH is resumed.

Remarks: For pregnant women with mechanical heart valves, the decision regarding the choice of anticoagulant regimen is so value and preference dependent (risk of thrombosis vs risk of fetal abnormalities) that we consider the decision to be completely individualized. Women of childbearing age and pregnant women with mechanical valves, should be counseled about potential maternal and fetal risks associated with various anticoagulant regimens, including continuation of vitamin K antagonists with substitution by LMWH or UFH close to term, substitution of vitamin K antagonists by LMWH or UFH until the 13th week and then close to term, and use of LMWH or UFH throughout pregnancy. Usual long-term anticoagulants should be resumed postpartum when adequate hemostasis is assured.

12.1.2. In women judged to be at very high risk of thromboembolism in whom concerns exist about the efficacy and safety of UFH or LMWH as dosed above (eg, older generation prosthesis in the mitral position or history of thromboembolism), we suggest vitamin K antagonists throughout pregnancy with replacement by UFH or LMWH (as above) close to delivery rather than one of the regimens above (Grade 2C)**.**

Remarks: Women who place a higher value on avoiding fetal risk than on avoiding maternal complications (eg, catastrophic valve thrombosis) are likely to choose LMWH or UFH over vitamin K antagonists.

12.1.3. For pregnant women with prosthetic valves at high risk of thromboembolism, we suggest the addition of low-dose aspirin, 75 to 100 mg/d (Grade 2C)**.**

This article is devoted to the use of antithrombotic therapy in pregnant women. Anticoagulant therapy is indicated during pregnancy for the prevention and treatment of VTE; for the prevention and treatment of systemic embolism in patients with mechanical heart valves; and, in combination with aspirin, for the prevention of recurrent pregnancy loss in women with antiphospholipid antibodies (APLAs).

The use of anticoagulation for prevention of pregnancy complications in women with hereditary thrombophilia is becoming more frequent. Given the absence of proven-effective therapy in women with unexplained recurrent pregnancy loss, there is also growing pressure to intervene with antithrombotic therapy in affected women with no known underlying thrombophilia. The use of anticoagulant therapy during pregnancy is challenging because of the potential for fetal and maternal complications.

1.0 Methods

Table 1 describes both the question definition (ie, population, intervention, comparator, and outcomes) and the eligibility criteria for studies considered in each section of the recommendations that follow. We consider the desirable and undesirable fetal and maternal consequences of antithrombotic therapy in the following populations: (1) breast-feeding women, (2) women using assisted reproductive technology, (3) women undergoing cesarean section, (4) pregnant women with newly diagnosed VTE, (5) pregnant women with prior VTE, (6) pregnant women with asymptomatic thrombophilia, (7) pregnant women with a history of pregnancy complications (including pregnancy loss, preeclampsia, fetal growth restriction, and placental abruption), and (8) pregnant women with mechanical heart valves.

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Table 1—*[Section 1.0] Structured Clinical Questions*

Section	Informal Question	PICO Question: Population	Intervention	Comparator	Outcome	Methodology
Maternal complications of antithrombotic therapy (section 2.0)	• Adverse maternal outcomes of commonly used antithrombotic agents while pregnant	• Pregnant women	• Unfractionated heparin • Low-molecular-weight heparin • Other relevant agents[a]	• No antithrombotic therapy or • Other antithrombotic therapy	• Maternal bleeding (total major) • Maternal bleeding (major: fatal + intracranial) • Maternal bleeding (major: nonfatal + nonintracranial) • Maternal bleeding (minor) • Maternal heparin-induced thrombocytopenia • Maternal heparin-associated osteoporosis • Maternal skin reaction (allergic)	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Fetal complications of antithrombotic therapy during pregnancy (section 3.0)	• Safety of antithrombotic therapy during pregnancy	• Fetuses and children of women using antithrombotic therapy during pregnancy	• Vitamin K antagonists • Unfractionated heparin • Low-molecular-weight heparin • Other relevant agents[a]	• No antithrombotic therapy exposure or • Other antithrombotic agent	• Fetal hemorrhage • Pregnancy loss • Congenital malformations • Developmental delay • Levels or results of coagulation testing in umbilical cord blood • Birth weight (centile); number small for dates	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Use of antithrombotic therapy in nursing mothers (section 4.0)	• Safety of antithrombotic therapy while breast-feeding	• Breast-fed infants of women receiving antithrombotic therapy	• Vitamin K antagonists • Unfractionated heparin • Low-molecular-weight heparin • Other relevant agents[a]	• No antithrombotic therapy exposure or • Other antithrombotic agent	• Infant hemorrhage • Levels or results of coagulation testing in breast milk • Levels or results of coagulation testing in plasma of breast-fed infants	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Prevention of VTE with assisted reproductive technology (section 5.0)	• Risk of VTE in women undergoing assisted reproduction -No additional risk factors -Prior VTE -Thrombophilia[b]	• Women using assisted reproductive technology to become pregnant	• No prophylaxis	• No intervention	• Proportion of pregnancies that are successful • DVT • Pulmonary embolism • Mortality • Major bleeding[c] • Bleeding during oocyte retrieval and embryo transfer	• Control arms of randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies

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Table 1—*Continued*

		PICO Question				
Section	Informal Question	Population	Intervention	Comparator	Outcome	Methodology
	• Choice, duration, and (if appropriate) route/dose of prophylaxis	• Women using assisted reproductive technology to become pregnant	• Low-molecular-weight heparin • Unfractionated heparin • Graduated compression stockings • Other relevant agents[a]	• No prophylaxis or • Other intervention	• DVT • Pulmonary embolism • Mortality • Major bleeding[c] • Bleeding during oocyte retrieval and embryo transfer	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Prevention of VTE following cesarean section (section 6.0)	• Risk of VTE following cesarean section in women with -No additional risk factors -Prior VTE -Thrombophilia[b] -Other comorbid conditions	• Pregnant women undergoing cesarean section	• No prophylaxis	• No intervention	• DVT • Pulmonary embolism • Embolism • Mortality • Major bleeding[d] • Epidural hematoma	• Control arms of randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
	• Choice, duration, and (if appropriate) route/dose of prophylaxis	• Pregnant women undergoing cesarean section	• Low molecular weight heparin • Unfractionated heparin • Graduated compression stockings • Intermittent pneumatic compression • Combined mechanical and pharmacologic prophylaxis • Other relevant agents[a]	• No prophylaxis or • Other antithrombotic strategy	• DVT • Pulmonary embolism • Mortality • Major bleeding: total[d] • Major bleeding[d] • Epidural hematoma	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies • Decision analysis
Treatment of proven acute VTE during pregnancy (section 7.0)	• Choice, route, and dose of antithrombotic therapy	• Pregnant women with proven acute VTE	• Vitamin K antagonists • Unfractionated heparin • Low-molecular-weight heparin • Other relevant agents[a]	• No treatment or • Other antithrombotic therapy or • Therapy in nonpregnant population with acute VTE	• Symptomatic recurrent DVT or pulmonary embolism • Fatal pulmonary embolism • Major bleeding • Postthrombotic syndrome	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
	• Duration of antithrombotic therapy	• Pregnant women with proven acute VTE	• Throughout pregnancy • Throughout pregnancy and 6 wk postpartum (at least 3 mo) • Throughout pregnancy and 6 wk postpartum (at least 6 mo) • Throughout pregnancy and indefinite postpartum	• Other duration	• Symptomatic recurrent DVT or pulmonary embolism • Fatal pulmonary embolism • Major bleeding	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies

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Table 1—*Continued*

Section	Informal Question	PICO Question: Population	PICO Question: Intervention	PICO Question: Comparator	PICO Question: Outcome	Methodology
	• Role of vena caval filters when antithrombotic therapy is contraindicated	• Pregnant women with proven acute VTE	• Venal caval filter	• No vena caval filter	• Symptomatic recurrent DVT or pulmonary embolism • Fatal pulmonary embolism • Major bleeding • Postthrombotic syndrome	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
	• Management of antithrombotic therapy around delivery	• Pregnant women with proven acute VTE	• Elective delivery[e] with discontinuation of antithrombotic therapy 24 to 48 h prior to delivery • No elective delivery,[e] transition to unfractionated heparin • No elective delivery,[e] transition to prophylactic dose of antithrombotic agent • No elective delivery[e] with discontinuation of antithrombotic therapy as soon as labor commences	•Other intervention	• Symptomatic recurrent DVT or pulmonary embolism • Major bleeding: total[d] • Epidural hematoma • Postthrombotic syndrome	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Prevention of recurrent VTE in pregnant women with prior VTE (section 8.0)	• Risk of recurrent VTE in pregnant women with: -A single unprovoked event -A single event that was associated with a transient risk factor (all, estrogen-related [OCP, pregnancy]) -Multiple prior events -Thrombophilia[b]	• Pregnant women with prior VTE	• No prophylaxis	• No intervention	• Symptomatic DVT, pulmonary embolism • Mortality • Major bleeding: total • Postthrombotic syndrome	• Control arms of randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
	• Choice and (if appropriate) route and dose of antithrombotic prophylaxis	• Pregnant women with prior VTE	• No antepartum prophylaxis, postpartum only -All relevant agents considered[a] • Antepartum and postpartum prophylaxis -All relevant agents considered[a]	• No prophylaxis	• Symptomatic recurrent DVT or pulmonary embolism • Major bleeding: total • Postthrombotic syndrome	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies

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Table 1—*Continued*

		PICO Question				
Section	Informal Question	Population	Intervention	Comparator	Outcome	Methodology
Prevention of pregnancy-related VTE in women with thrombophilia (section 9.0)	• Risk of pregnancy-related VTE in women with thrombophilia[b]	• Pregnant women with thrombophilia[b] and no prior VTE	• No prophylaxis	• No intervention	• Symptomatic DVT, pulmonary embolism • Mortality • Major bleeding	• Control arms of randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
	• Choice, duration, and (if appropriate) route/dose of prophylaxis	• Pregnant women with thrombophilia[b] and no prior VTE	• No antepartum prophylaxis, postpartum only -Low-molecular-weight heparin -Unfractionated heparin -Other relevant agents[a] -Graduated compression stockings -Combined mechanical and pharmacologic prophylaxis • Antepartum and postpartum prophylaxis -Similar agents as above	• No prophylaxis or • Other intervention	• Symptomatic DVT, pulmonary embolism • Mortality • Major bleeding	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Prevention of pregnancy complications in women with thrombophilia[b] (section 10.0)	• Risk of pregnancy complications in women with thrombophilia[b]	• Pregnant women with thrombophilia[b] and a history of pregnancy complications -Recurrent early pregnancy loss[f] -Late pregnancy loss (single)[g] -Late pregnancy loss (multiple)[h] -Pre-eclampsia -Intrauterine growth restriction -Placental abruption	• No prophylaxis	• No intervention	• Recurrent pregnancy complication (as defined under patient population) • Symptomatic DVT, pulmonary embolism • Mortality • Major bleeding	• Control arm of randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies

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Table 1—*Continued*

Section	Informal Question	PICO Question: Population	PICO Question: Intervention	PICO Question: Comparator	PICO Question: Outcome	Methodology
	• Choice and (if appropriate) route and duration of antithrombotic prophylaxis	• Pregnant women with thrombophilia[b] (antiphospholipid antibodies vs congenital thrombophilia vs specific congenital thrombophilia) and a history of pregnancy complications -Recurrent early pregnancy loss[f] -Late pregnancy loss (single)[g] -Late pregnancy loss (multiple)[h] -Preeclampsia -Intrauterine growth restriction -Placental abruption	• Aspirin • Unfractionated heparin (± aspirin) • Low-molecular-weight heparin (± aspirin)	• No prophylaxis or • Other antithrombotic strategy	• Recurrent pregnancy complication (as defined under patient population) • Symptomatic DVT, pulmonary embolism • Mortality • Major bleeding	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Prevention of recurrent preeclampsia or recurrent pregnancy loss in women without known thrombophilia[b] (section 11.0)	• Choice and (if appropriate) route and duration of antithrombotic prophylaxis	• Pregnant women with no known thrombophilia[b] and prior preeclampsia • Pregnant women with no known thrombophilia and at least two prior pregnancy losses	• Aspirin • Unfractionated heparin (± aspirin) • Low-molecular-weight heparin (± aspirin)	• No prophylaxis	• Recurrent preeclampsia • Recurrent pregnancy loss	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies
Prevention of thromboembolism in pregnant women with mechanical heart valves (section 12.0)	• Risk of thromboembolism in pregnant women with mechanical heart valves	• Pregnant women with mechanical heart valves	• No antithrombotic therapy	• No intervention	• Maternal thromboembolism • Major bleeding: total • Major bleeding: maternal death • Congenital malformations • Fetal/neonatal hemorrhage • Pregnancy loss	• Control arm of randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies

(Continued)

Ref. 2

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Table 1—*Continued*

		PICO Question				
Section	Informal Question	Population	Intervention	Comparator	Outcome	Methodology
	• Choice and (if appropriate) route and dose of antithrombotic therapy	• Pregnant women with mechanical heart valves	• Vitamin K antagonists throughout pregnancy • Unfractionated heparin throughout pregnancy • Low-molecular-weight throughout pregnancy • Vitamin K antagonists substituted with unfractionated heparin during first trimester (at or before 6 wk) • Vitamin K antagonists substituted with low-molecular-weight heparin during first trimester (at or before 6 wk) • Vitamin K antagonists substituted with unfractionated heparin after 6 wk • Vitamin K antagonists substituted with low molecular weight heparin after 6 wk • Aspirin throughout pregnancy	• No antithrombotic therapy or • Other antithrombotic strategy	• Maternal thromboembolism • Major bleeding maternal death • Congenital malformations • Fetal/neonatal hemorrhage • Pregnancy loss	• Randomized controlled trials • Observational studies -Case series -Cohort studies -Case-control studies

PICO = population, intervention, comparator, outcome.

[a]Other relevant agents included in comparisons were selected based on their relevance for a particular question and may include any or all of the following: unfractionated heparin, low-molecular-weight heparin, fondaparinux, danaparoid, direct thrombin inhibitor, novel oral anticoagulants (eg, apixaban, dabigatran, rivaroxaban), aspirin, and thrombolysis.

[b]Thrombophilia is one or a combination of the following: congenital, including antithrombin deficiency, protein C deficiency, protein S deficiency, activated protein C resistance, factor V Leiden, prothrombin gene mutation, persistently elevated factor VIII levels, or antiphospholipid antibodies, including elevated anticardiolipin antibody titers, nonspecific inhibitor/lupus anticoagulant, and antibodies to β_2-glycoprotein I.

[c]For this question, major bleeding would also include bleeding during oocyte harvest and embryo transfer.

[d]For this question, major bleeding would also include epidural hematoma.

[e]Elective delivery refers to planned delivery/scheduled delivery and may include induction of vaginal delivery or cesarean section.

[f]Preferred as defined by three early losses prior to 12 wk; if not able to extract by this definition, then authors' definition and comment were used.

[g]Preferred as defined by single loss at 12 wk or later; if not able to extract by this definition, then authors' definition and comment were used.

[h]Preferred as defined by two or more losses at 12 wk or later; if not able to extract by this definition, then authors' definition and comment were used.

In addition to considering fetal outcomes (eg, pregnancy loss, congenital malformations) and maternal outcomes (eg, mortality, VTE, major maternal hemorrhage), we also consider burden of treatment as an important outcome for pregnant women taking long-term low-molecular-weight heparin (LMWH) or warfarin. When considered relevant, we report deaths (preferably as disease and treatment-specific mortality). Maternal thromboembolism includes DVT and pulmonary embolism (PE) in sections discussing the treatment and prevention of VTE and systemic embolization and valve thrombosis in sections discussing the management of pregnant women with mechanical heart valves. Major nonfatal maternal hemorrhage is defined as a symptomatic bleeding complication noted during pregnancy or within 6 weeks postpartum that involves bleeding into a critical site (intracranial, intraspinal, intraocular resulting in vision changes, retroperitoneal, pericardial, intramuscular with compartment syndrome, or placental abruption), causing a fall in hemoglobin level of ≥ 20 g/L, and bleeding leading to transfusion of two or more units of whole blood or red cells. This definition is in part based on the definition recommended by the International Society on Thrombosis and Haemostasis.[1] Where major bleeding was not explicitly defined in primary research articles, the authors' definition was accepted. Fetal loss refers to loss at any time after confirmation of a viable intrauterine pregnancy, not including elective termination.

A comprehensive English-language literature search (January 2005-January 2010) was conducted to update our existing literature base. We followed the approach articulated by Grades of Recommendations, Assessment, Development, and Evaluation for formulation of recommendations.[2-4] In making recommendations, we have placed the burden of proof with those who would claim a benefit of treatment. Therefore, when there is uncertain benefit and a probability of important harm associated with therapy, we generally recommend against intervention.

There is a paucity of high-quality studies addressing risk factors for the outcomes discussed in this article as well as for the risks and benefits of antithrombotic therapy during pregnancy. Most recommendations, therefore, are based on low- to moderate-quality evidence and mirror our limited confidence in relative effect estimates from studies of antithrombotic treatment during pregnancy. To obtain baseline risk estimates for pregnancy complications, we summarize available observational studies of pregnant women, including case reports and case series of pregnant women in the absence of studies with a cohort design. We then apply the baseline risk estimates to the relative risk estimates to establish anticipated absolute benefits and harms of intervention. In the absence of direct evidence from randomized trials of reasonable quality, indirect evidence from randomized trials in nonpregnant patients is considered applicable to the present patient population (eg, we extrapolate the effect of thromboprophylaxis with LMWH on the incidence of VTE in patients undergoing general surgery to women undergoing cesarean section).

When describing the various regimens of unfractionated heparin (UFH) and LMWH, we use the following short forms:

- Adjusted-dose UFH: UFH subcutaneously every 12 h in doses adjusted to target a midinterval activated partial thromboplastin time (aPTT) into the therapeutic range
- Prophylactic LMWH: for example, dalteparin 5,000 units subcutaneously every 24 h, tinzaparin 4,500 units subcutaneously every 24 h, nadroparin 2,850 units subcutaneously every 24 h, or enoxaparin 40 mg subcutaneously every 24 h (although at extremes of body weight, modification of dose may be required)
- Intermediate-dose LMWH: for example, dalteparin 5,000 units subcutaneously every 12 h or enoxaparin 40 mg subcutaneously every 12 h
- Adjusted-dose LMWH: weight-adjusted or full-treatment doses of LMWH given once daily or bid (eg, dalteparin 200 units/kg or tinzaparin 175 units/kg once daily or dalteparin 100 units/kg every 12 h or enoxaparin 1 mg/kg every 12 h)

Postpartum anticoagulation refers to vitamin K antagonists for 6 weeks with a target INR of 2.0 to 3.0, with initial UFH or LMWH overlap until the INR is ≥ 2.0 or prophylactic- or intermediate-dose LMWH for 6 weeks. The term "clinical vigilance" refers to patient and physician alertness to the signs and symptoms of VTE and awareness of the need for timely and appropriate objective investigation of women with symptoms suspicious of DVT or PE. A family history of VTE refers to DVT or PE in a first-degree relative.

1.1 The Implications of Women's Preferences and Values During Pregnancy

In considering women's choices regarding risks and benefits of antithrombotic therapy in pregnancy, two considerations are of particular importance. First, treatment decisions during pregnancy and breast-feeding have implications not only for the health and life of the mother but also for the health and life of the fetus or child. Second, many women prefer to see pregnancy as a normal part of a healthy woman's life course rather than as a medical condition. On the background of these considerations, many factors, including the frequency and type of medication administration; pain, discomfort, and possible side effects; and the need, frequency, and type of testing associated with a given regimen, will affect women's choices.

The weight given to harmful effects (eg, maternal bleeding events, congenital malformations) and burden of treatment (eg, self-injecting with LMWH for 9 months) compared with beneficial effects (eg, avoiding VTE or pregnancy loss) affects trade-offs between benefits and harms of antithrombotic treatment in pregnancy. A systematic review of patient preferences for antithrombotic treatment did not identify any studies of pregnant women.[5] The findings of this systematic review, and the value and preference rating exercise described in Guyatt et al[4] suggest that one VTE should be viewed as more or less equivalent to one major extracranial bleed. Our clinical experience and preliminary results from a cross-sectional interview study (S. M. Bates, MDCM, personal communication, March 27, 2011) to determine the willingness of women with prior VTE who are either pregnant, actively planning a pregnancy, or who may in the future consider pregnancy to receive LMWH prophylaxis during pregnancy for prevention of recurrent VTE suggest that many, but not all women will choose long-term prophylaxis when confronted with the burden of self-injecting with LMWH over several months. Therefore, in general, we only make weak recommendations for long-term prophylaxis with LMWH.

In addition, the burden of long-term prophylaxis or treatment with LMWH or warfarin throughout pregnancy will have an impact on the choice of antithrombotic therapy. Clinical experience suggests that many, but not all women give higher priority to the impact of any treatment on the health of their unborn baby than to effects on themselves, placing a low value on avoiding the pain, cost, and inconvenience of heparin therapy in order to avoid the small risk of even a minor abnormality in their child. Attempts to balance the burden of long-term prophylaxis against the disutility associated with VTE or major bleeding events are further complicated by the fact that all pregnant women will experience the disutility of long-term prophylaxis, whereas only a minority will avoid VTE with treatment (because the baseline risk of such events generally is low).

Recommendations in this article, therefore, reflect our belief that although average women considering antithrombotic therapy

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will also want to avoid medicalizing their pregnancy, they will put an extremely high value on avoiding fetal risk. For women who do not share these values, some of the recommendations in this article may not apply. For most recommendations, optimal decision-making will require that physicians educate patients about their treatment options, including their relative effectiveness, the consequences for both mother and baby, the method of administration and monitoring, the likely side effects, and the uncertainty associated with the estimates of all these effects. Once educated, women can participate in the selection of the treatment regimen that best matches their preferences and values.

2.0 Maternal Complications of Anticoagulant Therapy

Maternal complications of anticoagulant therapy are similar to those seen in nonpregnant patients and include bleeding (for all anticoagulants) as well as heparin-induced thrombocytopenia (HIT), heparin-associated osteoporosis, bruising, local allergic reactions, and pain at injection sites for heparin-related compounds.

2.1 UFH Therapy

During pregnancy, UFH can be used for both prevention and treatment of thromboembolism. Prophylactic UFH is typically administered subcutaneously two to three times per day either in fixed doses or doses adjusted to a target a specific anti-Xa UFH level (prophylactic- or intermediate-dose UFH). When used in therapeutic doses, UFH is administered either intravenously by continuous infusion with dose adjustment to achieve a target therapeutic aPTT or subcutaneously by bid injections in doses sufficient to achieve a therapeutic aPTT 6 h after injection.

During pregnancy, the aPTT response to UFH often is attenuated likely because of increased levels of heparin-binding proteins, factor VIII, and fibrinogen.[6] Consequently, the use of an aPTT range that corresponds to therapeutic heparin levels in nonpregnant patients might result in higher dosing (and heparin levels) in pregnant women than in nonpregnant patients. However, it is not clear whether this translates into excessive bleeding because the reported rates of bleeding using the standard aPTT range appear to be low. In a retrospective cohort study of 100 pregnancies in 77 women,[7] the rate of major antepartum bleeding in pregnant women treated with UFH was 1% (95% CI, 0.2%-5.4%), which is consistent with reported rates of bleeding associated with heparin therapy in nonpregnant patients[8] and with warfarin therapy[9,10] when used for the treatment of DVT.

Therapeutic doses of subcutaneous UFH can cause a persistent anticoagulant effect, which can complicate its use prior to delivery. In a small cohort study, prolongation of the aPTT persisted for up to 28 h after the last injection of adjusted-dose subcutaneous heparin.[11] The mechanism for this prolonged effect is unclear. A similar effect has not been noted with IV UFH.

Thrombocytopenia during pregnancy is not uncommon, and pregnancy-specific causes[12] should be differentiated from IgG-mediated thrombocytopenia or HIT, which occurs in ~3% of nonpregnant patients receiving UFH.[13] The diagnosis, prevention, and treatment of HIT are described in Linkins et al[14] in these guidelines. In pregnant women who develop HIT and require ongoing anticoagulant therapy, use of the heparinoid danaparoid sodium is recommended because it is an effective antithrombotic agent[15] that does not cross the placenta[16-18] and has low cross-reactivity with UFH[19]; therefore, it rarely produces HIT (danaparoid was withdrawn from the US market in 2002). Although there are reports of fondaparinux[20,21] being used for this indication in pregnancy, experience with this agent during pregnancy is too limited to recommend fondaparinux over danaparoid.

Long-term treatment with UFH has been reported to cause osteoporosis in both laboratory animals and humans.[22-30] A number of studies have attempted to quantify the risk of osteoporosis during prolonged treatment (> 1 month) with UFH. Symptomatic vertebral fractures have been reported to occur in ~2% to 3% of the patient population, and significant reductions of bone density have been reported in up to 30%.[22,23] A small study (n = 40) reported an even higher percentage of fractures (15%) when older nonpregnant patients were treated with bid subcutaneous injections of 10,000 units UFH for a period of 3 to 6 months.[26]

Adverse skin reactions to UFH include bruising, urticarial rashes, erythematous well-circumscribed lesions (because of a delayed type 4 hypersensitivity reaction), skin necrosis (often due to vasculitis), and HIT. The true incidence of skin reactions caused by UFH is unknown.[31]

2.2 LMWH Therapy

Despite a paucity of supportive data from controlled trials or even large prospective observational studies, LMWH is now commonly used for prophylaxis and treatment of maternal thromboembolism. This change in practice is based largely on the results of large trials in nonpregnant patients, showing that LMWHs are at least as safe and effective as UFH for the treatment of VTE[32,33] and acute coronary syndromes[34] as well as for prophylaxis in high-risk patients.[35]

Retrospective analyses and systematic reviews suggest that the incidence of bleeding in pregnant

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women receiving LMWH is low.[36-38] A systematic review of 64 studies that included 2,777 pregnancies in which LMWH was used reported that the frequencies of significant bleeding were 0.43% (95% CI, 0.22%-0.75%) for antepartum hemorrhage, 0.94% (95% CI, 0.61%-1.37%) for postpartum hemorrhage, and 0.61% (95% CI, 0.36%-0.98%) for wound hematoma, giving an overall frequency of 1.98% (95% CI, 1.50%-2.57%).[38] The risk of HIT appears much lower with LMWH than with UFH.[13,37,38]

Evidence suggests that LMWHs carry a lower risk of osteoporosis than UFH. In a study by Monreal and colleagues[26] in which 80 patients (men and women; mean age, 68 years) with DVT were randomized to either subcutaneous dalteparin 5,000 units bid (intermediate dose) or subcutaneous UFH 10,000 units bid for a period of 3 to 6 months, the risk of vertebral fractures with UFH (six of 40 [15%] patients; 95% CI, 6%-30%) was higher than with dalteparin (one of 40 [3%] patients; 95% CI, 0%-11%). In another randomized trial of 44 pregnant women allocated to prophylactic doses of dalteparin (n = 21) or UFH (n = 23),[27] bone density did not differ between women receiving dalteparin and those in a concurrent nonrandomized cohort of healthy pregnant women but was significantly lower in those receiving UFH. A prospective observational study in which 55 pregnant women treated with prophylactic LMWH and aspirin and 20 pregnant untreated volunteers reported similar results.[39] Finally, in an a priori substudy of an ongoing randomized comparison of prophylactic LMWH (subcutaneous dalteparin 5,000 units/d) with placebo for prevention of pregnancy complications, there was no difference between the two groups with respect to mean bone mineral density.[40]

Despite these reassuring data, there have been case reports[41-44] of symptomatic osteoporosis occurring with LMWH. Osteoporosis may be due to individual susceptibility, reflecting the presence of risk factors for osteoporosis, a variable effect of different LMWH preparations or doses on bone density, or a combination of both. Risk factors that make women susceptible to this complication when exposed to LMWH in pregnancy remain to be identified.

Adverse skin reactions similar to those seen with UFH can also occur with LMWH, although the frequency appears reduced in patients receiving the latter. The reported incidence ranges from 1.8% to 29%.[38,45] Most LMWH-induced skin lesions are benign; however, HIT should be excluded.[46]

Recommendation

2.2.1. For pregnant patients, we recommend LMWH for the prevention and treatment of VTE, instead of UFH (Grade 1B).

3.0 Fetal Complications of Antithrombotic Therapy During Pregnancy

3.1 Vitamin K Antagonist Exposure In Utero

Vitamin K antagonists cross the placenta and have the potential to cause fetal wastage, bleeding in the fetus, and teratogenicity.[47-58] In a systematic review of the literature published between 1966 and 1997 that examined fetal and maternal outcomes of pregnant women with prosthetic valves, Chan and colleagues[49] provided pooled estimates of risks associated with the following approaches: (1) use of vitamin K antagonists throughout pregnancy, (2) replacement of vitamin K antagonists with UFH from 6 to 12 weeks, and (3) UFH use throughout pregnancy (Tables S1, S2) (tables that contain an "S" before the number denote supplementary tables not contained in the body of the article and available instead in an online data supplement; see the "Acknowledgments" for more information). The authors found that the use of vitamin K antagonists throughout pregnancy was associated with congenital anomalies in 35 of 549 live births (6.4%; 95% CI, 4.6%-8.9%). A subsequent systematic review covering the years 2000 to 2009 (Tables S1, S2) reported a slightly lower risk estimate (21/559 [3.7%]; 95% CI, 1.9%-4.8%).[50]

In both reviews, the most common fetal anomaly was coumarin or warfarin embryopathy consisting of midfacial hypoplasia and stippled epiphyses. Limb hypoplasia has been reported in up to one-third of cases of embryopathy.[51] Embryopathy typically occurs after in utero exposure to vitamin K antagonists during the first trimester of pregnancy.[48] The results of a recently published multicenter European study not included in the systematic reviews, in which the pregnancies of 666 consenting women who contacted one of 12 Teratology Information Services between 1988 and 2004 seeking advice about gestational exposure to vitamin K antagonists were prospectively followed, also suggests that the risk of coumarin embryopathy is not high.[58] Although the frequency of major birth defects after any first trimester exposure to vitamin K antagonists was increased compared with that seen in a control group of 1,094 women counseled during pregnancy about exposures known to be nonteratogenic (4.8% vs 1.4%, respectively; OR, 3.86; 95% CI, 1.86-8.00), there were only two cases of embryopathy among 356 live births (0.6%). Both cases involved exposure to phenprocoumon until at least the end of the first trimester.

The substitution of heparin at or prior to 6 weeks appears to eliminate the risk of embryopathy, raising the possibility that vitamin K antagonists are safe with regard to embryopathy during the first 6 weeks of gestation. In the systematic review by Chan and colleagues,[49] none of the 125 live births (95% CI,

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0%-3.0%) in which vitamin K antagonists were replaced with UFH at or before 6 weeks gestation or UFH used throughout pregnancy was associated with congenital fetal anomalies. In the European multicenter Teratology Information Services study, there were no cases of embryopathy among 235 live births when vitamin K antagonists were discontinued before week 8 after the first day of the last menstrual period.[58]

Vitamin K antagonists have also been associated with CNS abnormalities after exposure during any trimester.[48] Two patterns of CNS damage have been described: dorsal midline dysplasia (agenesis of the corpus callosum, Dandy-Walker malformation, and midline cerebellar atrophy) and ventral-midline dysplasia leading to optic atrophy.[48] These complications are uncommon.[48,49]

Although one cohort study reported that the use of coumarins during the second and third trimester was not associated with major risks for abnormalities in growth and long-term development of offspring, the authors noted an increased risk of minor neurodevelopmental problems (OR, 1.7; 95% CI, 1.0-3.0) in children exposed to coumarins in the second and third trimester of pregnancy compared with age-matched nonexposed children (14% vs 8%, respectively).[59] However, these minor neurodevelopmental problems are likely of minor importance because there were no differences in mean IQ or performance on tests for reading, spelling, and arithmetic between exposed and nonexposed children.[60]

Vitamin K antagonists have been linked to an increased risk of pregnancy loss[49,50,58,61] and can cause fetal hemorrhagic complications likely because the fetal liver is immature and fetal levels of vitamin K-dependent coagulation factors are low. Fetal coagulopathy is of particular concern at the time of delivery when the combination of the anticoagulant effect and trauma of delivery can lead to bleeding in the neonate. The risk of delivering an anticoagulated infant can be reduced by substituting UFH or LMWH for vitamin K antagonists approximately 3 weeks prior to planned delivery[61] and discontinuing these medications shortly before delivery. Others have advocated the use of planned cesarean section at 38 weeks with only a brief (2 to 3 day) interruption of anticoagulant therapy.[62] This approach resulted in good neonatal and maternal outcomes in a study of 30 babies. Cesarean section is not without risk and is not recommended for other conditions associated with an increased risk of neonatal intracranial hemorrhage at the time of delivery (eg, immune thrombocytopenia purpura).

3.1.1 Thromboprophylaxis in Women Using Vitamin K Antagonists and Planning Pregnancy: Physicians should counsel women receiving vitamin K antagonist therapy and contemplating pregnancy about the risks of vitamin K antagonist therapy before pregnancy occurs. If pregnancy is still desired, the following two options can reduce the risk of warfarin embryopathy:

1. Performance of frequent pregnancy tests and substitution of adjusted-dose LMWH or UFH for warfarin when pregnancy is achieved or
2. Replacement of vitamin K antagonists with LMWH or UFH before conception is attempted

Both approaches have limitations. The first assumes that vitamin K antagonists are safe during the first 4 to 6 weeks of gestation. Although the second approach minimizes the risks of early miscarriage associated with vitamin K antagonist therapy, it lengthens the duration of exposure to heparin and, therefore, is costly and exposes the patient to a greater burden of treatment associated with the use of parenteral anticoagulants.

3.2 UFH Exposure In Utero

UFH does not cross the placenta[63] and, therefore, does not have the potential to cause fetal bleeding or teratogenicity; although bleeding at the uteroplacental junction is possible. Several studies provide high-quality evidence that UFH therapy is safe for the fetus.[7,47,64]

3.3 LMWH Exposure In Utero

As determined by measurement of anti-Xa activity in fetal blood, LMWH also does not cross the placenta.[65,66] There is no evidence that LMWH causes teratogenicity or increases the risk of fetal bleeding.[36]

3.4 Danaparoid Exposure In Utero

Animal experiments and human case reports suggest negligible transport of danaparoid across the placenta[16-18,67] Thus, there is no demonstrable fetal toxicity with maternal danaparoid use. However, the quality of evidence available to support that claim is low. (Note: Danaparoid was withdrawn from the US market in 2002.)

3.5 Pentasaccharide Exposure In Utero

Although no placental passage of fondaparinux was demonstrated in a human cotyledon (small lobe on the uterine or maternal surface of the placenta) model,[68] anti-Xa activity (at approximately one-tenth the concentration of maternal plasma) was found in the umbilical cord plasma of five newborns of mothers treated with fondaparinux.[69] Although there have been a small number of reports of the successful use of this agent in pregnant woman,[70-76] most of these involve second trimester or later exposure. Thus, the quality of evidence regarding supporting use of fondaparinux in pregnancy is very low. Potential deleterious effects on the fetus cannot be excluded.

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3.6 Parenteral Direct Thrombin Inhibitor Exposure In Utero

Investigations have documented placental transfer of r-hirudin in rabbits and rats.[77,78] Although small numbers of case reports have documented successful outcomes with r-hirudin use in pregnancy,[77,79,80] there are insufficient data to evaluate its safety. Three case reports have been published describing the use of argatroban late in pregnancy.[81-83] There are no published reports on the use of bivalirudin.

3.7 New Oral Direct Thrombin and Anti-Xa Inhibitor Exposure In Utero

Pregnant women were excluded from participating in clinical trials evaluating these new agents. There are no published reports describing the use of new oral direct thrombin inhibitors (eg, dabigatran) or anti-Xa inhibitors (rivaroxaban, apixaban) in pregnancy. The Summaries of Product Characteristics for dabigatran and rivaroxaban describe animal reproductive toxicity.[84,85] The human reproductive risks of these medications are unknown.

3.8 Aspirin Exposure In Utero

Aspirin crosses the placenta, and animal studies have shown that aspirin may increase the risk of congenital anomalies. Several systematic reviews have examined the safety of aspirin use during pregnancy (Tables S1-S3).[86-88] A meta-analysis of 31 randomized studies comparing antiplatelet agents with either placebo or no antiplatelet agents in 32,217 pregnant women at risk for developing preeclampsia[86] reported that aspirin therapy was not associated with an increase in the risk of pregnancy loss, neonatal hemorrhage, or growth restriction. However, in a meta-analysis of eight studies that evaluated the risk of congenital anomalies with aspirin exposure during the first trimester, aspirin use was associated with a twofold increase in the risk for gastroschisis (OR, 2.37; 95% CI, 1.44-3.88).[87] The validity of this risk estimate is questionable because of a significant risk of bias in the contributing studies.

One population-based study did note an increased risk of miscarriage with aspirin use that was greatest when aspirin was taken around the time of conception[89]; however, the number of aspirin users was small, aspirin doses were unknown, and users may have had conditions associated with an increased risk of pregnancy loss.[90] A meta-analysis of seven randomized trials in which women started aspirin later in pregnancy (Tables S1, S3) failed to establish or refute an increase in risk of miscarriage with aspirin compared with placebo (risk ratio [RR], 0.92; 95% CI, 0.71-1.19 for first or second trimester exposure; RR, 1.3; 95% CI, 0.63-2.69 for first trimester exposure only).[88]

3.9 Thrombolysis During Pregnancy

Although investigations with ^{131}I-labeled streptokinase or tissue plasminogen activator showed minimal transplacental passage,[91] concerns remain about the use of thrombolytic therapy during pregnancy due to maternal and placental effects. Although there have been reports of successful thrombolysis in pregnancy (most involving streptokinase),[91-94] the number of cases is small. Given this and limitations of available data regarding the safety of this intervention in pregnancy, the use of thrombolytic therapy is best reserved for life-threatening maternal thromboembolism.[95]

Recommendations

3.0.1. For women receiving anticoagulation for the treatment of VTE who become pregnant, we recommend that LMWH over vitamin K antagonists during the first trimester (Grade 1A)**, in the second and third trimesters** (Grade 1B)**, and during late pregnancy when delivery is imminent** (Grade 1A)**.**

3.0.2. For women requiring long-term vitamin K antagonists who are attempting pregnancy and are candidates for LMWH substitution, we suggest performing frequent pregnancy tests and substituting LMWH for vitamin K antagonists when pregnancy is achieved rather than switching to LMWH while attempting pregnancy (Grade 2C)**.**

Remarks: Women who place little value on avoiding the risks, inconvenience, and costs of LMWH therapy of uncertain duration while awaiting pregnancy and a high value on minimizing the risks of early miscarriage associated with vitamin K antagonist therapy will probably choose LMWH while attempting pregnancy.

3.0.3. For pregnant women, we suggest limiting the use of fondaparinux and parenteral direct thrombin inhibitors to those with severe allergic reactions to heparin (including HIT) who cannot receive danaparoid (Grade 2C)**.**

3.0.4. For pregnant women, we recommend avoiding the use of oral direct thrombin (eg, dabigatran) and anti-Xa (eg, rivaroxaban, apixaban) inhibitors (Grade 1C)**.**

4.0 Use of Anticoagulants in Breast-feeding Women

In order for a drug to pose a risk to the breast-fed infant, not only must it be transferred and excreted into breast milk but also it must be absorbed from

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the infant's gut. Drugs that are poorly absorbed are unlikely to affect the neonate. Lipid-soluble drugs with a low molecular weight that are not highly protein bound are more likely to be transferred into breast milk.[96]

4.1 Use of Vitamin K Antagonists in Breast-feeding Women

Despite a lack of data suggesting any harmful effect to breast-feeding infants, many obstetricians remain reluctant to prescribe warfarin to lactating women. This might reflect concerns that less polar, more lipophilic vitamin K antagonists rarely used in North America (eg, phenindione, anisindione, and phenprocoumon) might be excreted into breast milk.[97] Warfarin, the oral anticoagulant prescribed for most patients in North America, is polar, nonlipophilic, and highly protein bound. There have been two convincing reports demonstrating that warfarin is not detected in breast milk and does not induce an anticoagulant effect in the breast-fed infant when nursing mothers consume the drug.[98,99] Acenocoumarol, which is commonly used in Europe, has similar properties (Tables S4, S5).[100,101] Therefore, the use of warfarin and acenocoumarol in women who require postpartum anticoagulant therapy is safe.

4.2 Use of UFH and LMWH in Breast-feeding Women

Because of its high molecular weight and strong negative charge, UFH does not pass into breast milk and can be safely given to nursing mothers.[102] In a case series of 15 women receiving 2,500 International Units of LMWH after cesarean section, there was evidence of excretion of small amounts of LMWH into the breast milk in 11 patients (Tables S4, S5).[103] However, given the very low bioavailability of oral heparin, there is unlikely to be any clinically relevant effect on the nursing infant.

4.3 Use of Danaparoid in Breast-feeding Women

Very little is known about the passage of danaparoid into breast milk. A small number of case reports have reported no or very low anti-Xa activity in the breast milk of danaparoid-treated women.[77] Because danaparoid is not absorbed by the GI tract after oral intake, it is unlikely that any anticoagulant effect would appear in breast-fed infants.

4.4 Use of Fondaparinux in Breast-feeding Women

According to the manufacturer's prescribing information, fondaparinux was found to be excreted in the milk of lactating rats.[104] There are no published data on the excretion of fondaparinux into human milk, and the effects on the nursing infant are unknown. As a negatively charged oligosaccharide, only minor amounts of fondaparinux are expected to pass the intestinal epithelial barrier after oral administration, and significant absorption by the nursing infant is unlikely.[105] However, the manufacturer recommends that caution be used when administering fondaparinux to breast-feeding women.

4.5 Use of Parenteral Direct Thrombin Inhibitors in Breast-feeding Women

In a single-case report, no r-hirudin was detected in the breast milk of a nursing mother with a therapeutic plasma hirudin level.[106] Enteral absorption of r-hirudin appears to be low.[78] Therefore, it is unlikely that exposed infants would experience a significant anticoagulant effect, even if small amounts of r-hirudin appear in breast milk.

4.6 Use of New Oral Direct Thrombin and Factor Xa Inhibitors in Breast-feeding Women

Breast-feeding women were excluded from trials evaluating new oral direct thrombin and anti-Xa inhibitors, and there are no clinical data on the effect of these agents on breast-fed infants. The Summary of Product Characteristics for rivaroxaban notes that animal data indicate that this agent is secreted into breast milk.[85] The manufacturers of dabigatran and rivaroxaban both recommend against using these medications in breast-feeding women.[84,85]

4.7 Use of Aspirin in Breast-feeding Women

Although aspirin is a polar, acidic drug that is poorly lipid soluble and highly bound to plasma proteins, maternal aspirin ingestion is associated with excretion of salicylates into breast milk.[107] There are, therefore, potential risks of platelet dysfunction and GI bleeding in nursing infants of mothers using high doses of this drug.[107,108] Metabolic acidosis has been reported in breast-fed infants of mothers taking several grams of aspirin per day.[109,110] Theoretically, nursing infants of mothers taking aspirin could be at risk for developing Reye syndrome.[107] The use of low-dose aspirin (<100 mg/d) late in pregnancy was not associated with significant effects on neonatal platelet function.[111,112] In a prospective study of 15 breast-feeding mothers taking aspirin therapy, no negative effects were noted (Tables S4, S5).[113]

Recommendations

4.0.1. For lactating women using warfarin, acenocoumarol, or UFH who wish to breast-feed, we recommend continuing the use of warfarin, acenocoumarol, or UFH (Grade 1A).

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4.0.2. For lactating women using LMWH, danaparoid, or r-hirudin who wish to breast-feed, we recommend continuing the use of LMWH, danaparoid, or r-hirudin (Grade 1B)**.**

4.0.3. For breast-feeding women, we suggest alternative anticoagulants rather than fondaparinux (Grade 2C)**.**

4.0.4. For breast-feeding women, we recommend alternative anticoagulants rather than oral direct thrombin and factor Xa inhibitors (Grade 1C)**.**

4.0.5. For lactating women using low-dose aspirin for vascular indications who wish to breast-feed, we suggest continuing this medication (Grade 2C)**.**

5.0 VTE in Patients Using Assisted Reproductive Technology

Assisted reproductive technology, which refers to all treatments or procedures involving in vitro handling of human oocytes and sperm or embryos for the purpose of achieving pregnancy,[114,115] may be associated with VTE. Data regarding the frequency of VTE, however, comprise predominantly of case reports, case series, and relatively small cohort studies (Table S6).[116-121] In two large retrospective series of patients undergoing in vitro fertilization, thrombosis complicated 0.1% (95% CI, 0%-0.3%)[116] and 0.3% (95% CI, 0%-0.8%)[117] of cycles. A hospital-based case-control study demonstrated a fourfold increase in antenatal VTE with assisted reproductive technology for singleton pregnancies and a sixfold incidence in twin pregnancies but no statistically significant association with postpartum VTE.[121] Thus, although in vitro fertilization appears to be a risk factor for antepartum thromboembolism, the overall absolute incidence of symptomatic thrombosis appears to be low.

The risk of thrombosis may be higher in women with ovarian hyperstimulation syndrome, with an incidence of thrombosis of up to 4.1% (95% CI, 1.1%-13.7%) in severe cases.[116] In a review of thrombosis associated with assisted reproductive technology, Chan and colleagues[122] identified 61 reports of venous thrombosis (49 cases involving the veins of the neck and arm) and 35 arterial events. Ovarian hyperstimulation syndrome was reported in 90% of arterial cases and 78% of venous events. In 98% of cases, thrombosis occurred after ovulation induction. Venous events were delayed compared with those involving the arterial circulation (42.4 days after embryo transfer and 10.7 days post-transfer, respectively).[122]

5.1 Prevention of VTE in Patients Undergoing Assisted Reproductive Technology

The bleeding risk most relevant to this population is intraabdominal and vaginal bleeding. The estimates of normal blood loss during uncomplicated oocyte retrieval vary, ranging from approximately 230 mL in one prospective cohort of 220 women[123] to 13 mL (range, 0-98 mL) in a study of 83 women.[124] Although patient-important vaginal bleeding appears to occur in up to 2% to 3% of patients, significant intraabdominal bleeding is much less common ($\leq$0.5% procedures) (Table S7).[120,125-132] Whether these risks are increased with antithrombotic prophylaxis is uncertain.

All studies that address the impact of prophylaxis in in vitro fertilization have important limitations, and the number of patients who have received anticoagulants is too small to draw any conclusions about safety and efficacy (Table S8).[133-135] Therefore, we used indirect evidence from a meta-analysis of thromboprophylaxis in patients undergoing hip arthroplasty[136] to estimate the relative effects LMWH prophylaxis in assisted reproductive technology. Table 2 and Table S9 summarize the quality of evidence and anticipated absolute effects of thrombosis prophylaxis in women with and without ovarian hyperstimulation syndrome. We rate the quality of evidence as low due to indirectness of the population and intervention and due to considerable imprecision in risk estimates for major bleeding events and VTE. In women with severe ovarian hyperstimulation syndrome, thromboprophylaxis may result in 26 fewer VTE per 1,000 women treated (number needed to treat [NNT] of 39 [given an estimated baseline risk of VTE of 4%]), with no increased risk of significant bleeding. However, in women without ovarian hyperstimulation syndrome in whom the baseline risk of VTE is estimated to be ~0.2%, the use of LMWH prophylaxis is of very limited value (NNT, 781).

Data regarding the risk of VTE in women with thrombophilia or prior VTE who undergo assisted reproduction are lacking. Given the low baseline risk of VTE associated with assisted reproduction, if the magnitude of relative risk increases is similar to that reported with pregnancy-related VTE (sections 8 and 9), women with low-risk thrombophilias or prior VTE associated with major transient risk factors will receive only very small benefit from prophylaxis.

Dosage and duration of thromboprophylaxis after assisted reproductive therapy has not been well studied. If LMWH is used in women who develop ovarian hyperstimulation, extension of prophylaxis for 4 to 8 weeks postresolution of hyperstimulation[114] or throughout any resultant pregnancy and into the postpartum period[115] has been suggested given that

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Table 2—*[Section 5.1.1, 5.1.2] Summary of Findings: Prophylactic-Dose LMWH vs No Thromboprophylaxis for Women Who Undergo Assisted Reproductive Therapy*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects During Pregnancy: Risk Without Prophylaxis	Anticipated Absolute Effects During Pregnancy: Risk Difference With LMWH (95% CI)
Symptomatic VTE, DVT, and pulmonary embolism	1,953 (6 RCTs), 27-35 d postoperative	Low due to indirectness[b] and imprecision[a]	RR 0.36 (0.20-0.67)	Without severe ovarian hyperstimulation syndrome	
				2 VTE per 1,000[c]	1 fewer VTE per 1,000 (from 2 fewer to 0 fewer)
				With severe ovarian hyperstimulation syndrome	
				40 VTE per 1,000[c]	26 fewer per 1,000 (from 32 fewer to 13 fewer)
Major bleed	5,456 (7 RCTs), 3 wks-9 mo	Low due to indirectness[b] and imprecision[a]	RR 0.43 (0.11-1.65)	30 bleeding events per 1,000[c]	No significant difference 17 fewer bleeding events per 1,000 (from 27 fewer to 20 more)

GRADE = Grades of Recommendations, Assessment, Development, and Evaluation; LMWH = low-molecular-weight heparin; RCT = randomized controlled trial; RR = risk ratio.

[a]Rated down for imprecision due to imprecise control group risk estimates for bleeding events and for VTE in the subset of women with ovarian hyperstimulation (Tables S6-S8).

[b]The population did not include pregnant women. Different doses of LMWH were used, treatment was initiated variably before or after surgery with a duration of ~7 d (in hospital). Outcomes were variably reported; meta-analysis also provides other outcomes such as mortality, asymptomatic DVT, and specific bleed outcomes (wound hematoma, transfusion). Follow-up varied between trials from 3 wk to 9 mo.

[c]Control group risk for VTE and major bleed come from observational studies of women undergoing assisted reproductive technology, with many studies following women until delivery (Tables S6-S8).

most reported events have developed days to weeks (range, 2 days-11 weeks) after resolution of ovarian hyperstimulation.[115] However, given the lack of a clear association between assisted reproductive technology and postpartum events,[117,121] continuing anticoagulant prophylaxis after delivery is less likely to be of benefit.

Recommendations

5.1.1. For women undergoing assisted reproduction, we recommend against the use of routine thrombosis prophylaxis (Grade 1B)**.**

5.1.2. For women undergoing assisted reproduction who develop severe ovarian hyperstimulation syndrome, we suggest thrombosis prophylaxis (prophylactic LMWH) for 3 months postresolution of clinical ovarian hyperstimulation syndrome rather than no prophylaxis (Grade 2C)**.**

Remarks: Women who are averse to taking medication for very small benefit and those who consider self-injecting a considerable burden will be disinclined to use LMWH for extended thrombosis prophylaxis. Given that the absolute benefit decreases as time from the hyperstimulation event increases, such women will be very disinclined to continue prophylaxis throughout the entire resultant pregnancy.

6.0 VTE Following Cesarean Section

6.1 Risk of VTE Following Cesarean Section

The puerperium is the time of maximal daily risk of pregnancy-associated VTE.[137,138] Several observational studies have assessed the risk of VTE after cesarean section, with absolute risk estimates ranging from < 1 in 1,000 up to 18 of 1,000 cesarean deliveries.[121,139-148] However, studies based on hospital records and disease coding may result in an underestimation of the true incidence of symptomatic VTE.[149] A Norwegian study of 59 low-risk women undergoing elective cesarean section who underwent screening for DVT using triplex ultrasonography (compression ultrasonography, color Doppler echocardiography, and spectral Doppler echocardiography) 2 to 5 days after delivery and followed up for 6 weeks reported that none had symptomatic or asymptomatic VTE (95% CI, 0%-6.1%).[144] A small prospective study in which patients after cesarean section underwent screening ultrasounds at hospital discharge and 2 weeks postpartum and were followed for 3 months reported a symptomatic event rate of five of 1,000 (95% CI, 0.1%-2.8%).[147] This is consistent with estimates based on hospital discharge data antedating the use of thromboprophylaxis.[138,140]

Observational studies provide evidence concerning risk factors for VTE in pregnant women (Tables S10, S11)[121,146,148,150-152]; these are likely to be relevant in women undergoing cesarean section. In assessing risk in this setting, the number of risk factors, the magnitude of risk associated with these factors, and their impact when occurring together are all relevant. Table 3 provides an overview of major and minor risk factors we suggest clinicians use to identify women at increased risk of VTE after cesarean section. The presence of one major or at least two minor risk factors will indicate whether patients qualify for

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Table 3—*[Section 6.2.1-6.2.4] Risk Factors for VTE Resulting in a Baseline Risk of Postpartum VTE of > 3%*

Major risk factors (OR > 6): presence of at least one risk factor suggests a risk of postpartum VTE > 3%
Immobility (strict bed rest for ≥ 1 week in the antepartum period)
Postpartum hemorrhage ≥ 1,000 ml with surgery
Previous VTE
Preeclampsia with fetal growth restriction
Thrombophilia
Antithrombin deficiency[a]
Factor V Leiden (homozygous or heterozygous)
Prothrombin G20210A (homozygous or heterozygous)
Medical conditions
Systemic lupus erythematosus
Heart disease
Sickle cell disease
Blood transfusion
Postpartum infection
Minor risk factors (OR > 6 when combined): presence of at least two risk factors or one risk factor in the setting of emergency cesarean section suggests a risk of postpartum VTE of > 3%
BMI > 30 kg/m^2
Multiple pregnancy
Postpartum hemorrhage > 1 L
Smoking > 10 cigarettes/d
Fetal growth restriction (gestational age + sex-adjusted birth weight < 25th percentile)
Thrombophilia
Protein C deficiency
Protein S deficiency
Preeclampsia

Data from Jacobsen et al,[121] Jacobsen et al,[144] Lindqvist et al,[146] Simpson et al,[148] Knight,[150] Robertson et al,[151] and James et al.[152]
[a]Although the OR in a systematic review was 4.69, CIs were wide and numbers small. Further, other retrospective studies have calculated ORs of 282 (95% CI, 31-2,532) for type 1 antithrombin deficiency and 28 (95% CI, 5.5-142) for type 2 deficiency.[153] Thus, antithrombin deficiency has been left as a major risk factor.

our weak recommendation for thrombosis prophylaxis (section 6.2). Extrapolating from high-risk populations,[154-156] the combination of LMWH prophylaxis with mechanical methods may be of benefit when multiple major risk factors for VTE are present. Further, when major risk factors continue in the puerperium, consideration should be given to extending prophylaxis for the 6 weeks during which pregnancy-associated prothrombotic changes may persist.[137]

6.2 Thromboprophylaxis Following Cesarean Section

A recent systematic review[157] identified four studies (830 women) comparing prophylaxis with LMWH[158,159] or UFH[160,161] with placebo. There was no statistically significant difference between groups with respect to symptomatic VTE for LMWH vs placebo (two of 105 and zero of 105, respectively; RR, 2.97; 95% CI, 0.31-28.03) and UFH vs placebo (three of 297 and four of 333, respectively; RR, 0.85; 95% CI, 0.19-3.76).[157] However, the small number of study participants and outcome events provide insufficient evidence on which to make prophylaxis recommendations. A decision analysis model suggested that the benefits of LMWH prophylaxis exceed risks after cesarean section[141] but that this benefit was small in women with no risk factors and the low-quality evidence makes the assumptions that underlie the model questionable.

We use indirect evidence from patients undergoing general surgery[156] to generate anticipated absolute effects of LMWH on VTE and major bleeding events in pregnant women undergoing cesarean section. Table 4 and Table S12 show the quality of evidence and main findings from a meta-analysis of three trials comparing LMWH vs placebo in 4,890 patients undergoing general surgery.[162] We have rated down the quality of evidence because of indirectness. Extrapolating from general surgery patients (Table 4, Table S12), the balance of desirable and undesirable consequences would suggest prophylaxis for women with an absolute VTE risk of ≥ 30 of 1,000. With a baseline risk of five of 1,000 VTE after cesarean delivery, the presence or absence of risk factors will determine the absolute benefit of thrombosis prophylaxis. We categorize women into low risk (five of 1,000) and high risk (30 of 1,000); clinicians can use Table 3 to determine to which group their patient belongs.

Mechanical prophylaxis with elastic stockings or intermittent pneumatic compression are alternatives to pharmacologic prophylaxis in pregnant women at high risk of VTE and may be used with LMWH in women at particularly high risk of VTE. We consider evidence from a variety of populations undergoing general surgery to be applicable to pregnant women undergoing cesarean section and, therefore, refer the reader to Gould et al[156] in these guidelines for a detailed review of the evidence supporting the use of mechanical thromboprophylaxis with elastic stockings or intermittent pneumatic compression. In short, when compared with pharmacologic prophylaxis, mechanical prophylaxis is associated with less major bleeding (RR, 0.51; 95% CI, 0.40-0.64 for high-quality evidence) but a higher risk of VTE (RR, 1.8; 95% CI, 1.2-2.8 for low-quality evidence). Applying the baseline risk estimates for VTE and major bleeding events provided in Table 4 to 1,000 pregnant women at high risk of VTE after cesarean section, it follows that selecting mechanical prophylaxis over pharmacologic prophylaxis would result in 24 more VTE and seven fewer bleeding events. Although elastic stockings have been associated with skin breakdown when used poststroke (RR, 4.0; 95% CI, 2.4-6.9), this complication is much less likely to occur in young women. Elastic stockings and intermittent pneumatic compression may be inconvenient and cumbersome to use.

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Ref. 2

Table 4—[*Section 6.2.1-6.2.4] Summary of Findings: LMWH vs No Thromboprophylaxis for Prevention of VTE in Women Undergoing Cesarean Section*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects Over 6 wk Postpartum	
				Risk Without Prophylaxis	Risk Difference With LMWH (95% CI)
Symptomatic VTE, DVT, and pulmonary embolism	4,890 (3 RCTs), 3 wk-9 mo	Moderate due to indirectness[b]	RR 0.29 (0.11-0.73)	Low risk (see Table 13)	
				5 VTE per 1,000[a]	3 fewer VTE per 1,000 (from 4 fewer to 1 fewer)
				High risk (see Table 13)	
				40 VTE per 1,000[a]	21 fewer per 1,000 (from 27 fewer to 9 fewer)
Major bleed[c]	5,456 (7 RCTs), 3 wk-9 mo	Moderate due to indirectness[c]	RR 2.03 (1.37-3.01)	20 bleeding events per 1,000[d]	20 more bleeding events per 1,000 (from 8 more to 40 more)

See Table 2 legend for expansion of abbreviations.
[a]Control group risk estimates come from studies providing risk factors for VTE after cesarean section (Tables S10 and S11).
[b]Rated down for indirect study population (general surgery patients). We did not rate down for risk of bias, although only five of eight RCTs of LMWH vs placebo/no treatment reported mortality.
[c]Rated down for indirectness due to variable bleeding definitions in trials: bleeding leading to death, transfusion, reoperation, or discontinuation of therapy. Measured at end of therapy.
[d]Control group risk estimate comes from a decision analysis by Blondon et al.[141]

The optimal duration of prophylaxis after cesarean section is not established. If we extrapolate from general surgery,[156,163-166] treatment until discharge from the hospital, with extended prophylaxis for those with significant ongoing risk factors, may be appropriate. We express a preference for LMWH over UFH because of its favorable safety profile (see section 4.0).

There are no relevant cost-effectiveness data in this setting using UFH or LMWH; however, in one study modeling the cost-effectiveness of intermittent pneumatic compression, this intervention was considered cost-effective when the incidence of post-cesarean section DVT was at least 6.8 of 1,000[167] (Tables S13, S14). However, these devices are not readily available at all sites, and patients and nurses often find them to be inconvenient and cumbersome to use.

Recommendations

6.2.1. For women undergoing cesarean section without additional thrombosis risk factors, we recommend against the use of thrombosis prophylaxis other than early mobilization (Grade 1B)**.**

6.2.2. For women at increased risk of VTE after cesarean section because of the presence of one major or at least two minor risk factors (Table 3), we suggest pharmacologic thromboprophylaxis (prophylactic LMWH), or mechanical prophylaxis (elastic stockings or intermittent pneumatic compression) in those with contraindications to anticoagulants while in the hospital following delivery rather than no prophylaxis (Grade 2B)**.**

Remarks: The reduced bleeding risk with mechanical prophylaxis should be weighed against the inconvenience of elastic stockings and intermittent pneumaric compression.

6.2.3. For women undergoing cesarean section who are considered to be at very high risk for VTE and who have multiple additional risk factors for thromboembolism that persist in the puerperium, we suggest that prophylactic LMWH be combined with elastic stockings and/or intermittent pneumatic compression over LMWH alone (Grade 2C)**.**

6.2.4. For selected high-risk patients in whom significant risk factors persist following delivery, we suggest extended prophylaxis (up to 6 weeks after delivery) following discharge from the hospital (Grade 2C)**.**

7.0 Treatment of Proven Acute VTE During Pregnancy

PE remains a leading cause of maternal mortality in the western world,[168,169] and VTE in pregnancy is an important cause of maternal morbidity.[168,170,171] Results from studies in which either all or most patients underwent accurate diagnostic testing for VTE report that the incidence of VTE ranges from 0.6 to 1.7 episodes per 1,000 deliveries.[138,139,146,148,152,172] A meta-analysis showed that two-thirds of DVT occur antepartum, with these events distributed throughout all three trimesters.[173] In contrast, 43% to 60%

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of pregnancy-related episodes of PE appear to occur in the 4 to 6 weeks after delivery.[139,148] Because the antepartum period is substantially longer than the 6-week postpartum period, the daily risk of PE, as well as DVT, is considerably higher following delivery than antepartum.

7.1 Treatment of VTE During Pregnancy

Based on safety data for the fetus, heparin compounds are preferred over vitamin K antagonists for the treatment of VTE in pregnancy (see section 3.0). LMWH is the preferred option for most patients because of its better bioavailability, longer plasma half-life, more predictable dose response, and improved maternal safety profile with respect to osteoporosis and thrombocytopenia (see section 2.0).[35-38] Further, LMWH is a more convenient option because it can be given once daily, and unlike UFH, LMWH does not require aPTT monitoring.[6]

A systematic review of LMWH use in pregnancy[38] and subsequent observational studies[36,150,174] confirm the safety and efficacy of LMWH in this patient population when used for treatment of VTE. Our strong recommendation for LMWH over vitamin K antagonists in the treatment of VTE in pregnancy is further supported by evidence showing that in the nonpregnant population, LMWH is more effective than vitamin K antagonists in preventing recurrent VTE and postthrombotic syndrome without increasing the risk of major bleeding events.[175-178] Table 5 and Table S15 summarize the quality of evidence and main findings from a systematic review of nonpregnant patients deemed applicable to the present population of pregnant women with acute VTE. Given these results, we consider the burden of self-injecting with LMWH for several months and possibility of skin reactions of lesser importance.

If LMWH is used for treatment of acute VTE in pregnancy, a weight-adjusted dosing regimen should be used. LMWH requirements may alter as pregnancy progresses because the volume of distribution of LMWH changes and glomerular filtration rate increases in the second trimester. The latter has led some to recommend a bid LMWH dosing schedule. However, many clinicians use a once-daily regimen to simplify administration and enhance compliance. Observational studies have not demonstrated any increase in the risk of recurrence with the once-daily regimen over the bid regimen.[150,174]

The need for dose adjustments over the course of pregnancy remains controversial. Some suggest that dose should be increased in proportion to the change in weight.[181] On the basis of small studies showing the need for dose-escalation to maintain therapeutic anti-Xa LMWH levels,[182,183] some advocate the performance of periodic (eg, every 1-3 months) antifactor Xa LMWH levels 4 to 6 h after injection with dose adjustment to maintain a therapeutic anti-Xa level (0.6-1.0 units/mL if a bid regimen is used and higher

Table 5—*[Section 7.1.2] Summary of Findings: Should LMWH Rather Than VKA Be Used for Long-term Treatment of VTE in Pregnant Women?*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects During Pregnancy[b]	
				Risk With VKA	Risk Difference With LMWH (95% CI)
Recurrent symptomatic VTE, DVT, and pulmonary embolism	2496 (7 RCTs); median, 6 mo	Moderate due to risk of bias[a]	RR 0.62 (0.46-0.84)	30 VTE per 1,000[b]	11 fewer VTE per 1,000 (from 16 fewer to 5 fewer)
Major bleeding	2727 (8 RCTs); median, 6 mo	Moderate due to imprecision[c]	RR 0.81 (0.55-1.2)	20 bleeding events per 1,000[d]	4 fewer bleeding events per 1,000 (from 9 fewer to 4 more)
PTS self-reported leg symptoms and signs	100 (1 RCT); median, 3 mo	Low due to risk of bias[a] and indirectness[e]	RR 0.85 (0.77-0.94)	480 PTS per 1,000[f]	38 fewer bleeding events per 1,000 (from 110 fewer to 29 fewer)

Limited to LMWH regimens that used ≥50% of the acute treatment dose during the extended phase of treatment. Meta-analysis is based on RCTs as referenced in Kearon et al[178] in this guideline. PTS = postthrombotic syndrome; VKA = vitamin K antagonist. See Table 2 legend for expansion of other abbreviations.

[a]Risk of bias due to lack of blinding.

[b]Control group risk estimate for VTE with VKAs comes from cohort study by Prandoni et al,[179] adjusted to the 6-mo time frame considered applicable to the pregnancy period.

[c]Rated down for imprecision because CI includes both benefit and harm. Borderline decision not to rate down for risk of bias (considered this outcome less subjective, so lack of blinding not serious threat to validity).

[d]Control group risk estimate for major bleeding events comes from cohort studies by Prandoni et al[179] and Beyth et al,[180] adjusted to a 6-mo time frame considered applicable to the pregnancy period.

[e]Predictive value from 3 mo (follow-up in study) to long term is uncertain.

[f]Control group risk estimate for PTS comes from observational study of pregnant women (most mild).[171]

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if a once-daily regimen is chosen). However, other researchers have demonstrated that few women require dose adjustment when therapeutic doses of LMWH are used.[184-186] Given the absence of large studies using clinical end points that demonstrate an optimal therapeutic anti-Xa LMWH range or that dose adjustments increase the safety or efficacy of therapy, the lack of accuracy and reliability of the measurement,[187] the lack of correlation with risk of bleeding and recurrence,[188] and the cost of the assay, routine monitoring with anti-Xa levels is difficult to justify.

Where LMWH cannot be used or when UFH is preferred (eg, in patients with renal dysfunction), UFH can be used through one of two alternatives: (1) initial IV therapy followed by adjusted-dose subcutaneous UFH given every 12 h or (2) bid adjusted-dose subcutaneous UFH. With subcutaneous therapy, UFH doses should be adjusted to prolong a midinterval (6 h postinjection) aPTT into therapeutic range, although it is recognized that aPTT monitoring is less reliable in pregnancy.[6] As previously discussed, the use of fondaparinux is inadvisable in pregnancy (see section 3.5). In this guideline, Linkins et al[14] and Kearon et al[178] present evidence regarding platelet count monitoring for the detection of HIT and the role of compression stockings in the acute management of DVT.

It remains unclear whether the dose of LMWH (or UFH) can be reduced after an initial phase of therapeutic anticoagulation. Some suggest that full-dose anticoagulation should be maintained throughout pregnancy and the puerperium because of the ongoing risk of recurrent VTE. However, regimens in which the intensity of LMWH is reduced later during the course of therapy to an intermediate-dose regimen[26] or 75% of a full-treatment dose[177] have been used successfully in the nonpregnant population, including in cancer patients who have a much higher risk of recurrence. A similar approach when using LMWH in pregnancy may reduce the small risks of anticoagulant-related bleeding and heparin-induced osteoporosis. Although there have been no studies directly comparing full-dose LMWH with one of these modified dosing strategies in pregnant women, a modified dosing regimen may be useful in pregnant women at increased risk of either of these two complications.

No studies have assessed optimal duration of anticoagulant therapy for treatment of pregnancy-related VTE. In nonpregnant patients with VTE, evidence supports a minimum duration of 3 months treatment (see Kearon et al[178] in this guideline). We consider the additional fivefold to 10-fold increase in risk for VTE in pregnant women, coupled with the high rate of proximal thrombi (compared with the nonpregnant population), sufficient to recommend treatment throughout pregnancy and the postpartum period for a minimum total duration of 3 months.

The delivery options in women using anticoagulants are best considered by a multidisciplinary team. Several options are possible, including spontaneous labor and delivery, induction of labor, and elective cesarean section. The plan for delivery should take into account obstetric, hematologic, and anesthetic issues. In order to avoid an unwanted anticoagulant effect during delivery (especially with neuraxial anesthesia) in women receiving adjusted-dose subcutaneous UFH[11] or LMWH who have a planned delivery; twice-daily subcutaneous UFH or LMWH should be discontinued 24 h before induction of labor or cesarean section, whereas patients taking once-daily LMWH should take only 50% of their dose on the morning of the day prior to delivery (see Kunz et al[189] in this guideline). If spontaneous labor occurs in women receiving anticoagulation, neuraxial anesthesia should not be used. Where the level of anticoagulation is uncertain and where laboratory support allows for rapid assessment of heparin levels, then testing can be considered to guide anesthetic and surgical management. In women receiving subcutaneous UFH, careful monitoring of the aPTT is required and, if it is markedly prolonged, protamine sulfate[190] may be required to reduce the risk of bleeding. If bleeding occurs that is considered secondary to LMWH rather than to an obstetric cause, protamine sulfate may provide partial neutralization.[191]

Women with a very high risk for recurrent VTE (eg, proximal DVT or PE close to the expected date of delivery) may benefit by having a planned delivery by induction or cesarean section, as appropriate, so that the duration of time without anticoagulation can be minimized. Those at the highest risk of recurrence (eg, proximal DVT or PE within 2 weeks) can be switched to therapeutic IV UFH, which is then discontinued 4 to 6 h prior to the expected time of delivery or epidural insertion. Alternatively, a temporary inferior vena caval filter can be inserted and removed postpartum. However, the latter may be best restricted to women with proven DVT who have recurrent PE despite adequate anticoagulation because experience with these devices during pregnancy is limited,[192-194] and the risk of filter migration and inferior vena cava perforation may be increased during pregnancy.[193,194]

Recommendations

7.1.1. For pregnant women with acute VTE, we recommend therapy with adjusted-dose subcutaneous LMWH over adjusted-dose UFH (Grade 1B)**.**

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7.1.2. For pregnant women with acute VTE, we recommend LMWH over vitamin K antagonist treatment antenatally (Grade 1A)**.**

7.1.3. For pregnant women with acute VTE, we suggest that anticoagulants should be continued for at least 6 weeks postpartum (for a minimum total duration of therapy of 3 months) in comparison with shorter durations of treatment (Grade 2C)**.**

7.1.4. For pregnant women receiving adjusted-dose LMWH or UFH therapy and where delivery is planned, we recommend discontinuation of the heparin at least 24 h prior to induction of labor or cesarean section (or expected time of neuraxial anesthesia) rather than continuing LMWH up until the time of delivery (Grade 1B)**.**

8.0 Prevention of VTE in Pregnant Women With Prior DVT or PE

Compared with individuals without a history of VTE, patients with previous events are at increased risk of future episodes of DVT and PE.[195] Women with a history of VTE have a threefold to fourfold higher risk of VTE during subsequent pregnancies than outside pregnancy.[196] Thromboprophylaxis during pregnancy involves long-term parenteral LMWH, which is expensive, inconvenient, and painful to administer. Although bleeding, osteoporosis, and HIT are very uncommon in patients receiving prophylactic LMWH,[37-40,197] injection site skin reactions may occur.[45] The threshold for recommending postpartum prophylaxis is lower than for antepartum prophylaxis because of the shorter length of required treatment (ie, 6 weeks) and the higher average daily risk of VTE in the postpartum period.[137,173] Given the distribution of DVT throughout all three trimesters,[173] antepartum prophylaxis, if used, should be instituted early in the first trimester.

8.1 Prior VTE and Pregnancy

Cohort studies evaluating the risk of recurrent VTE during pregnancy in women with a history of VTE in whom no prophylaxis is given have shown variable results (Table S16). The higher risk estimates from retrospective studies of nonconsecutive patients in which objective testing was not used routinely to confirm the diagnosis of recurrent VTE likely represent overdiagnosis.[198,199] Prospective studies provide lower estimates.[200-203]

The largest prospective study to date investigated 125 pregnant women with a single previous episode of objectively diagnosed VTE in whom antepartum heparin was withheld and anticoagulants (usually warfarin with a target INR of 2.0-3.0 with an initial short course of UFH or LMWH) were given in the postpartum period for 4 to 6 weeks.[203] In this study, the incidence of antepartum recurrence was 2.4% (95% CI, 0.2%-6.9%), whereas that during the postpartum period was 2.5% (95% CI, 0.5%-7.0%). The advanced median gestational age at enrollment (~15 weeks) and the exclusion of women with known thrombophilia might have resulted in an underestimation of the risk of pregnancy-related recurrent VTE.

In subsequently published large retrospective cohort studies, the probability of antepartum VTE in women not given prophylaxis was ~6%, whereas for postpartum VTE, the observed incidence ranged from 6% to 8%.[204,205] Differences in study population (inclusion of women with more than one prior episode of VTE and inclusion of pregnancies not ending in live birth [ie, miscarriages]) and failure to independently adjudicate recurrent events might account for the higher risk of recurrence. However, as shown in Table S16, the overall risk of recurrent VTE antepartum in both prospective and retrospective studies was $<10\%$, and CIs around the risk estimates of individual studies are overlapping.

Data regarding prognostic factors for recurrent VTE during pregnancy are inconsistent. A post hoc subgroup analysis of the prospective cohort study described previously identified women without thrombophilia who had a temporary risk factor (including oral contraceptive therapy or pregnancy) at the time of their prior VTE event as being at low risk of recurrence, with no recurrent events in 44 patients (0%; 95% CI, 0.0%-8.0%).[203] Antepartum recurrences occurred in three of 51 women with abnormal thrombophilia testing, a previous episode of thrombosis that was unprovoked, or both (5.9%; 95% CI, 1.2%-16.0%).

In the retrospective studies, the association between the presence or absence of temporary risk factors or of a definable thrombophilia and the risk of recurrent VTE associated with pregnancy was not consistent (Table S16). In these studies, it appears that women who had their first episode of VTE provoked by use of oral contraceptives or related to pregnancy or the postpartum period had a higher risk of recurrent VTE in a subsequent pregnancy than women whose first VTE was unprovoked or associated with a nonhormonal transient risk factor, although these differences did not reach statistical significance in the individual studies.[204,205] These findings are consistent with those from a large population-based cohort study that used administrative data[206] in which women who had their first VTE associated with pregnancy or the postpartum period had a higher risk of recurrence

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during a subsequent pregnancy than women with an unprovoked first VTE (ie, 4.5% vs 2.7%; RR, 1.71; 95% CI, 1.0-2.8).

8.2 Prevention of Recurrent VTE in Pregnant Women

A systematic review of the effects of thromboprophylaxis in pregnant women[157] identified two randomized controlled trials that evaluated the safety and efficacy of prophylaxis (compared with placebo or no treatment) in pregnant women with prior VTE.[158,202] Both studies have major methodologic weaknesses, including very small sample sizes (n = 40 and n = 16).[158,202] A third, unblended randomized trial compared LMWH prophylaxis with UFH prophylaxis in a selected group of pregnant women with prior VTE[207] (Table S17).

Several observational studies have evaluated the risk of recurrent VTE with various treatment regimens[36-38,44,199,204,208-212] (Table S18). Some of these studies stratified patients according to their perceived risk of recurrence. The estimates of the risk of recurrent VTE while using some form of pharmacologic prophylaxis range from 0% to 15%, with the higher results seen in an older study that may have overestimated the recurrence rate because objective diagnostic testing was not used.[199]

Given the low quality of the direct evidence, we use indirect evidence about the relative effects of thromboprophylaxis from other patient populations to inform our recommendations for antenatal prevention of VTE. Table 6 and Table S19 summarizes the quality of the evidence and main findings from a systematic review of thromboprophylaxis in orthopedic patients at high risk for VTE.[136] Our choice of indirect evidence is based on similarities in risk of VTE, the type and duration of intervention (extended prophylactic-dose LMWH), and outcomes (symptomatic VTE and major bleeding events). Our baseline risk estimates are based on observational studies of pregnant women with previous VTE (Table S16). We have categorized patients into groups at low risk (major transient risk factor for VTE), intermediate (hormone- or pregnancy-related or unprovoked VTE), or high risk (multiple prior unprovoked VTE or persistent risk factors, such as paralysis) during pregnancy. Clinicians can use these risk groups to determine the anticipated absolute effects of treatment with LMWH in their patients. Given the evidence of similar absolute risks for VTE antepartum and postpartum outlined

Table 6—*[Section 8.2.2, 8.2.3] Summary of Findings: Antepartum and Postpartum Prevention of VTE With Prophylactic-Dose LMWH vs No Prophylaxis in Pregnant Women With Prior VTE*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects During Pregnancy: Risk Without Prophylaxis	Anticipated Absolute Effects During Pregnancy: Risk Difference With LMWH (95% CI)
Symptomatic VTE, DVT, and pulmonary embolism	1,953 (6 RCTs), 27-35 d postoperative	Low due to indirectness[c] and imprecision[a]	RR 0.36 (0.20-0.67)	Low risk (transient risk factor)	
				20 VTE per 1,000[a]	13 fewer VTE per 1,000 (from 16 fewer to 7 fewer)
				Intermediate and high risk (pregnancy- or estrogen-related, idiopathic or multiple prior VTE but discontinued VKAs)	
				80 VTE per 1,000[a]	51 fewer VTE per 1,000 (from 65 fewer to 30 fewer)
Major bleeding[b]	1,953 (6 RCTs), 27-35 d postoperative	Low due to indirectness[c] and imprecision[d]	RR 0.43 (0.11-1.65)	Antepartum period	
				5 bleeding events per 1,000[e]	No significant difference; 3 fewer bleeding events per 1,000 (from 3 fewer to 3 more)
				Postpartum period	
				20 bleeding events per 1,000[e]	No significant difference; 11 fewer bleeding events per 1,000 (from 18 fewer to 13 more)

See Table 2 and 5 legends for expansion of abbreviations.

[a]Control group risk estimates for VTE in the antepartum and postpartum period come from studies summarized in Table S16. Quality of evidence is rated down because of imprecision in these risk estimates. We consider the distribution of VTE antepartum and postpartum to be equal.

[b]Nonfatal maternal hemorrhage (according to section 1.0) defined as a symptomatic bleeding complication noted during pregnancy or within 6 wk postpartum that involved bleeding into a critical site, bleeding causing a fall in hemoglobin level of ≥ 2 g/dL, or bleeding leading to transfusion of ≥ 2 units of whole blood or red cells.

[c]Population is indirect (ie, did not include pregnant women). Different doses of LMWH were used. Treatment was initiated variably before or after surgery with a duration of ~7 days (in hospital). Outcomes variably reported. Meta-analysis also provides other outcomes such as mortality, asymptomatic DVT, and specific bleed outcomes (wound hematoma, transfusion). Follow-up varied between trials from 3 wk to 9 mo.

[d]Wide CIs for absolute effect of LMWH in high-risk group included benefit and harm.

[e]Control group risk estimate for major bleeding events comes from a systematic review by Greer et al.[38]

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previously herein, the absolute effects of LMWH shown in Table 6 and Table S19 are applicable both to the 9-month antepartum period and the 6-week postpartum period.

LMWH is the preferred agent for prophylaxis (see section 2.0). Dose regimens include subcutaneous enoxaparin 40 mg every 24 h,[27,158] dalteparin 5,000 units every 24 h,[207] and dose-adjusted LMWH to achieve a peak anti-Xa level of 0.2 to 0.6 units/mL[213-215] (Table S18). Although all of the studies evaluating these regimens reported low recurrence rates, most were cohort studies and, therefore, no comparative data from untreated controls are available. Further, because different doses of anticoagulant prophylaxis have not been compared directly, the optimal dose of LMWH is unknown. Although indirect evidence (Table 6, Table S19) suggests that prophylactic-dose LMWH is effective (ie, RR of 0.36) in high-risk settings, some investigators have reported recurrent pregnancy-associated VTE in pregnant women prescribed prophylactic LMWH[36,37,204,208,216] However, it is unclear whether these represent true failures or were due to compliance issues with long-term daily subcutaneous injections.

Women who have an indication for long-term vitamin K antagonists, mostly because of multiple episodes of VTE, are considered at very high risk of recurrent VTE during pregnancy and the postpartum period. Dose-adjusted LMWH is a rational option for anticoagulant therapy during pregnancy, with resumption of long-term vitamin K antagonists after delivery. Alternatively, a reduced therapeutic-dose regimen (~75% of the usual therapeutic dose) may represent a reasonable option given evidence of the superior effectiveness of LMWH compared with vitamin K antagonists observed in the treatment of VTE in cancer patients.[177]

Increased renal clearance of LMWH during pregnancy has led to suggestions that clinicians monitor the anticoagulant effect of prophylactic-dose LMWH using anti-Xa levels.[209,214] However, the appropriate target range for prophylaxis is uncertain, and there is no evidence to support any specific target range. Moreover, routine monitoring of anti-Xa levels is expensive, inconvenient, and possibly unreliable[187,217] (see Garcia et al[188] in this guideline).

An alternate strategy for DVT prevention is repeated screening during the antepartum period with noninvasive tests for DVT, such as compression ultrasonography. This strategy generally is not justified for two reasons. First, if we postulate rates of recurrent VTE of 5%, given an ultrasound sensitivity of 96% and specificity of 98%, we would anticipate that 28% of positive results would be false positives. Second, there is no evidence to guide the timing of screening, and it is possible that a clinically important recurrence could occur between ultrasounds. We recommend that women should be investigated aggressively if symptoms suspicious of DVT or PE occur. That said, the performance of a baseline compression ultrasound of a previously affected leg prior to or early on in pregnancy may be useful to help differentiate residual thrombosis from new disease in women presenting with symptoms during pregnancy (see Bates et al[218] in this guideline).

Recommendations

8.2.1. For all pregnant women with prior VTE, we suggest postpartum prophylaxis for 6 weeks with prophylactic- or intermediate-dose LMWH or vitamin K antagonists targeted at INR 2.0 to 3.0 rather than no prophylaxis (Grade 2B)**.**

8.2.2. For pregnant women at low risk of recurrent VTE (single episode of VTE associated with a transient risk factor not related to pregnancy or use of estrogen), we suggest clinical vigilance antepartum rather than antepartum prophylaxis (Grade 2C)**.**

8.2.3. For pregnant women at moderate to high risk of recurrent VTE (single unprovoked VTE, pregnancy- or estrogen-related VTE, or multiple prior unprovoked VTE not receiving long-term anticoagulation), we suggest antepartum prophylaxis with prophylactic- or intermediate-dose LMWH rather than clinical vigilance or routine care (Grade 2C)**.**

8.2.4. For pregnant women receiving long-term vitamin K antagonists, we suggest adjusted-dose LMWH or 75% of a therapeutic dose of LMWH throughout pregnancy followed by resumption of long-term anticoagulants postpartum rather than prophylactic-dose LMWH (Grade 2C)**.**

9.0 Prevention of VTE in Pregnant Women With Thrombophilia and No Prior VTE

9.1 Risk of Pregnancy-Related VTE in Women With Thrombophilia

A number of studies have examined the association between hereditary thrombophilias and pregnancy-related VTE. Table 7 presents estimated and observed pooled risks for pregnant women with thrombophilia in the absence and presence of a positive family history.

In a systematic review of nine studies that assessed the risk of VTE in pregnant women with inherited thrombophilias but not necessarily a family history of

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Table 7—*[Section 9.2.1-9.2.4] Risk of Pregnancy-Related VTE in Women With Thrombophilia Stratified by Family History for VTE*

Thrombophilic Defect, n/No. Women With Thrombophilia	Estimated Relative Risk, OR (95% CI)[a]	Observed or Estimated Absolute Risk of VTE Antepartum and Postpartum Combined, % Pregnancies (95% CI)[b,c]
Antithrombin/protein C/protein S deficiency combined		
Family studies, 7/169[219]	…	4.1 (1.6-8.3)
Antithrombin deficiency		
Family studies, 1/33[219]	…	3.0 (0.08-15.8)
Nonfamily studies, 8/11[151]	4.7 (1.3-17.0)	0.7 (0.2-2.4)
Protein C deficiency		
Family studies, 1/60[219]	…	1.7 (0.4-8.9)
Nonfamily studies, 23/32[151]	4.8 (2.2-10.6)	0.7 (0.3-1.5)
Protein S deficiency		
Family studies, 5/76[219]	…	6.6 (2.2-14.7)
Nonfamily studies, 16/28[151]	3.2 (1.5-6.9)	0.5 (0.2-1.0)
Factor V Leiden, heterozygous		
Family studies, 26/828[220-222, 223]	…	3.1 (2.1-4.6)
Nonfamily studies, 96/226[151]	8.3 (5.4-12.7)	1.2 (0.8-1.8)
Factor V Leiden, homozygous		
Family studies, 8/57[224-226]	…	14.0 (6.3-25.8)
Nonfamily studies, 29/91[153]	34.4 (9.9-120.1)	4.8 (1.4-16.8)
Prothrombin G20201A mutation, heterozygous		
Family studies, 6/228[227,228]	…	2.6 (0.9-5.6)
Nonfamily studies, 42/61[151]	6.8 (2.5-18.8)	1.0 (0.3-2.6)
Prothrombin G20201A mutation, homozygous		
Family studies, n/a	…	…
Nonfamily studies, 2/2[151]	26.4 (1.2-559.3)	3.7 (0.2-78.3)

[a]Data from Robertson et al[151]; number of VTE cases in women with the thrombophilia in question vs VTE cases in women without the specified thrombophilia.
[b]In the family studies, number of women with VTE out of number of women with thrombophilia. Observed absolute risks for family studies are risks observed in cohorts of families from a proband with symptomatic VTE and thrombophilia. Study numbers are pooled. Incidence is derived by adding number of events and dividing by number of pregnancies.
[c]Estimated absolute risks for nonfamily studies are derived by multiplying the pooled ORs with their corresponding 95% CIs from Robertson et al[151] with the overall baseline VTE incidence (ie, antepartum and until 6 wk postpartum combined) of 1.40 per 1,000 from a group of women aged 25 to 34 y (I. A. Greer, MD, personal communication, November 2010).

VTE, all with the exception of homozygosity for the thermolabile methylene tetrahydrofolate reductase variant (MTHFR C677T) were associated with a statistically significant increase in the risk of pregnancy-related VTE (Table 7).[151] The highest risks were associated with homozygosity for factor V Leiden (OR, 34.4; 95% CI, 9.9-120.1) or the prothrombin G20210A variant (OR, 26.4; 95% CI, 1.2-559.3). The most common inherited thrombophilias (ie, heterozygosity for factor V Leiden [OR, 8.3; 95% CI, 5.4-12.7], prothrombin G20210A variant [OR, 6.8; 95% CI, 2.5-18.8]) were associated with lower risks. Deficiencies of the endogenous anticoagulants were associated with similar risk increases (ORs for antithrombin, protein S, and protein C deficiency, 4.7 [95% CI, 1.30-17.0], 4.8 [95% CI, 2.2-10.7], and 3.2 [95% CI, 1.5-6.0], respectively).

In a subsequently published meta-analysis undertaken to provide an estimate of the association of the factor V Leiden mutation with pregnancy-related VTE that used slightly different study entry criteria, the risk estimate obtained from case-control studies was similar to that in the first systematic review (OR, 8.6; 95% CI, 4.8-12.6).[229] However, cohort studies, which are likely to be more reliable, showed a lower pooled OR of 4.5 (95% CI, 1.8-10.9).[229] Given a background incidence of VTE during pregnancy of ~1/1,000 deliveries, the absolute risk of VTE in women without a prior event or family history remains low (in the range of 5-12/1,000 deliveries) for most of the inherited thrombophilias, except perhaps for homozygous carriers of the factor V Leiden or the prothrombin mutations where the OR from case-control studies suggest baseline risks of pregnancy-related VTE of ~4%.

Regardless of the presence of thrombophilia, a positive family history of VTE increases the risk for VTE twofold to fourfold.[230] Several family-based cohort studies found that women with inherited thrombophilia and a positive family history who have not had a previous episode of VTE have a risk of developing a first VTE during pregnancy or the postpartum period of between 1.7% for protein C deficiency[219] and 14.0% for homozygous carriers of the factor V

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Leiden mutation[224-226] (Table 7).[219-228,231,232] These estimates are, however, imprecise, particularly for the less common thrombophilias (see wide CIs in Table 7).

Although the deficiencies of the natural anticoagulants (and in particular, antithrombin deficiency) are usually labeled as high-risk thrombophilias, this perception is based on older studies with important methodological limitations. For instance, Conard et al[233] reported a very high risk of pregnancy-related VTE in women with antithrombin and protein C or protein S deficiency, but many patients included in this report had a history of recurrent VTE, and all episodes of VTE were not objectively confirmed. More rigorous recent studies included in Table 7 do not support the high risk of recurrence from previous studies. Two small studies that investigated the risk in women with both the factor V Leiden and prothrombin mutations found similar risk estimates to those seen in single heterozygous carriers.[226,234] Based on these estimates, we suggest that serious consideration of prophylaxis is warranted only in (1) homozygous carriers of the factor V Leiden or prothrombin gene mutations (regardless of family history) and (2) women with the other inherited thrombophilias with a family history of VTE.

Acquired thrombophilias have been less well studied, but repeated positivity at least 12 weeks apart for APLAs (lupus anticoagulants [nonspecific inhibitors], moderate- or high-titer IgG or IgM anticardiolipin antibodies [> 40 GPL or MPL or > 99th percentile], or moderate- of high-titer IgG or IgM anti-β_2-glycoprotein I antibodies [> 99th percentile]) is associated with an increased risk of VTE.[235,236] The VTE risk in women with APLAs and no previous venous thrombosis is uncertain.[237,238]

Hyperhomocysteinemia is associated with an increased risk of VTE in nonpregnant women.[239] However, it does not appear that homozygosity for MTHFR C667T (the genetic abnormality most commonly associated with hyperhomocysteinemia) alone leads to an increased risk of VTE in pregnant women.[151] As clinical events in homozygotes are likely to reflect the interaction of the genotype with a relative deficiency of vitamins, such as B12 and folic acid, the absence of an association of this genotype with gestational VTE may reflect pregnancy-related physiological reduction in homocysteine levels and the effects of folic acid supplements that are now taken widely by women in pregnancy for prevention of neural tube defects.[240]

9.2 Prevention of Pregnancy-Related VTE in Women With Thrombophilia

Because of a paucity of high-quality evidence measuring the effectiveness and safety of antithrombotic agents in preventing VTE in this population, we used indirect evidence to inform our treatment recommendations. Given the low risk for VTE in women with thrombophilia but no family history, we restricted our analysis to women with thrombophilia and a family history of VTE (Table 8, Table S20). We estimated the baseline VTE incidence (ie, antepartum and until 6 weeks postpartum combined) as 1.40 of 1,000 (I. A. Greer, MD, personal communication, November 8, 2010). Evidence about relative effects of treatment is taken from a meta-analysis of thromboprophylaxis in patients undergoing hip arthroplasty.[136] We have rated the quality of evidence as low because of indirectness of the population and intervention as well as the considerable imprecision in baseline risk estimates for VTE in women with thrombophilias.

Estimates of absolute effects are relatively large in women with a positive family history of VTE who are homozygous for the factor V Leiden mutation—47 fewer VTE/1,000 antepartum and 47 fewer VTE of 1,000 postpartum when prophylaxis is used, with no increased risk of major bleeding (Table 8, Table S20). In women with a positive family history for VTE and antithrombin, protein C, or protein S deficiency, these figures are approximately 13 of 1,000 antepartum and 13 of 1,000 postpartum. For the other thrombophilias, the estimated number of VTE prevented is 10 of 1,000 both antepartum and postpartum. The evidence is, however, low quality and includes imprecise estimates.

The increased risk in women with thrombophilia and a family history of VTE begins early in pregnancy[173]; therefore, when antepartum prophylaxis is used, it should be commenced as early as possible in the first trimester. The burden of self-injecting with LMWH over several months and the risk of skin reactions weigh into our weak recommendation for antepartum thromboprophylaxis. For postpartum prophylaxis, we consider vitamin K antagonist therapy targeted to an INR of 2.0 to 3.0 an appropriate alternative to LMWH, except in patients with protein C or S deficiency who are at risk for developing warfarin-induced skin necrosis.[241-243]

Recommendations

9.2.1. For pregnant women with no prior history of VTE who are known to be homozygous for factor V Leiden or the prothrombin 20210A mutation and have a positive family history for VTE, we suggest antepartum prophylaxis with prophylactic- or intermediate-dose LMWH and postpartum prophylaxis for 6 weeks with prophylactic- or intermediate-dose LMWH or vitamin K antagonists targeted at INR 2.0 to 3.0 rather than no prophylaxis (Grade 2B)**.**

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Table 8—*[Section 9.2.1-9.2.4] Summary of Findings: Antepartum and Postpartum Prophylactic-Dose LMWH vs No Thromboprophylaxis for Pregnant Women With a Known Thrombophilia*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects Antepartum and Postpartum (Different Risk Estimates for Bleeding Events): Risk Without Prophylaxis	Risk Difference With LMWH (95% CI)
Symptomatic VTE, DVT, and pulmonary embolism	1,953 (6 RCTs), 27-35 d postoperative	Low due to indirectness[b] and imprecision[a]	RR 0.36 (0.20-0.67)	Positive family history VTE and heterozygous factor V Leiden or prothrombin 202010A	
				15 VTE per 1,000[c]	10 fewer VTE per 1,000 (from 12 fewer to 5 fewer)
				Positive family history VTE and antithrombin, protein C, or protein S deficiency	
				20 VTE per 1,000[c]	13 fewer VTE per 1,000 (from 16 fewer to 6 fewer)
				Positive family history VTE and homozygous factor V Leiden or prothrombin 20210A	
				70 VTE per 1,000[c]	47 fewer per 1,000 (from 56 fewer to 31 fewer)
				No family history of VTE but homozygous factor V Leiden or prothrombin 20210A	
				20 VTE per 1,000[c]	13 fewer VTE per 1,000 (from 16 fewer to 6 fewer)
Major bleeding	5,456 (7 RCTs), 3 wks-9 mo	Moderate due to indirectness[b]	RR 0.43 (0.11-1.65)	Antepartum period	
				5 bleeding events per 1,000[d]	No significant difference; 3 fewer bleeding events per 1,000 (from 3 fewer to 3 more)
				Postpartum period	
				20 bleeding events per 1,000[d]	No significant difference; 11 fewer bleeding events per 1,000 (from 18 fewer to 13 more)

See Table 2 legend for expansion of abbreviations.
[a]Imprecision in control group risk estimates for all thrombophilias (see Table S20) results in imprecise anticipated absolute effects.
[b]The population did not include pregnant women. Different doses of LMWH were used; treatment was initiated variably before or after surgery with a duration of ~7 days in hospital and 25 d out of hospital. Outcomes were variably reported.
[c]Control group risk estimate for VTE comes from observational studies summarized in Table S20. Our antepartum risk estimate is based on assumed equal distribution of antepartum and postpartum VTE events based on data from observational studies (I. A. Greer, MD, personal communication, November 8, 2010).
[d]Control group risk estimate for major bleeding events antepartum comes from systematic review by Greer.[38]

9.2.2. For pregnant women with all other thrombophilias and no prior VTE who have a positive family history for VTE, we suggest antepartum clinical vigilance and postpartum prophylaxis with prophylactic- or intermediate-dose LMWH or, in women who are not protein C or S deficient, vitamin K antagonists targeted at INR 2.0 to 3.0 rather than routine care (Grade 2C)**.**

9.2.3. For pregnant women with no prior history of VTE who are known to be homozygous for factor V Leiden or the prothrombin 20210A mutation and who do not have a positive family history for VTE, we suggest antepartum clinical vigilance and postpartum prophylaxis for 6 weeks with prophylactic- or intermediate-dose LMWH or vitamin K antagonists targeted at INR 2.0 to 3.0 rather than routine care (Grade 2B)**.**

9.2.4. For pregnant women with all other thrombophilias and no prior VTE who do not have a positive family history for VTE, we suggest antepartum and postpartum clinical vigilance rather than pharmacologic prophylaxis (Grade 2C)**.**

10.0 Thrombophilia and Pregnancy Complications

Various pregnancy complications have been linked to thrombophilic states. Unfortunately, adverse pregnancy outcomes are not infrequent in the general population. Fifteen percent of clinically recognized pregnancies end in miscarriage, but total reproductive loss may be as high as 50%.[244] Five percent of women experience two or more losses, and 1% to 2% have three or more consecutive losses. Other placental-mediated pregnancy complications include preeclampsia, fetal growth restriction, and placental abruption.

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Successful pregnancy outcome depends on trophoblast invasion into the uterine vasculature and on the development and maintenance of an adequate uteroplacental circulatory system. Inadequate placentation and damage to the spiral arteries with impaired flow, an increased maternal inflammatory response, and prothrombotic changes may lead to placental-medicated pregnancy complications.[245] Animal studies suggest that the hemostatic system plays an important role in placental and fetal development, although hypercoagulability is unlikely to be the sole mechanism by which thrombophilia increases the risk of pregnancy failure. It is more likely that effects on trophoblast differentiation and early placentation may be involved through as yet unknown mechanisms. Interestingly, both aspirin and heparin appear to influence these early trophoblast and placentation mechanisms in vitro as well as in a hypercoagulability mouse model.[246-248]

10.1 Risk of Pregnancy Complications in Women With Thrombophilia

Pregnancy complications occur with increased frequency in women with APLAs. APLA syndrome can be diagnosed in women who test positive for lupus anticoagulant (nonspecific inhibitor) or moderate- to high-titer antibodies to IgG or IgM anticardiolipin (> 40 GPL or MPL or > 99th percentile) or IgG or IgM β_2-glycoprotein I (> 99th percentile) on two occasions at least 12 weeks apart and who experience at least one unexplained fetal death (later than 10 weeks of gestation); three or more unexplained consecutive miscarriages (before 10 weeks of gestation); or one or more premature births of a morphologically normal neonate before the 34th week of gestation because of eclampsia, severe preeclampsia, or placental insufficiency.[235]

There is convincing evidence that APLAs are associated with an increased risk of recurrent and late pregnancy loss.[151,249-253] Lupus anticoagulants (nonspecific inhibitors) are more strongly related to pregnancy loss than are the other antibodies against phospholipids; although associations have also been seen with moderate- to high-titer IgG and IgM antibodies (> 5 SDs above normal, > 99th percentile, or > 20 GPL/MPL units).[253] The importance of anti-β_2-glycoprotein I antibodies is not clearly established.[253] Furthermore, there is less agreement on the association between the presence of APLAs and the occurrence of other pregnancy complications, including preeclampsia, placental abruption, and intrauterine growth restriction.[151,243,254-268]

The association between inherited thrombophilic disorders and miscarriage, first observed in women from families with venous thrombosis, has been confirmed in many studies.[151,228,269-277] A single late fetal loss and severe preeclampsia are also associated with inherited thrombophilia,[151,274,275] whereas the presence of an association is controversial in women with fetal growth restriction or placental abruption.[151,277]

Table 9 summarizes the findings of a meta-analysis of 25 studies (predominantly case control)[151] examining the association between thrombophilia and various pregnancy complications. The wide CIs around the point estimates of some associations illustrate the uncertainty of the findings, particularly for the less-prevalent thrombophilias. In a meta-analysis limited to prospective cohort studies,[277] the pooled OR for pregnancy loss in women with factor V Leiden (absolute risk, 4.2%) compared with women without this mutation (absolute risk, 3.2%) was 1.52 (95% CI, 1.06-2.19). The meta-analysis was unable to establish or refute an association between the presence of factor V Leiden and preeclampsia (OR, 1.23; 95% CI, 0.89-1.70) or fetal growth restriction (OR, 1.0; 95% CI, 0.80-1.25). Results also failed to demonstrate or exclude an association between the prothrombin mutation and either preeclampsia (OR, 1.25; 95% CI, 0.79-1.99), fetal growth restriction (OR, 1.25; 95% CI, 0.92-1.70), or pregnancy loss (OR, 1.13; 95% CI, 0.64-2.01). Given these results, it remains unclear whether screening for inherited thrombophilias is in the best interests of women with pregnancy complications.

10.2 Prevention of Pregnancy Complications in Women With Thrombophilia

Clinicians are increasingly using antithrombotic therapy in women at risk for these complications (Tables S21, S22).[278-299] With respect to acquired thrombophilias, of the interventions examined in a systematic review[252] (up to date in February 2005) that summarized the data from 13 randomized or quasi-randomized trials, including a total of 849 pregnant women with APLA and a history of at least two unexplained pregnancy losses, only UFH combined with aspirin (two trials, n = 150) reduced the incidence of pregnancy loss.[278,279] Consistent findings of a third study (n = 72),[290] when included, yielded an relative risk of 0.44 (95% CI, 0.30-0.66) for UFH combined with aspirin compared with aspirin alone (Table 10, Table S23). The use of higher-dose UFH and aspirin did not decrease the risk of pregnancy loss compared with low-dose UFH and aspirin (one trial, n = 50; RR, 0.83; 95% CI, 0.29-2.38).[252,280] On its own, aspirin (three trials, n = 71) failed to demonstrate or exclude an effect on pregnancy loss compared with usual care[281] or placebo[282,283] (RR, 1.05; 95% CI, 0.66-1.68).[252] In one trial, the combination of LMWH with aspirin had also failed to demonstrate or exclude an effect on pregnancy loss when compared with

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Table 9—*[10.2.1,10.2.2] Association Between Pregnancy Complications and Thrombophilia*

Type of Thrombophilia	Early Loss	Recurrent First Trimester Loss	Nonrecurrent Second Trimester Loss	Late Loss	Preeclampsia	Placental Abruption	Fetal Growth Restriction
Factor V Leiden (homozygous)	2.71 (1.32-5.58)	[a]	[a]	1.98 (0.40-9.69)	1.87 (0.44-7.88)	8.43 (0.41-171.20)	4.64 (0.19-115.68)
Factor V Leiden (heterozygous)	1.68 (1.09-2.58)	1.91 (1.01-3.61)[a]	4.12 (1.93-8.81)[a]	2.06 (1.10-3.86)	2.19 (1.46-3.27)	4.70 (1.13-19.59)	2.68 (0.59-12.13)
Prothrombin gene mutation (heterozygous)	2.49 (1.24-5.00)	2.70 (1.37-5.34)	8.60 (2.18-33.95)	2.66 (1.28-5.53)	2.54 (1.52-4.23)	7.71 (3.01-19.76)	2.92 (0.62-13.70)
MTHFR C677T (homozygous)	1.40 (0.77-2.55)	0.86 (0.44-1.69)	NA	1.31 (0.89-1.91)	1.37 (1.07-1.76)	1.47 (0.40-5.35)	1.24 (0.84-1.82)
Antithrombin deficiency	0.88 (0.17-4.48)	NA	NA	7.63 (0.30-196.36)	3.89 (0.16-97.19)	1.08 (0.06-18.12)	NA
Protein C deficiency	2.29 (0.20-26.43)	NA	NA	3.05 (0.24-38.51)	5.15 (0.26-102.22)	5.93 (0.23-151.58)	NA
Protein S deficiency	3.55 (0.35-35.72)	NA	NA	20.09 (3.70-109.15)	2.83 (0.76-10.57)	2.11 (0.47-9.34)	NA
Anticardiolipin antibodies	3.40 (1.33-8.68)	5.05 (1.82-14.01)	NA	3.30 (1.62-6.70)	2.73 (1.65-4.51)	1.42 (0.42-4.77)	6.91 (2.70-17.68)
Lupus anticoagulants (nonspecific inhibitor)	2.97 (1.03-9.76)	NA	14.28 (4.72-43.20)	2.38 (0.81-6.98)	1.45 (0.70-4.61)	NA	NA
Hyperhomocysteinemia	6.25 (1.37-28.42)	4.21 (1.28-13.87)	NA	0.98 (0.17-5.55)	3.49 (1.21-10.11)	2.40 (0.36-15.89)	NA

Data are presented as OR (95% CI) and derived from Robertson et al.[151] MTHFR = methylene tetrahydrofolate reductase variant; NA = not available.
[a]Homozygous and heterozygous carriers were grouped together; it is not possible to extract data for each state.

aspirin alone (RR, 0.78; 95% CI, 0.39-1.57).[252,284] Similar results were obtained when LMWH and aspirin was compared with IV γ-globulin (RR, 0.37; 95% CI, 0.12-1.16).[252,281]

A subsequent meta-analysis that combined data from randomized trials testing the efficacy of a combination of heparin (either UFH or LMWH) and aspirin vs aspirin alone in patients with APLAs and recurrent pregnancy loss[292] included an additional LMWH study published since the first systematic review.[293] When data from five trials (n = 334) were combined, the frequency of live births was significantly higher in the aspirin and heparin group (74.3%) than in those randomized to aspirin alone (55.8%) (RR, 1.3; 95% CI, 1.0-1.7; NNT to achieve one live birth, 5.6).[292] When studies that used LMWH and UFH were analyzed separately, there was just a trend of higher birth rate in patients receiving aspirin and LMWH (RR, 1.1; 95% CI, 0.9-1.3). Although the relative effectiveness of UFH vs LMWH with respect to prevention of recurrent pregnancy loss in women with APLAs is not established, the results of two small pilot studies (n = 26 and n = 50) suggest that the combination of LMWH and aspirin might at least be equivalent to UFH and aspirin in preventing recurrent pregnancy loss (RR, 0.44 [95% CI, 0.17-1.00][285] and 0.8 [95% CI, 0.26-2.48][286] in women receiving LMWH vs UFH, respectively). Given the absence of evidence that women with APLA syndrome and a single late pregnancy loss, preeclampsia, or fetal growth restriction benefit from the addition of UFH or LMWH to aspirin, we do not recommend for or against screening for APLAs in women with these pregnancy complications.

The data addressing the use of antithrombotic therapy in women with inherited thrombophilia and pregnancy loss consists of predominantly small uncontrolled trials or observational studies with important methodological limitations.[288,289,294-302] Gris et al[297] reported that enoxaparin in women with factor V Leiden, the prothrombin gene mutation, or protein S deficiency and one previous pregnancy loss increased the live birth rate (86%) compared with low-dose aspirin alone (29%); however, the methodology and results of this randomized trial have generated much controversy,[300,303-305] and we have not included it in the evidence we used to make recommendations. A subsequent cohort study found the live birth rate of subsequent pregnancies after a single pregnancy loss at or later than 12 weeks gestation in carriers of factor V Leiden or the prothrombin mutation was, without intervention, 68% (95% CI, 46%-85%).[306]

Tables S21 and S22 summarize the methodology and results of randomized trials and nonrandomized observational studies (excluding those that used a historical control group). These data do not provide

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Table 10—*[Section 10.2.1,10.2.3] Summary of Findings: Should UFH Plus Aspirin or Aspirin Alone Be Used for Pregnant Women With APLA and Recurrent Pregnancy Loss*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects During Pregnancy: Risk With Aspirin	Anticipated Absolute Effects During Pregnancy: Risk Difference With UFH + Aspirin (95% CI)
Pregnancy loss	212 (3 RCTs), not reported	Moderate due to risk of bias[b]	RR 0.44 (0.33- 0.66)	500 losses per 1,000[a]	283 fewer losses per 1,000 (from 353 fewer to 172 fewer)
IUGR[c]	134 (3 RCTs), not reported	Low due to risk of bias[b] and imprecision[d]	RR 1.71 (0.48-6.17)	56 IUGR per 1,000[a]	No significant difference; 39 more IUGR per 1,000 (from 29 fewer to 287 more)
Preeclampsia not clearly defined	134 (3 RCTs), not reported	Low due to risk of bias[b] and imprecision[d]	RR 0.43 (0.09-2.08)	74 cases per 1,000[a]	No significant difference; 30 fewer cases per 1,000 (from 67 fewer to 80 more)

Data from unpublished meta-analysis based on three trials.[278,279,290] Major bleeding is a critical outcome that was not reported in the three trials. APLA = antiphospholipid antibody; IUGR = intrauterine growth restriction; UFH = unfractionated heparin. See Table 2 legend for expansion of other abbreviations.
[a]Control group risk estimates with aspirin come from the meta-analysis of three trials.
[b]Risk of bias due to issues of randomization, allocation concealment, and blinding.
[c]Estimated fetal weight below the 10th percentile for gestational age.
[d]Wide CIs include benefit and harm.

evidence that LMWH improves pregnancy outcome in women with inherited thrombophilia and recurrent pregnancy loss.

Recommendations

10.2.1. For women with recurrent early pregnancy loss (three or more miscarriages before 10 weeks of gestation), we recommend screening for APLAs (Grade 1B).

10.2.2. For women with a history of pregnancy complications, we suggest not to screen for inherited thrombophilia (Grade 2C).

10.2.3. For women who fulfill the laboratory criteria for APLA syndrome and meet the clinical APLA criteria based on a history of three or more pregnancy losses, we recommend antepartum administration of prophylactic- or intermediate-dose UFH or prophylactic LMWH combined with low-dose aspirin, 75 to 100 mg/d, over no treatment (Grade 1B).

10.2.4. For women with inherited thrombophilia and a history of pregnancy complications, we suggest not to use antithrombotic prophylaxis (Grade 2C).

11.0 Management of Women With a History of Preeclampsia or Recurrent Fetal Loss and No Thrombophilia

Preeclampsia is associated with microvascular fibrin deposition indicative of activation of platelets and coagulation[307] as well as widespread endothelial dysfunction.[308-310] The manifestations of this disease are protean,[310] and preeclampsia should not be considered as a single disease entity but rather as a maternal response to abnormal placentation.[311,312] Women with a thrombophilic disorder, whether it be acquired or heritable, may be more likely to develop preeclampsia, but for heritable thrombophilias, this association is largely based on retrospective case-control (Table 9) and cohort studies[151]; prospective investigations have not confirmed these findings.[275,313]

11.1 Prevention of Recurrent Preeclampsia in Women With No Thrombophilia

The observations of endothelial dysfunction and platelet dysfunction in preeclampsia led to the hypothesis that antiplatelet agents might prevent or delay the development of this condition. Systematic review results suggest that the use of antiplatelet agents (primarily low-dose aspirin) is associated with modest reductions in the relative risk of preeclampsia. Table 11[314,315] and Table S24 summarize the evidence and main findings from the most recent Cochrane review of 43 randomized trials with 32,590 women,[314] providing moderate-quality evidence of a significant reduction (RR, 0.83; 95% CI, 0.77-0.89) in the risk of preeclampsia associated with the use of antiplatelet agents. The relative effect of antiplatelet therapy appears to be similar in women at low and high risk for preeclampsia (ie, no evidence of subgroup effect). However, as shown in Table 11 and Table S24, the baseline risk of preeclampsia determines the absolute effect of antiplatelet therapy, and women at low risk have a substantially lower benefit (NNT, 100) than

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Table 11—*[11.1.1] Summary of Findings: Should Aspirin Rather Than No Treatment Be Used for Prevention of Preeclampsia in Women Without Thrombophilia*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects During Pregnancy: Risk Without Antiplatelet Therapy	Anticipated Absolute Effects During Pregnancy: Risk Difference With Antiplatelet Therapy (95% CI)
Preeclampsia defined as proteinuric preeclampsia in Cochrane Systematic Review	32,590 (43 RCTs), not reported	Moderate due to inconsistency[b]	RR 0.83 (0.77-0.89)	Low risk for preeclampsia[c]	
				60 cases per 1,000[a]	10 fewer cases per 1,000 (from 14 fewer to 7 fewer)
				High risk for preeclampsia[c]	
				210 cases per 1,000[a]	36 fewer losses per 1,000 (from 46 fewer to 23 fewer)
Major bleeding events[d]	95,000 (6 RCTs), 3.8-10 y	Moderate due to indirectness[e]	RR 1.54 (1.30-1.82)	15 bleeding events per 1,000[f]	8 more bleeding events per 1,000 (from 5 more to 12 more)

Data from Duley et al[314] and ATT Collaboration.[315] See Table 2 legend for expansion of abbreviations.
[a]Control group risk estimates for preeclampsia is based on control event rates in studies included in subgroup analyses in the meta-analysis.
[b]Heterogeneity ($I^2 = 46\%$, $P < .001$) might be related to different types and doses of antiplatelet agents, the lack of placebo in the control group in many of the trials, different populations of pregnant women concerning risk of preeclampsia, and effect of treatment.
[c]High risk was defined in the systematic review: Women who were either normotensive or had chronic hypertension without superimposed preeclampsia at trial entry were classified as being at high risk if they had one or more of the following: previous severe preeclampsia, diabetes, chronic hypertension, renal disease, or autoimmune disease. Low risk constitutes women without these characteristics.
[d]Major antenatal nonfatal hemorrhage.
[e]Rated down for indirectness due to population (primary prevention cardiovascular disease).[315] The Cochrane Review does not report the effects of antiplatelet therapy on major bleeding events in pregnant women.
[f]Control group risk estimate for major bleeding events antepartum from systematic review by Greer et al.[38]

women at high risk (NNT, 28). Current data from the Cochrane review do not show a difference in effect when low-dose aspirin is started before or after 20 weeks gestation.[314]

What constitutes high risk for preeclampsia is not always immediately clear, as available studies have used different risk stratification schemes. In identifying levels of risk, studies quantifying the risk of preeclampsia[312,316,317] suggested a relative risk of more than sevenfold with APLAs and previous preeclampsia and an approximately twofold increase in relative risk associated with a BMI ≥ 35 kg/m², preexisting diabetes, twin pregnancy, and a family history of preeclampsia. According to the Cochrane systematic review, women who were either normotensive or had chronic hypertension without superimposed preeclampsia at trial entry were classified as being at high risk if they had one or more of the following: previous severe preeclampsia, diabetes, chronic hypertension, renal disease, or autoimmune disease.[314]

Some have suggested anticoagulant therapy with LMWH or UFH for women at very high risk for preeclampsia. An effect of anticoagulant therapy on the risk of preeclampsia is biologically plausible not only because of a reduction in thrombosis formation but also because LMWH has been shown to have an antiapoptotic effect on trophoblasts,[248,318] a potential trigger for preeclampsia. However, an observational study of 58 women with previous preeclampsia and an underlying thrombophilia found no difference in the risk of preeclampsia between those treated with LMWH and low-dose aspirin vs those treated with low-dose aspirin alone or no prophylactic therapy.[319] In a randomized trial of 80 nonthrombophilic women considered to be at increased risk for preeclampsia on the basis of both a history and an underlying angiotensin-converting enzyme insertion/deletion polymorphism that examined the effect of prophylactic LMWH (dalteparin 5,000 units/d) on the pregnancy outcome, maternal BP, and uteroplacental flow,[320] women receiving LMWH had a lower incidence of adverse outcomes, with a 74.1% reduction in preeclampsia (RR, 0.26; 95% CI, 0.08-0.86) and a 77.5% reduction in fetal growth restriction (RR, 0.14; 95% CI, 0.03-0.56). A subsequent pilot study of 116 pregnant women with no detectable thrombophilia and previous severe preeclampsia, small for gestational age baby, placental abruption, or intrauterine fetal demise randomized to prophylactic-dose dalteparin or no dalteparin reported that dalteparin was associated with a lower rate of a composite of one or more of severe preeclampsia, birth weight in the fifth percentile or less, or major abruption (adjusted OR, 0.15; 95% CI, 0.03-0.70).[321]

The results of these studies need to be interpreted with some caution. First, it is not clear whether the positive effects of LMWH on prevention of preeclampsia in women with underlying angiotensin-converting enzyme insertion/deletion polymorphisms are broadly generalizable. Second, the pilot study

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was stopped before reaching its planned sample size of 276 when an interim analysis performed because of slow accrual suggested a statistically significant decrease in the primary outcome, potentially exaggerating the treatment effect.[322,323]

Recommendation

11.1.1. For women considered at risk for preeclampsia, we recommend low-dose aspirin throughout pregnancy, starting from the second trimester, over no treatment (Grade 1B)**.**

11.2 Women Without Known Thrombophilia and at Least Two Prior Pregnancy Losses

A Cochrane systematic review from 2009 that examined the use of aspirin and anticoagulation for recurrent pregnancy loss in women without APLA syndrome[324] identified two randomized trials: one comparing aspirin to placebo (n = 54)[283] and the other comparing enoxaparin to aspirin (n = 107).[325] Neither of the studies found significant differences in live birth rates, which ranged from 81% to 84%. Another systematic review, published in 2010, of LMWH vs aspirin or LMWH vs no treatment/placebo identified five randomized trials (n = 757).[326] The studies reviewed varied in terms of definition of early or late pregnancy loss, thrombophilic risk factors, and number of prior pregnancy losses. No meta-analysis was performed in the systematic review due to clinical heterogeneity of the studies. Risk ratios for pregnancy loss in the individual studies ranged from 0.95 to 3.0. The authors of this systematic review concluded that there was low-quality evidence, suggesting no effect of LMWH or aspirin. Two randomized trials have subsequently been published that provide relevant evidence on the effects of LMWH plus aspirin vs aspirin or placebo/no treatment on recurrent idiopathic pregnancy loss.[327,328]

11.2.1 LMWH and Aspirin vs No Treatment or Placebo: Table 12 and Table S25 summarize the quality of evidence and main findings from our meta-analysis of the two randomized trials that included 538 women with at least two miscarriages.[327,328] The meta-analysis provides moderate-quality evidence that LMWH and aspirin do not reduce miscarriage or increase major bleeding events in women with at least two recurrent miscarriages.

Women with three or more pregnancy losses might benefit from anticoagulant therapy. Two randomized trials of women with three or more pregnancy losses reported a substantial benefit of LMWH therapy on miscarriages.[329,330] However, both of these studies had important methodologic limitations, including a lack of blinding[329] or uncertain blinding,[330] relatively high rates of loss to follow-up,[329,330] lack of prospective trial registration,[329,330] and an unexpectedly low live birth rate in the placebo arm.[330] These findings are challenged by findings from the more recent high-quality randomized trials described previously in the present article. In one of these studies, a prespecified subgroup analysis of women with three or more miscarriages showed no evidence of a different relative effect of LMWH and aspirin vs placebo (test for interaction $P = .85$).[327] The other study provided data for the same subgroup of women and found no difference in effect (27% miscarriages in treatment group vs 24% in control group), although no formal subgroup analysis was performed.[326] We consider these findings more credible than those of the two lower-quality randomized

Table 12—*[Section 11.2.1] Summary of Findings: Should LMWH and Aspirin Rather Than No Treatment Be Used for Prevention of Recurrent Pregnancy Loss in Women Without Thrombophilia*

				Anticipated Absolute Effects During Pregnancy	
Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Risk Without Treatment	Risk Difference With LMWH + Aspirin (95% CI)
Miscarriage	496 (2 RCTs), 9 mo	Moderate due to imprecision[b]	RR 1.01 (0.84-1.38)	300 cases of miscarriage per 1,000[c]	No significant difference; 3 more cases per 1,000 (from 48 fewer to 114 more)
Major bleeding events[d]	294 (1 RCTs), 9 mo	Moderate due to imprecision[b]	RR 1.00 (0.42-2.33)	15 bleeding events per 1,000[e]	No significant difference; 0 more bleeding events per 1,000 (from 9 fewer to 20 more)

Data from unpublished meta-analysis[1] of two RCTs by Kaandorp et al[327] and Clark et al.[328] See Table 2 for expansion of abbreviations.
[a]Wide CIs include benefit and harm.
[b]Meta-analysis performed in RevMan version 5 with fixed-effects model for heterogeneity.
[c]Control group risk for miscarriage comes from study event rates in the two available randomized trials.[327,328]
[d]Antepartum maternal major hemorrhage. Bleeding outcomes variably reported in the two trials. We use data from Clark et al[328] on serious adverse events and antepartum hemorrhage both to generate relative risks and baseline risks for anticipated absolute effects. Kaandorp et al[327] reported nosebleed, GI problems, hematuria, and bleeding gums. There were no major bleeding events (S. Middeldorp, MD, personal communication, October 2010).
[e]Control group risk estimate for major bleeding events antepartum with aspirin comes from systematic review by Greer et al.[38]

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trials; however, a possible deleterious effect of aspirin on pregnancy outcome when used in combination with LMWH cannot be excluded.

11.2.2 Aspirin vs Placebo: Table 13 and Table S26 summarize the main findings from a randomized comparison of 104 women allocated to aspirin and 103 women with two or more unexplained recurrent miscarriages allocated to placebo.[327] This trial provides moderate-quality evidence that aspirin does not improve live birth rates among women with two or more unexplained recurrent miscarriages. Similarly, the randomized trial of low-dose aspirin vs placebo[283] that included 54 women with three or more pregnancy losses did not find a significant difference in miscarriages (RR, 1.00; 95% CI, 0.78-1.29).

Recommendation

11.2.1. For women with two or more miscarriages but without APLA or thrombophilia, we recommend against antithrombotic prophylaxis (Grade 1B)**.**

12.0 Maternal and Fetal Risks Related to Anticoagulation During Pregnancy for Mechanical Prosthetic Valves

Patients with a mechanical heart valve not receiving antithrombotic therapy face a high risk of valve thrombosis and death or systemic embolism (see Whitlock et al[331] in this guideline). However, as outlined in section 3.0, the use of vitamin K antagonists during pregnancy carries potential for risks to the fetus, especially if these drugs are administered during the first trimester or at term. Although LMWH or UFH can be substituted for vitamin K antagonists, doubt has been raised about their effectiveness for prevention of systemic embolism in this setting. Unfortunately, properly designed trials have not been performed, and even the small amount of data available is limited by significant heterogeneity for valve type; valve position; valve area; and presence of comorbid conditions, such as atrial fibrillation.

12.1 Anticoagulant Management of Mechanical Prosthetic Valves in Pregnant Women

Tables S27 and S28 present the available data regarding maternal outcomes in this setting.[49,50,332-340] In a systematic review of observational studies between 1966 and 1997 that reported on outcomes with various anticoagulant regimens in pregnant women with mechanical prosthetic valves, the regimen associated with the lowest risk of valve thrombosis/systemic embolism was the use of vitamin K antagonists throughout pregnancy (3.9%).[49] The use of UFH in the first trimester and near term was associated with a higher risk of valve thrombosis (9.2%).[49] The risk of thromboembolic complications was highest when UFH was used throughout pregnancy (33.3%),[49] and events occurred in women receiving both IV and adjusted-dose subcutaneous UFH and in those treated with low-dose heparin. Although these data suggest that vitamin K antagonists are more effective than UFH for thromboembolic prophylaxis of pregnant women with mechanical heart valves, some of the thromboembolic events in women treated with UFH might be explained by inadequate dosing, use of an inappropriate target aPTT range, or differences in risk profile in the patient populations treated with UFH vs those treated with vitamin K antagonists.

LMWH has advantages over UFH in terms of the maternal side effect profile, and there is increasing use of LMWH in pregnant women with prosthetic heart

Table 13—*[Section 11.2.1] Summary of Findings: Should Aspirin Rather Than No Treatment Be Used for Prevention of Recurrent Pregnancy Loss in Women Without Thrombophilia*

Outcomes	Participants (Studies), Follow-up	Quality of the Evidence (GRADE)	Relative Effect (95% CI)[a]	Anticipated Absolute Effects During Pregnancy: Risk Without Aspirin	Anticipated Absolute Effects During Pregnancy: Risk Difference With Aspirin (95% CI)
Miscarriage	202 (1 RCT), 9 mo	Moderate due to imprecision[a]	RR 1.16 (0.80-1.69)	300 cases of miscarriage per 1,000[b]	No significant difference; 48 more cases per 1,000 (from 60 fewer to 207 more)
Major bleeding events[c]	95 000 (6), 3.8-10 y	Moderate due to indirectness[d]	RR 1.54 (1.30-1.82)	15 bleeding events per 1,000[e]	8 more bleeding events per 1,000 (from 5 more to 12 more)

Data from Kaandorp et al,[327] the only study identified that compared aspirin to placebo in this population, and ATT Collaboration,[315] for relative effect estimate of major bleeding events. See Table 2 legend for expansion of abbreviations.
[a]Wide CIs include benefit and harm of aspirin on miscarriage.
[b]Baseline risk for miscarriage comes from study event rates in the two available randomized trials.[327,328]
[c]Major antenatal nonfatal hemorrhage.
[d]Rated down for indirectness due to population (primary prevention cardiovascular disease).[315] There were no major bleeding events in the Anticoagulants for LIving Fetuses (ALIFE) Study[327] (S. Middeldorp, MD, personal communication, October 2010).
[e]Control group risk estimate for major bleeding events antepartum comes from systematic review by Greer et al.[38]

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valves. The safety of LMWH for this indication was questioned in a warning from an LMWH manufacturer.[341] This warning was based on postmarketing reports of valve thrombosis in an undisclosed number of patients receiving this LMWH as well as on clinical outcomes in an open randomized study comparing LMWH (enoxaparin) with warfarin and UFH in pregnant women with prosthetic heart valves.[341] Because of two deaths in the LMWH arm, the study was terminated after 12 of the planned 110 patients were enrolled.

In a systematic review of observational studies published between 2000 and 2009, the use of LMWH (or UFH) during the first trimester and near term or throughout pregnancy was associated with a higher risk of valve thrombosis or maternal thromboembolism (7.2% and 13.4%, respectively) than the use of vitamin K antagonists alone (2.9%).[50] Maternal bleeding risks were similar across the various treatment regimens.

In a review of case series and cohort studies between 1996 and 2006 involving pregnant women with mechanical heart valves who were converted to LMWH prior to pregnancy or by the end of the first trimester, maternal valve thrombosis or thromboembolism occurred in 17 of 76 (22.4%) pregnancies.[332] Another systematic review of LMWH use in pregnant women with mechanical prosthetic heart valves that used slightly different eligibility criteria found that valve thrombosis occurred in seven of 81 pregnancies (8.6%; 95% CI, 2.5%-14.8%), and the overall thromboembolic rate was 10 of 81 pregnancies (12.4%; 95% CI, 5.2%-19.5%).[333] However, nine of the 10 patients with thromboembolic complications received a fixed dose of LMWH, and in two of these, a fixed low dose was used. Among 51 pregnancies in which anti-Xa LMWH levels were monitored and doses adjusted according to the result, only one patient was reported to have experienced a thromboembolic complication. Two subsequent case series[334,335] and one cohort study without internal control[336] that evaluated LMWH given every 12 h and adjusted to maintain therapeutic peak anti-Xa LMWH levels reported risks of maternal valve thrombosis or systemic thromboembolism ranging between 4.3% and 16.7%.

As outlined in Table S29, the use of UFH or LMWH throughout pregnancy essentially eliminates the risk of congenital malformation.[49,50,332-336] Most published studies suggest that risks of malformation are also low (<2%) if UFH or LMWH are substituted for vitamin K antagonists during the first trimester (preferably before the 6th week of gestation).[49,50,332-336] The number of pregnancy losses appears higher in those patients who receive either vitamin K antagonists or a heparin throughout pregnancy than in those in whom UFH or LMWH are substituted for vitamin K antagonists in the first trimester and at term.[49,50,332-340]

Thus, it appears that there is no single optimal treatment approach for managing pregnant women with mechanical prosthetic valves. Given the limited and sometimes conflicting data, several approaches remain acceptable (Table 14). The decision about which regimen to use should be made after full discussion with the patient. Additional risk factors for thromboembolism as well as patient preference should be taken into consideration. For example, women at very high risk (eg, first-generation mechanical valve in the mitral position, history of thromboembolism, associated atrial fibrillation) may prefer vitamin K antagonist use throughout pregnancy. If warfarin is used, the dose should be adjusted as recommended by Whitlock et al.[331] If subcutaneous UFH is used, it should be initiated in high doses (17,500-20,000 units every 12 h) and adjusted to prolong a 6-h postinjection aPTT into the therapeutic range. If LMWH is used, it should be administered bid and dosed to achieve the manufacturer's peak anti-Xa level 4 h after subcutaneous injection. Extrapolating from data in nonpregnant patients with mechanical valves receiving warfarin therapy,[342] for the same high-risk women, the addition of aspirin 75 to 100 mg/d can be considered in an attempt to reduce the risk of thrombosis, recognizing that it increases the risk of bleeding.

Recommendations

12.1.1. For pregnant women with mechanical heart valves, we recommend one of the following anticoagulant regimens in preference to no anticoagulation (all Grade 1A)**:**

(a) Adjusted-dose bid LMWH throughout pregnancy. We suggest that doses be adjusted

Table 14—*[12.1.1-12.1.3] Recommended Anticoagulant Regimens in Pregnant Women With Mechanical Heart Valves*

Adjusted-dose bid LMWH throughout pregnancy, with doses adjusted to achieve the manufacturer's peak anti-Xa LMWH 4 h postsubcutaneous injection (Grade 1A).
Adjusted-dose UFH throughout pregnancy administered subcutaneously every 12 h in doses adjusted to keep the midinterval aPTT at least twice control or attain an anti-Xa heparin level of 0.35-0.70 units/mL (Grade 1A).
UFH or LMWH (as above) until the 13th week with substitution by vitamin K antagonists until close to delivery when UFH or LMWH is resumed (Grade 1A).
For women judged to be at very high risk of thromboembolism in whom concerns exist about the efficacy and safety of UFH or LMWH as dosed above (eg, older-generation prosthesis in the mitral position or history of thromboembolism), vitamin K antagonists throughout pregnancy with replacement by UFH or LMWH (as above) close to delivery (Grade 2C).

aPTT = activated partial thromboplastin time. See Table 2 and 10 legends for expansion of abbreviations.

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to achieve the manufacturer's peak anti-Xa LMWH 4 h postsubcutaneous injection; or (b) Adjusted-dose UFH throughout pregnancy administered subcutaneously every 12 h in doses adjusted to keep the midinterval aPTT at least twice control or attain an anti-Xa heparin level of 0.35 to 0.70 units/mL; or (c) UFH or LMWH (as above) until the 13th week with substitution by vitamin K antagonists until close to delivery when UFH or LMWH is resumed.

Remarks: For pregnant women with mechanical heart valves, the decision regarding the choice of anticoagulant regimen is so value and preference dependent (risk of thrombosis vs risk of fetal abnormalities) that we consider the decision to be completely individualized. Women of childbearing age and pregnant women with mechanical valves should be counseled about potential maternal and fetal risks associated with various anticoagulant regimens, including continuation of vitamin K antagonists with substitution by LMWH or UFH close to term, substitution of vitamin K antagonists by LMWH or UFH until the 13th week and then close to term, and use of LMWH or UFH throughout pregnancy. Usual long-term anticoagulants should be resumed postpartum when adequate hemostasis is assured.

12.1.2. In women judged to be at very high risk of thromboembolism in whom concerns exist about the efficacy and safety of UFH or LMWH as dosed above (eg, older-generation prosthesis in the mitral position or history of thromboembolism), we suggest vitamin K antagonists throughout pregnancy with replacement by UFH or LMWH (as above) close to delivery rather than one of the regimens above (Grade 2C)**.**

Remarks: The recommendation for women at very high risk of thromboembolism places a higher value on avoiding maternal complications (eg, catastrophic valve thrombosis) than on avoiding fetal complications. Women who place a higher risk on avoiding fetal risk will choose LMWH or UFH over vitamin K antagonists.

12.1.3. For pregnant women with prosthetic valves at high risk of thromboembolism, we suggest the addition of low-dose aspirin 75 to 100 mg/d (Grade 2C)**.**

13.0 Recommendations for Research

Although new information has been published since our last review, the available evidence in this article is still generally of low quality. Most recommendations are based on observational studies and extrapolation from other populations. There is an urgent need for appropriately designed studies to inform us of the risk of recurrent pregnancy-associated VTE and of first VTE in thrombophilic women and those undergoing cesarean section and assisted reproductive technology. Further research is needed to optimize regimens for the prevention of VTE and mechanical valve thrombosis. Given the uncertainty of baseline estimates for both the risks of the various conditions discussed in this article and the benefits of prophylactic and therapeutic interventions, knowledge of pregnant women's values and treatment preferences are crucial when making recommendations. Although investigators have explored patient values and preferences with respect to antithrombotic therapy in other contexts, no studies have been performed in pregnant women.

Although the performance of clinical trials involving pregnant women is challenging, there is a clear need for methodologically strong studies in this patient population. All pregnant women are best protected when evidence about conditions that affect them is gathered in a scientifically rigorous manner that maximizes participant safety.[343]

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Dr Bates: contributed as Deputy Editor.
Dr Greer: contributed as a panelist.
Dr Middeldorp: contributed as a panelist.
Dr Veenstra: contributed as a resource consultant.
Dr. Prabulos: contributed as a front line clinician.
Dr Vandvik: contributed as Topic Editor.
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Endorsements: This guideline is endorsed by the American Association for Clinical Chemistry, the American College of Clinical Pharmacy, the American Society of Health-System Pharmacists, the American Society of Hematology, and the International Society of Thrombosis and Hematosis. Additionally, the guidelines presented in this article have been endorsed by the American College of Obstetricians and Gynecologists.
Additional Information: The supplement Tables can be found in the Online Data Supplement at http://chestjournal.chestpubs.org/content/141/2_suppl/e691S/suppl/DC1.

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A nonsense polymorphism in the protein Z-dependent protease inhibitor increases the risk for venous thrombosis

Javier Corral, Rocio González-Conejero, Jose Manuel Soria, Jose Ramón González-Porras, Elena Pérez-Ceballos, Ramón Lecumberri, Vanessa Roldán, Juan Carlos Souto, Antonia Miñano, David Hernández-Espinosa, Ignacio Alberca, Jordi Fontcuberta and Vicente Vicente

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A nonsense polymorphism in the protein Z–dependent protease inhibitor increases the risk for venous thrombosis

Javier Corral, Rocio González-Conejero, Jose Manuel Soria, Jose Ramón González-Porras, Elena Pérez-Ceballos, Ramón Lecumberri, Vanessa Roldán, Juan Carlos Souto, Antonia Miñano, David Hernández-Espinosa, Ignacio Alberca, Jordi Fontcuberta, and Vicente Vicente

The protein Z–dependent protease inhibitor (ZPI) is a hemostatic serpin with anticoagulant activity. As for antithrombin, deficiency of ZPI could have relevant thrombotic consequences. We have studied 6 genetic modifications affecting the *ZPI* gene, identifying 5 haplotypes. Haplotype H5 is featured by a stop codon at position 67. The relevance of these genetic modifications and haplotypes in venous thrombosis was evaluated in a case-control study including 1018 patients and 1018 age- and sex-matched controls. Surprisingly, the H5 haplotype was found in 0.9% of controls, supporting that the Arg67Stop change is a low frequency nonsense polymorphism. The prevalence of this haplotype increased significantly in patients (3.0%), one of whom was in a homozygous state. Multivariate analysis confirms that carriers have a 3.3-fold risk of developing venous thrombosis ($P = .002$; 95% CI: 1.5-7.1). Moreover, we observed a significant association of this polymorphism with familial history of thrombosis ($P < .001$). Our study supports that the *ZPI* Arg67Stop nonsense polymorphism might be an independent genetic risk factor for venous thrombosis. This polymorphism has slightly lower prevalence but similar thrombotic risk than the *FV* Leiden or prothrombin 20210A. Although further studies are required, all available data support that the *ZPI* is a candidate to play a significant role in thrombosis and should be evaluated in thrombophilic studies. (Blood. 2006;108:177-183)

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Introduction

Initially, venous thrombosis was considered a monogenic disease, as rare mutations affecting some anticoagulant proteins (antithrombin, protein C, and protein S) identified in thrombophilic families significantly increased (more than 8-fold) the risk for venous thrombosis.[1] However, the consensus about the relevance in venous thrombosis of 2 polymorphisms (factor V Leiden and prothrombin 20210) supported that venous thrombosis is a multigenic disease.[2] Accordingly, common genetic variations with mild associated risk could play a role in this disorder.[2] Unfortunately, despite a considerable effort during 2 decades evaluating hundreds of polymorphisms affecting hemostatic proteins, no further genetic risk factors for venous thrombosis have been clearly identified.[3] One possible explanation of these frustrating results is that the screening of candidate polymorphisms has been exclusively focused on classic hemostatic proteins. It would be interesting to evaluate further candidates that might play a role in hemostasis.

The precise role of the protein Z–dependent protease inhibitor (ZPI) is uncertain, but in vitro studies suggest that it might be a potent anticoagulant. Its name derives from the role that protein Z (PZ) plays in enhancing its function. ZPI is a 72-kDa member of the serpin superfamily of serin protease inhibitors (SERPINA10) with tyrosine-serine as the P1-P1′ residues.[4] Nevertheless, ZPI efficiently inhibits both factor Xa and factor XIa by 2 different mechanisms.[5] In plasma, PZ completely complexes all ZPI. In the presence of procoagulant phospholipids and calcium, the PZ-ZPI complex produces rapid inhibition of factor Xa ($t_{1/2} < 10$ seconds, at a rate 1000-fold faster than ZPI alone).[5] Factor XIa inhibition by ZPI is not dependent on PZ, phospholipids, or calcium, but is enhanced by heparin.[5] In contrast to other anticoagulant serpins, the inhibitory complex FXa-ZPI seems to be quite unstable, and the inactivation of FXIa also produced cleaved ZPI. Accordingly, the ZPI activity is consumed during the in vitro coagulation of plasma.[5] A recent paper supports that the ZPI may be an unusual physiologic regulator of both the intrinsic FXase and the prothrombinase complexes.[6] The possible relevance of this system in regulating the coagulation response and in thrombosis arises from the *PZ* knock-out mice, which indicated that PZ appears to dampen the prothrombotic response. Moreover, the *PZ*-deficient mice exhibited prothrombotic tendency in combination with factor V Leiden.[7] Recently, one nonsense mutation within the *ZPI* gene (Trp303Stop) was identified in 3.8% of patients with venous thrombosis but not in controls.[8] All of these data support a relevant role of this molecule in venous thrombosis. Therefore, this gene is a candidate to exhibit other genetic changes that might influence the risk of venous thrombosis.

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Patients, materials, and methods

Patients and controls

Our study included 1018 consecutive and unrelated white patients with a first, objectively confirmed episode of venous thromboembolism before the age of 75 years. These patients were enrolled from the files of the anticoagulation clinics in 4 hospitals in Spain: Hospital General Universitario (Murcia), Hospital de la Santa Creu i Sant Pau (Barcelona), Hospital Clínico Universitario (Salamanca), and Clínica Universitaria de Navarra (Pamplona). All cases were diagnosed appropriately by clinical probability, D-dimer levels, compression ultrasonography, ventilation perfusion lung scan, and, when necessary, phlebography or pulmonary angiography. Patients with known malignant disorders were excluded. The control group of our study included 1018 unrelated people without a history of vascular or thromboembolic disease. These controls were randomly selected among 2 sources: blood donors and traumatology and ophthalmology patients matched by age, sex, race, and geographic distribution with 1018 cases.

All included subjects gave their informed consent to enter the study, which was performed in accordance with the Declaration of Helsinki as amended in Edinburgh in 2000. Approval for this study was obtained from the institutional review boards of Hospital General Universitario (Murcia, Spain), the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), Hospital Clínico Universitario (Salamanca, Spain) and Clínica Universitaria de Navarra (Pamplona, Spain).

Demographic parameters (age and sex) were recorded in all subjects. Moreover, a list of other clinical data (familial history of venous thrombosis, recurrence, type of thrombotic event, location of the thrombosis, and other thrombotic risk factors such as use of oral contraceptives, hormone replacement therapy, phospholipid antibodies, and protein C, protein S, or antithrombin deficiency) were recorded.

Genetic analysis

Blood was collected from the antecubital vein into citrate tubes (0.1 volume 0.106 M trisodium citrate), and genomic DNA was purified. Amplification of the 4 coding exons of the *ZPI* gene (exons 2-5) was performed by polymerase chain reaction (PCR) using the primers described by Van de Water et al,[8] with minor modifications. The 1277G>A (Gly250Ser) polymorphism was genotyped by single-strand conformation polymorphism (SSCP). The 1276C>T (Tyr249) polymorphism was determined by SSCP and confirmed by PCR-ASRA using the *Rsa*I enzyme. The 1438G>A (Trp303Stop) mutation was evaluated by SSCP and PCR–allelic specific restriction assay (ASRA) with *Hin*fI. The 603A>G (Lys25Arg), 647A>G (Ser40Gly), and 728C>T (Arg67Stop) polymorphisms were evaluated by SSCP and PCR-ASRA with *Eco*130I, *Msp*I, and *Sat*I, respectively. Confirmation of the identified genotypes for all polymorphisms was performed by sequence analysis.

Moreover, the factor V *(FV)* Leiden and prothrombin (PT) 20210G>A polymorphisms were determined in all patients and controls as described.[9]

Statistical analysis

Results are expressed as median value and interquartile ranges for continuous variables and as percentages for categoric variables. Univariate statistical analysis was performed by the chi-square test. The strength of the association of major risk factors and the polymorphism with the occurrence of disease was estimated by calculation of the odds ratio (OR) with EpiInfo software (Centers for Disease Control and Prevention, Atlanta, GA) and the Cornfield method for the calculation of 95% confidence intervals (CIs).[10] Multivariate analysis was performed using logistic regression enter method, with the SPSS statistical package for Windows 8.0 software (Chicago, IL). Differences with a 2-tailed *P* value less than .05 were considered statistically significant.

We tested the null hypothesis that Hardy-Weinberg equilibrium holds using the chi-square method. We calculated the *P* value by simulation with 100 000 replicates.

Linkage disequilibrium (LD) between each pair of SNPs was estimated by the pairwise correlation (r) among genotypes. Working with SNPs, this measure is equivalent to the correlation of Weir[11] (Appendix A in Meng et al[12]).

Haplotype analysis with unrelated individuals has to face up to the problem of ambiguity of haplotypes; that is, many individuals are consistent with different haplotype configurations. We used the likelihood approach described previously to estimate the haplotypes.[13] This method, in addition to estimating haplotype frequencies, computes the posterior probabilities of the pairs of haplotypes that are consistent with the observed markers for each subject. Then, to evaluate the association of the affection status with haplotypes, we applied the score method that uses those posterior probabilities to account for haplotype ambiguities.[14] Based on generalized linear models, this approach can be applied to binary, ordinal, and quantitative traits and, at the same time, allow adjustment for nongenetic covariates, which may be critical when analyzing genetic complex traits. In our case, we used *FV* Leiden and PT as covariates.

All genetic calculations were done with the statistical environment R (The R Project for Statistical Computing, http://www.r-project.org).

Results

Identification of genetic variations in the *ZPI* gene

In a previous study of our group, we evaluated the *ZPI* Trp303Stop mutation in 218 Spanish patients with venous thrombosis by PCR-ASRA and SSCP. No patient carried the Trp303Stop nonsense mutation.[15] However, the SSCP analysis and ulterior sequence revealed 2 variations in the published sequence of *ZPI* exon 3: 1276C>T silent mutation (Tyr249) and 1277G>A point mutation, responsible for the Gly250Ser missense change (nucleotide and amino acid numbering as per mRNA GenBank entry AF181467) (Figure 1A). Of interest, these mutations are consecutive. The first modification, also identified by Van de Water et al,[8] was recognized as a possible SNP in the NCBI SNPs database (ID: rs2232707).[16] The second change was also identified in the New Zealand cohort,[8] and it was present in 8 patients with venous thrombosis from our preliminary study. This change was also present in the NCBI SNPs database (ID: rs2232708).[16]

Additionally, we sequenced all coding exons of the *ZPI* gene (exons 2-5) of 5 carriers of the *ZPI* Gly250Ser polymorphism using primers and conditions previously reported.[8] No subject had further genetic modifications on exons 3, 4, and 5. However, we identified 3 genetic changes in the 5′-end of exon 2 (Figure 1B), a region homologous to that of another hemostatic serpin: heparin cofactor II,[17] which encodes the acidic tail of the N-terminus of the ZPI molecule. All Gly250Ser carriers simultaneously had a 603A>G transition (responsible for the Lys25Arg missense modification). Of interest, 4 subjects also had a 728C>T point mutation, which causes a nonsense change (Arg67Stop) in heterozygous state. Finally, only one patient also had the 647A>G (Ser40Gly) variation (Figure 1B). All of these modifications were also identified by Van de Water et al[8] and are present in the NCBI SNPs database ID: Lys25Arg, rs941590; Arg67Stop, rs2232698; Ser40Gly, rs941591.[16]

Unfortunately, the frequency and the role in venous thrombosis of these genetic modifications were not clearly defined. Accordingly, we extensively evaluated these 5 SNPs and the Trp303Stop mutation of the *ZPI* gene on a large cohort of patients with venous thrombosis and controls.

Case-control study

Demographic, clinical, and genetic data of cases and controls are presented in Table 1. These features did not significantly differ in the samples from the 4 hospitals (data not shown).

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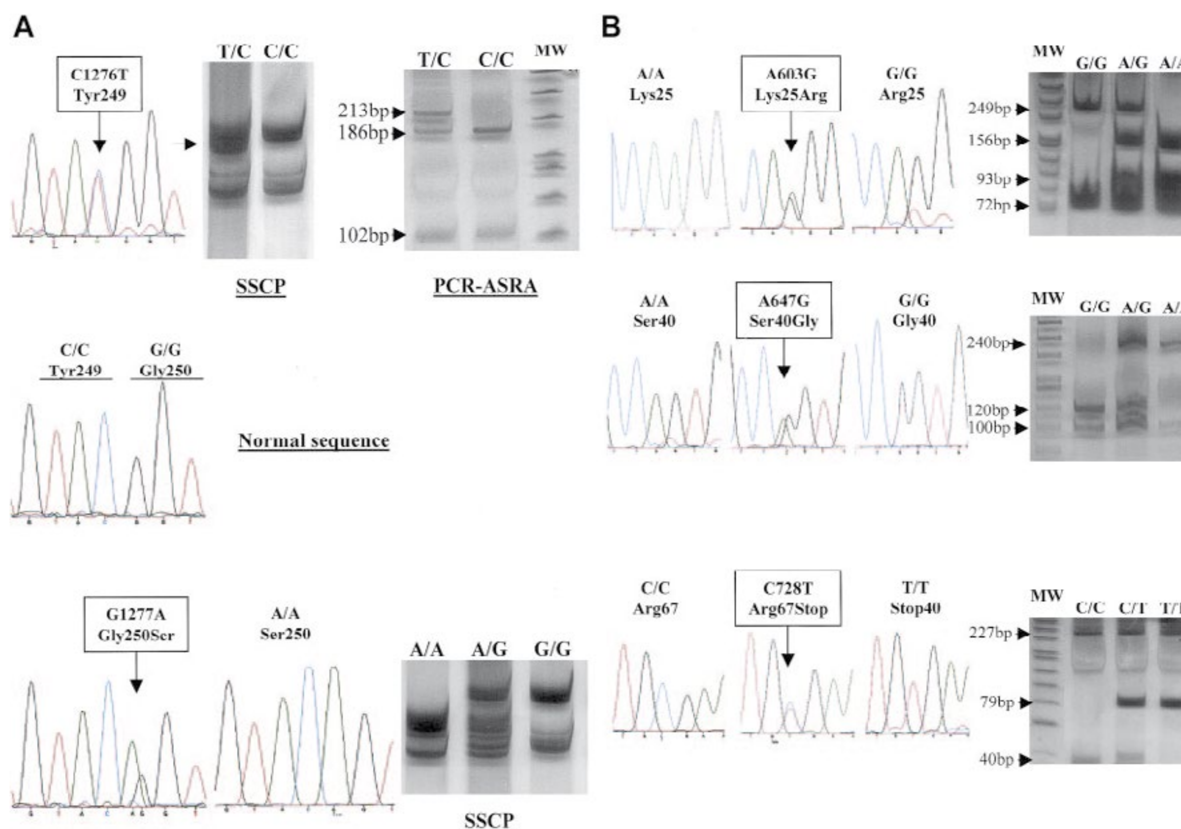

Figure 1. Identification of genetic variations in the ZPI encoding gene. (A) Variations in exon 3. (B) Variations in 5′-end of exon 2. MW indicates molecular weight marker (ϕX174 DNA/*Hin*fI Marker; Promega, Madison, WI).

The median age of patients at the time of the first thrombotic event was 45 years (interquartile ranges, 33-58), and the ratio of males to females was 1:1. Approximately two thirds of the patients suffered a single thrombotic episode (280 of 1018 had recurrent episodes of venous thrombosis). Moreover, 218 patients reported risk factors such as use of oral contraceptives, antiphospholipid antibodies, or hormone replacement therapy, and deficiency of antithrombin, protein C, or protein S. Finally, 177 patients (17.4%) reported familial history of thrombosis.

The prevalence of the classic prothrombotic polymorphisms was similar to that described in other series. Thus, the *FV* Leiden was present in 145 patients (3 in homozygous state; 14.2%), and

Table 1. Characteristics of patients with venous thrombosis and control subjects

	Patients	Control subjects	*P**; OR (95% CI)
N	1018	1018	
Age, y (range)	49 (36–62)	47 (35–63)	.618
Male sex, no	509	509	.999
***FV* Leiden, no.**			< .001; 5.29 (3.5–8.0)
−/−	873	987	
+/− & +/+	142 & 3	31 & 0	
***PT* 20210, no.**			< .001; 4.20 (2.7–6.7)
G/G	917	992	
G/A & A/A	94 & 7	26 & 0	
***ZPI* 603 (Lys25Arg), no.**			.113
A/A	295	328	
A/G & G/G	522 & 201	525 & 165	
***ZPI* 647 (Ser40Gly), no.**			.959
A/A	759	758	
A/G & G/G	246 & 13	249 & 11	
***ZPI* 728 (Arg67Stop), no.**			< .001; 3.40 (1.5–7.8)
C/C	988	1009	
C/T & T/T	29 & 1	9 & 0	
***ZPI* 1277 (Gly250Ser), no.**			< .001; 3.01 (1.6–5.8)
G/G	977	1004	
G/A & A/A	40 & 1	14 & 0	

*Crude analysis; OR is given when $P < .05$.

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Table 2. Clinical features of patients according to the *ZPI, FV* Leiden and prothrombin polymorphisms

	No. (%)	Age at first episode, y (range)	Recurrence, no. (%)	Other risk factors, no. (%)	Familial history, no. (%)
***ZPI* Lys25Arg**					
Carrier	723 (71.0)	45 (33-58)	203 (68.8)	164 (22.7)	138 (19.1)
Noncarrier	295 (29.0)	47 (33-60)	77 (26.1)	54 (18.3)	39 (13.2)*
***ZPI* Ser40Gly**					
Carrier	259 (25.4)	45 (33-56)	70 (27.0)	58 (22.4)	38 (14.7)
Noncarrier	759 (74.6)	45 (33-59)	210 (27.7)	160 (21.0)	139 (18.3)
***ZPI* Arg67Stop**					
Carrier	30 (2.9)	49 (37-63)	11 (36.7)	9 (33.3)	11 (40.0)
Noncarrier	988 (97.1)	45 (33-58)	269 (27.2)	209 (21.2)	166 (16.8)†
***ZPI* Gly250Ser**					
Carrier	41 (4.0)	49 (33-63)	11 (29.3)	10 (26.8)	14 (36.6)
Noncarrier	977 (96.0)	45 (33-58)	269 (27.5)	208 (21.3)	163 (16.7)†
***FV* Leiden**					
Carrier	145 (14.2)	44 (30-56)	42 (29.0)	31 (21.4)	30 (20.7)
Noncarrier	873 (85.8)	45 (33-59)	238 (27.3)	187 (21.4)	147 (16.8)
***PT* 20210A**					
Carrier	101 (9.9)	45 (32-56)	22 (21.8)	21 (20.8)	16 (15.8)
Noncarrier	917 (90.1)	45 (33-59)	258 (28.1)	197 (21.5)	161 (17.6)

*$P < .05$.
†$P < .005$.

the *PT* 20210A allele was identified in 101 patients (7 in homozygous state; 9.9%) (Table 1). Controls had a significantly lower prevalence of these prothrombotic polymorphisms: 31 (3.0%) were heterozygous for the *FV* Leiden and 26 (2.6%), for the *PT* 20210A allele (Table 1). Accordingly, these polymorphisms increased 5.3-fold (*FV* Leiden) and 4.2-fold (*PT* 20210A) the risk of venous thrombosis ($P < .001$; 95% CI: 3.5-8.0 and $P < .001$; 95% CI: 2.7-6.7, respectively). The age of the first episode of venous thrombosis, the percentage of recurrence, the incidence of additional risk factors, and the history of familial thrombosis associated with these polymorphisms are shown in Table 2.

In our study, no patient or control carried the *ZPI* Trp303Stop mutation.

The *ZPI* 1276C>T silent mutation (Tyr249) was identified in only one patient with venous thrombosis. This patient suffered from an idiopathic deep venous thrombosis at the age of 58 years and reported no familial history of thrombosis. This mutation was not identified in controls.

The prevalence of other *ZPI* polymorphisms (1277G > A [Gly250Ser]; 603A>G [Lys25Arg]; 647A>G [Ser40Gly]) in patients and controls is reported in Table 1.

Finally, the *ZPI* 728C>T transition, responsible for a nonsense change (Arg67Stop), has to be considered as a low prevalence polymorphism as it was identified in heterozygous state in 9 controls (0.9%). Of interest, this nonsense polymorphism was more frequent among patients with venous thrombosis (3.0%, Table 1). Remarkably, one patient was homozygous for this nonsense variation (Figure 1B). This patient suffered the first episode of deep venous thrombosis at the age of 62 years when she was under hormone replacement therapy, and reported familial history of thrombosis. According to our results, this polymorphism increased 3.4-fold the risk of venous thrombosis ($P < .001$; 95% CI: 1.5-7.8) (Table 1).

The clinical features of carriers and noncarriers of these polymorphisms are shown in Table 2. No significant differences were observed when attending to the age of the first episode, recurrence, presence of additional risk factors, or familial history of venous thrombosis for all tested polymorphisms except an increased familial history of venous thrombosis among carriers of the *ZPI* Lys25Arg, Arg67Stop, and Gly250Ser variants (Table 2).

The allele and genotype frequencies for the present study are consistent with that predicted by Hardy-Weinberg equilibrium (data not shown).

ZPI haplotypes

Our results supported strong linkage disequilibrium between 4 polymorphisms of the *ZPI* gene (603A>G, 647A>G, 728C>T, and 1277G>A) (Table 3) that defined 5 different haplotypes (Figure 2). The most common haplotype, with an overall frequency of 0.58 in controls, was wild type at all 4 loci (H1: 603A, 647A, 728C, and 1277G) (Figure 2). All other haplotypes shared the 603G variant. Of interest, haplotype H5 had the 728T and 1277A variations. One patient had an H5-variant haplotype (H5v), probably caused by recombination. Figure 2 also suggests a model that might explain the origin of these haplotypes.

The distribution of haplotypes in patients and controls is reported in Table 4. The estimated haplotype frequencies are also shown in Table 4. We point out that the haplotype H5 was the only one showing a prevalence statistically higher in patients than in controls (Table 4).

Table 3. Linkage disequilibrium between each pair of *ZPI* SNPs in cases and controls

	FV Leiden	*ZPI* 603	*ZPI* 647	*ZPI* 728	*ZPI* 1277
Patients					
PT	−0.01	0.00	−0.01	0.01	0.01
FV Leiden	NA	−0.03	0.00	0.01	0.04
ZPI 603	NA	NA	0.40	0.13	0.18
ZPI 647	NA	NA	NA	−0.06	−0.05
ZPI 728	NA	NA	NA	NA	0.83
Controls					
PT	−0.03	0.04	−0.03	−0.02	−0.02
FV Leiden	NA	−0.00	−0.00	−0.02	−0.02
ZPI 603	NA	NA	0.41	0.08	0.09
ZPI 647	NA	NA	NA	−0.003	−0.05
ZPI 728	NA	NA	NA	NA	0.80

NA indicates not applicable.

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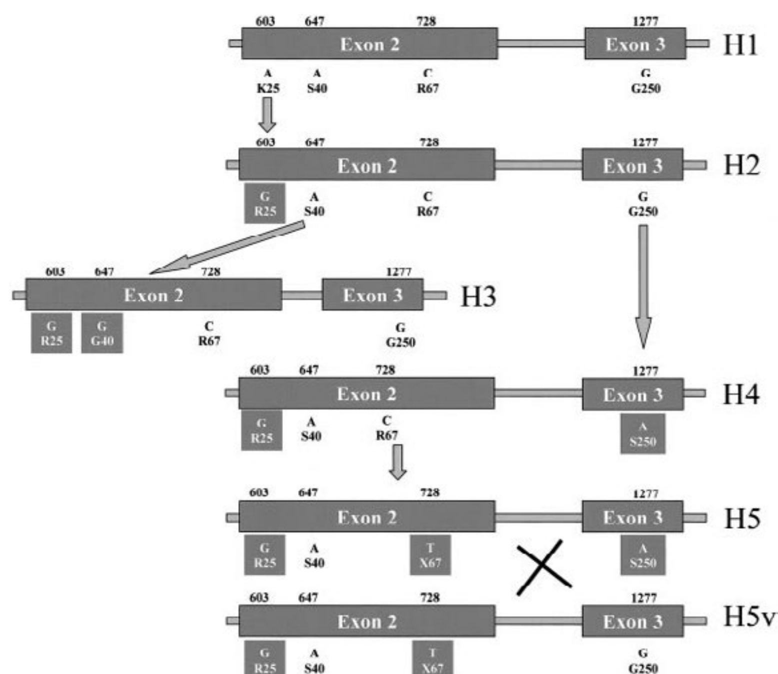

Figure 2. *ZPI* haplotypes identified in this study and a suggested model that explains the origin of these haplotypes.

The score test for association of traits with haplotypes allows the introduction of covariates in the model.[14] We use *FV* Leiden and *PT* as covariates. Moreover, the score statistic takes into account the uncertainty in the imputation of haplotypes to unrelated individuals. The result of this test suggests a clear association between the haplotypes and the disease ($P = .002$).

Multivariate analysis including the 2 classic prothrombotic polymorphisms (*FV* Leiden and *PT* 20210G>A) confirmed that the *ZPI* H5 haplotype is an independent risk factor for venous thrombosis ($P = .002$). The risk of venous thrombosis associated with the *ZPI* H5 haplotype (ie, the risk of the nonsense *ZPI* Arg67Stop polymorphism) is slightly lower than that of *FV* Leiden or prothrombin 20210G>A polymorphisms (OR = 3.32, 95% CI: 1.5-7.1; OR = 5.32, 95% CI: 3.6-7.9; OR = 4.24, 95% CI: 2.7-6.6, respectively).

Table 4. Distribution of *ZPI* haplotypes among patients with venous thrombosis and control subjects

	Patients, no. (% or frequency)	Controls, no. (% or frequency)
H1/H1 (reference group)	297 (29.2)	328 (32.2)
H1/H2	337 (33.1)	334 (32.8)
H1/H3	163 (16.0)	182 (17.9)
H1/H4	3 (0.3)	4 (0.4)
H1/H5 & H1/H5v	17 & 1 (1.8)	5 (0.5)*
H2/H2	90 (8.8)	83 (8.1)
H2/H3	77 (7.6)	66 (6.5)
H2/H4	5 (0.5)	1 (0.1)
H2/H5	9 (0.9)	3 (0.3)
H3/H3	13 (1.3)	11 (1.1)
H3/H4	3 (0.3)	0
H3/H5	2 (0.2)	1 (0.1)
H5/H5	1 (0.1)	0
H1 (reference group)	1115 (0.546)	1181 (0.580)
H2	608 (0.299)	570 (0.280)
H3	271 (0.134)	271 (0.133)
H4	11 (0.006)	5 (0.003)
H5 & H5v	30 & 1 (0.015 & 0.0005)	9 & 0 (0.004)*

*Statistical comparison versus reference group $P < .01$.

The clinical features of patients according to their *ZPI* haplotype are shown in Table 5. Statistical comparison with carriers of the common H1 haplotype in homozygous state (reference group) found that the H5 and H2 haplotypes were associated with familial history of thrombosis ($P < .001$) (Table 5).

Finally, no control simultaneously carried the *ZPI* H5 haplotype and *FV* Leiden or prothrombin polymorphisms, but 9 patients did (4 patients carried the *ZPI* Arg67Stop and the prothrombin 20210A variants [0.4%], and 5 patients presented simultaneously the *ZPI* Arg67Stop and the *FV* Leiden polymorphisms [0.5%]). Similarly, the simultaneous presence of *FV* Leiden and prothrombin variants was identified in 14 patients (1.4%) but not in the controls.

Discussion

The members of the serpin superfamily of serine protease inhibitors share a conserved structure and use a unique suicide substrate-like inhibitory mechanism. Serpins differ from other protease inhibitors in that they undergo a profound change in topology in order to entrap their target protease in an irreversible complex. This extremely efficient mechanism explains the selection of the serpins to control the proteolytic cascades of higher organisms. Thus, serpins have a central role in controlling proteinases in many biologic pathways such as fibrinolysis, inflammation, complement activation, apoptosis, angiogenesis, and blood coagulation.[18] Unfortunately, the molecular flexibility of serpins also renders these molecules susceptible to even minor factors (genetic or environmental) that cause dysfunction, by different mechanisms.[19] Thus, the same serpins that are crucial for a normal physiologic function could also be involved in diseases. Multiple serpins play a significant role in the hemostatic system: PAI-1, PAI-2, PCI, heparin cofactor II, α2-antiplasmin, and antithrombin. Mutations affecting some of these molecules, especially antithrombin, have been identified in patients with thrombosis.[20] The ZPI could be a new serpin to be included in the list of key molecules of the hemostatic system. This molecule plays a significant anticoagulant role as it potently inhibits FXa and FXIa.[5,6] Accordingly, impaired

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Table 5. Clinical features of patients according to the *ZPI* haplotypes

	No.	Age first episode, y, median (range)	Recurrence, no. (%)	Other risk factors, no. (%)	Familial history, no. (%)
H1/H1 (reference group)	297	46 (36–60)	77 (25.9)	54 (18.2)	40 (13.5)
H1/H2	337	45 (32–58)	95 (28.2)	77 (22.8)	69 (20.5)
H1/H3	163	45 (32–56)	46 (28.2)	42 (25.8)	21 (12.9)
H1/H4	3	21 (18–65)	0	0	0
H1/H5 & H1/H5v	17 & 1	47 (32–62)	3 (16.7)	7 (38.9)*	7 (38.9)*
H2/H2	90	43 (33–59)	28 (31.1)	21 (23.3)	20 (22.2)*
H2/H3	77	45 (34–53)	19 (24.7)	13 (16.9)	12 (15.6)
H2/H4	5	53 (36–67)	0	0	1 (20.0)
H2/H5	9	50 (38–65)	5 (55.6)	1 (11.1)	2 (22.2)
H3/H3	13	41 (28–61)	4 (30.8)	2 (15.4)	1 (7.7)
H3/H4	3	29 (25–62)	0	1 (33.3)	2 (66.7)
H3/H5	2	57 (50–65)	2 (100)	0	1 (50.0)
H5/H5	1	62	1 (100)	0	1 (100)
Haplotype carrier					
H2	608	45 (33–58)	147 (28.4)	111 (21.4)	104 (20.1)*
H3	271	45 (32–56)	71 (27.5)	58 (22.5)	37 (14.3)
H4	11	44 (25–64)	0	1 (9.1)	3 (27.3)
H5 & H5v	31	46 (32–62)	11 (36.7)	9 (30.0)	11 (36.7)†

*$P < .05$.
†$P < .001$.

function or reduced levels of ZPI might increase the risk of thrombosis. Thus, deficiency of its cofactor PZ seems to increase the risk of thrombosis.[7] Moreover, the PZ/ZPI system is impaired by antiphospholipid antibodies, increasing the thrombotic risk of patients with this autoimmune disease.[21] A recent report identifies nonsense mutations in the *ZPI* gene that significantly increase the risk of venous thrombosis.[8] Our results further support the relevance of this serpin on hemostasis and identify a genetic risk factor for venous thrombosis. The results of this case-control study suggest that the *ZPI* H5 haplotype, featured by a stop codon at position 67, might be an independent risk factor for venous thrombosis. The prevalence of the *ZPI* Arg67Stop polymorphism is slightly lower than that of the *FV* Leiden or *PT* 20210G>A, but the associated risk is similar. Moreover, this polymorphism is associated with familial history of thrombosis. Our data support that the *ZPI* Arg67Stop polymorphism could be included in the list of markers of thrombophilia. The study performed in New Zealand also identified this polymorphism.[8] However, further studies in other populations are required to confirm the thrombotic role of the *ZPI* Arg67Stop nonsense polymorphism. Another limitation of this study derives from the inclusion of patients with recurrence, which could produce a biased estimate of venous thromboembolic risk. The optimal design to assess recurrences is a prospective study after a first event. We have reanalyzed all the data considering only patients with a single episode of thrombosis ($n = 738$). This group was matched for age, race, and sex with controls, and the results were equivalent (multivariate analysis: *FV* Leiden: $P < .001$ [OR = 5.54, 95% CI: 3.4-9.0]; *PT* 20210G>A: $P < .001$ [OR = 5.37, 95% CI: 3.1-9.3]; and *ZPI* Arg67Stop: $P = .011$ [OR = 3.35, 95% CI: 1.3-8.6]).

All available information supports a relatively high incidence of nonsense mutations in the *ZPI* gene among patients with venous thrombosis: 4.4% in New Zealand population[8] and 3.0% in Spanish population. Accordingly, genetic modifications causing premature stop codons in the *ZPI* (polymorphisms or mutations) will probably increase the risk of venous thrombosis. The functional consequence of these nonsense mutations seems obvious. The stop codon, especially that located in the N-terminus region of the protein (position 67), should cause circulating deficiency of this serpin. Certainly, it is necessary to determine the levels of ZPI in carriers of these nonsense modifications to confirm such suggestion, but the expected ZPI deficiency of these patients might explain the increased risk of venous thrombosis. However, these results are partially conflictive with the recent report of the Leiden Thrombophilia Study (LETS).[22] This study did not detect a relationship between the plasmatic level of *ZPI* and venous thrombosis.[22] This apparent discrepancy might be explained by the relatively low frequency of nonsense changes (probably $< 4\%$ in the LETS study), most of them in heterozygous state. Thus, the normal levels of ZPI in noncarriers of the *ZPI* Arg67Stop polymorphism or the Trp303Stop mutation might mask any possible moderate deficiency of ZPI associated with this polymorphism in heterozygous state.

Our study identifies a nonsense polymorphism. Although it might seem that a genetic change causing a stop mutation never would have been evolutionary selected, there are old and recent references of this process. First, the origin of a stop codon might provide an evolutionary advantage that explains its selection. Thus, the common Arg577Stop nonsense polymorphism of the *ACTN3* gene has a beneficial effect on the function of skeletal muscle in generating forceful contractions at high velocity, increasing sprint performance,[23] or a stop codon polymorphism of Toll-like receptor 5 is associated with resistance to systemic lupus erythematosus.[24] Other nonsense polymorphisms, such as the Tyr319Stop polymorphism of the *SLC6A18* gene,[25] have no significant relevance. In contrast, there are examples of nonsense polymorphisms associating with different pathologies. Thus, the frequency of the 446G>T (Trp149Stop) polymorphism of the *ARLTS1*, a member of the ADP ribosylation family, was similar in controls and patients with sporadic tumors but was significantly more common among patients with familial cancer.[26] Finally, the common nonsense polymorphism affecting the complement *C9* gene (Arg95Stop), which is present in 6.7% of the Japanese population,[27] causes complement deficiency when present in homozygous state. Other polymorphisms affecting serpin-encoding genes might cause deficiency of the affected protein. The best example is the deficiency of α1-antitrypsin associated with the Z variant (Glu342Lys), which associates with emphysema and liver cirrhosis in homozygous

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state.[28] To the best of our knowledge, the *ZPI* Arg67Stop is the first nonsense polymorphism identified in serpins that increases the risk of venous thrombosis in heterozygous state.

Finally, we identified the first case homozygous for a nonsense mutation in the *ZPI* gene. Certainly, this patient suffered from recurrent venous thrombosis, but the first episode was quite late (at 62 years old) and there were additional risk factors present (hormone replacement therapy). Moreover, the carrier has no additional pathologies. The features of this *ZPI* knock-out patient are similar to that described for another anticoagulant serpin that shares additional structural similarities with the *ZPI*, the heparin cofactor II. Our group also identified the first case with heparin cofactor II homozygous deficiency that has no significant pathologic consequences.[29] In contrast, complete antithrombin deficiency is associated with embryonic lethality in mice and humans.[30] All of these data support that many serpins are not necessary for embryo development, but they are necessary to the control of serine proteases in the adult life. Moreover, an important redundancy between different serpins and other inhibitory mechanisms should exist to explain the absence of significant pathologies under complete deficiency of some serpins such as ZPI or heparin cofactor II.

In conclusion, our results support that the *ZPI* Arg67Stop nonsense polymorphism might be considered as a mild genetic risk factor for venous thrombosis. This polymorphism has lower prevalence but similar risk than the *FV* Leiden or prothrombin 20210A variants (ORs 3.32, 5.32, and 4.23, respectively). All available data support that the ZPI is a good candidate to play a significant role in thrombosis. Other genetic changes affecting this serpin, including other polymorphisms or unusual mutations, might be present in some of the so far idiopathic thrombosis, and consequently, functional and genetic analysis of the ZPI would be performed in thrombophilic studies.

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Antithrombin Cambridge II (A384S): an underestimated genetic risk factor for venous thrombosis

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The antithrombin A384S mutation has a relatively high frequency in the British population but has not been identified in other populations. This variant has been associated with cases of thrombotic disease, but its clinical relevance in venous thrombosis remained unclear. We have conducted a secondary analysis of the prevalence of the mutation in a large case-control study, including 1018 consecutive Spanish patients with venous thromboembolism. In addition, we evaluated its functional consequences in 20 carriers (4 homozygous). This mutation, even in the homozygous state, did not affect anti-Xa activity or antigen levels, and it only slightly impaired anti-IIa activity. Thus, routine clinical methods cannot detect this anomaly, and, accordingly, this alteration could have been underestimated. We identified this mutation in 0.2% of Spanish controls. Among patients, this variant represented the first cause of antithrombin anomalies. Indeed, 1.7% patients carried the A384S mutation, but 0.6% had any other antithrombin deficiency. The mutated allele was associated with an increased risk of venous thrombosis with an adjusted OR of 9.75 (95% CI, 2.2-42.5). This is the first study supporting that antithrombin A384S mutation is a prevalent genetic risk factor for venous thrombosis and is the most frequent cause of antithrombin deficiency in white populations. (Blood. 2007;109: 4258-4263)

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Introduction

Thrombosis is the leading cause of death in western societies, with an annual incidence of 1:1000 persons in the United States and Western Europe.[1] Because of the high incidence of mortality and morbidity, thrombotic disorders consume a significant portion of the national health care expenditure.[2-5] The negative impact of thrombosis could be significantly reduced if at-risk persons were identified and managed prior to clinical manifestation.[6]

Considerable progress has been made in determining the molecular bases of inherited thrombophilia in the past 40 years. Since Egeberg's description of antithrombin deficiency in 1965, a number of monogenic autosomal dominant disorders have been well established as risk factors for venous thromboembolic disease. At the beginning of the 1980s, deficiencies of other anticoagulant proteins, such as protein C and protein S, were also linked to venous thrombosis. All these disorders are rare, but they strongly increase the risk of venous thrombosis (10- to 20-fold). They follow a dominant inherited pattern with variable penetrance, suggesting that venous thrombosis might be a monogenic disease. However, all these deficiencies together accounted only for 4% to 8% of consecutive patients with venous thrombosis and 16% to 20% of families with thrombophilia. In 1994, the discovery of the factor V (FV) Leiden and, 2 years later, the identification of the genetic variation affecting the prothrombin gene (G20210A), dramatically changed the molecular view of venous thrombosis. These polymorphisms are relatively frequent in the normal population (2%-10%), but they mildly increase the risk of venous thrombosis (3- to 5-fold). Moreover, many symptomatic patients simultaneously carry more than one genetic risk factor, supporting that thrombophilia represents a polygenic rather than a monogenic clinical phenotype. In addition, genetic factors interact with environmental risk factors, clearly reflecting that thrombophilia is a complex and multifactorial trait.[7] However, there are still up to 60% of consecutive cases with venous thrombosis and 25% of families with thrombophilia with no recognized genetic risk factors. Unfortunately, despite considerable efforts during 2 decades evaluating hundreds of polymorphisms affecting hemostatic-related proteins, no further genetic risk factors for venous thrombosis have been clearly identified.[8]

The first mutation in the antithrombin gene was characterized in 1984.[9] Since then, more than 127 different mutations have been identified, which mostly occur in the heterozygous state.[10] All these data, together with animal models, support the key role of antithrombin in hemostasis, as the most important endogenous anticoagulant, and the pathologic consequences of its deficiency. The heterozygous state is a rare defect with an estimated prevalence in the healthy general populations between 1/1400 and 16/9669 (0.07%-0.16%).[11] Deficiency of this potent anticoagulant in thrombophilic patients is estimated between 1% and 8% and lower in consecutive patients with venous thrombosis.[12] As for any rare mutation, most of the identified mutations affecting the antithrombin gene appear as a single entry in the database.[10] Surprisingly, an antithrombin variant, A384S (antithrombin Cambridge II), had a high frequency in a study of blood donors in western Scotland (1/630),[13] but it had not been studied in other

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populations. This variant has been associated with cases of thrombotic diseases, but its clinical relevance in venous thrombosis remains unclear. The aim of this study was to evaluate the prevalence of this mutation, which determines a mild deficiency of the most important physiologic anticoagulant, in a Spanish population and its role in venous thrombosis in a large cohort of patients and controls.

Materials and methods

Patients and controls

Our study included 1018 unrelated white patients with venous thromboembolism. The control group of our study included 1018 unrelated people without a history of vascular or thromboembolic disease. The features of these groups were previously described.[14] Briefly, patients with a first objectively confirmed episode of venous thromboembolism before the age of 75 years who consecutively entered into the anticoagulation clinics from 4 Spanish hospitals in a time window between 2 and 4 years were enrolled. Objective diagnoses were done by clinical probability, D-dimer levels, compression ultrasonography, ventilation perfusion lung scan, and, when necessary, phlebography or pulmonary angiography. Patients with known malignant disorders were excluded. Controls were randomly and prospectively selected to match with the 1018 cases by age, sex, race, and geographic distribution.

Demographic parameters (age and sex) were recorded for all subjects. Relevant clinical data (family history of venous thrombosis, recurrence, type of thrombotic event, location of the thrombosis, and other thrombotic risk factors such as oral contraceptives, hormone replacement therapy) were recorded in patients. Functional protein S was assayed in automated coagulometers. Total and free protein S were assayed using enzyme-linked immunoabsorbent assay (ELISA) methods. Antithrombin (anti-Xa activity) and protein C were measured in automated coagulometers using chromogenic methods. Lupus anticoagulant was investigated by using the Exner method, and antiphospholipid antibodies were screened by ELISA methods that used cardiolipin or phosphatidylserine as antigen. The analysis of antibodies to β2-glycoprotein I was performed using ELISA methods.

All included subjects gave their informed consent to enter the study, which was approved by the ethics committees for each participating institution and performed in accordance with the Declaration of Helsinki, as amended in Edinburgh in 2000.

Genetic analysis

Blood samples were obtained by venipuncture collection into 1:10 volume of trisodium citrate (Vacutainer; Becton Dickinson, Meylon, France). Platelet-poor plasma fractions were obtained by centrifugation at 4°C for 20 minutes at 2200*g* (within 5 minutes after blood collection). Genomic DNA was purified by standard procedures. The exon 6 of the antithrombin gene was amplified by polymerase chain reaction (PCR) using the primers AT6F2, 5′-TGAGGAATTGCTGTGTCTGTG-3′, and AT6B, 5′-AGAGGTGCAAAGAATAAGAA-3′. The A384S mutation (antithrombin Cambridge II) was genotyped by PCR–allele-specific restriction assay (PCR-ASRA) using *Pvu*II (New England Biolabs, Hitchin, England). Confirmation of genotypes was performed by sequencing. PCR products were purified from 1.5% agarose gels using Ultraclean Gel Spin (MoBio, Solana Beach, CA). The sequence reaction was performed with the ABI Prism Big Dye Terminator Cycle sequencing kit on an automated sequencer type 377 (Perkin-Elmer Applied Biosystems, Washington Chesire, United Kingdom), with forward and reverse primers used for amplification. FV Leiden, prothrombin G20210A, and protein Z-dependent protease inhibitor (ZPI) R67X polymorphisms were determined in patients and controls, as described.[14,15]

Measurement of plasma antithrombin activity and levels

Antithrombin activity was determined by chromogenic methods in citrated plasma from 20 carriers of the antithrombin Cambridge II mutation, including 4 in homozygous state, and 17 controls without this mutation. Anti-Xa assays were performed with heparin, bovine FXa, and S-2765 chromogenic substrate (Instrumentation Laboratory, Milan, Italy). Anti-IIa assays were performed with heparin, bovine thrombin, and chromogenic substrate (ethyl-malonyl-S-Pro-Arg-pNA.AcOH–CBS 61.50-; STA Antithrombin; Diagnostica Stago, Asnières, France). Both assays were performed with unfractionated heparin and low molecular weight heparin (Bemiparin) (both from Laboratorios Rovi S.A., Madrid, Spain).

Antigen levels were measured by immunodiffusion (Laurell Technologies, New Wales, PA).

Results are expressed as a percentage of a pool generated with plasma from 100 healthy controls.

Statistical analysis

Continuous variables were expressed as mean ± SD and as percentages for categorical variables. Comparison between groups was done using an unpaired, Student *t* test or a chi-square test, as appropriate. Univariate statistical analysis was performed by the chi-square test. The strength of the association of major risk factors and the polymorphism with the occurrence of disease was estimated using 2 × 2 tables and calculating the odds ratio (OR) with the EpiInfo software (Centre for Disease Control, Atlanta, GA) and the Cornfield method for the calculation of 95% confidence intervals (CIs). Multivariate analysis was performed using multiple logistic regression and included all the significant covariates and age in a single step (Enter method), with the SPSS statistical package for Windows 8.0 software (Chicago, IL). Differences with a 2-tailed *P* value less than .05 were considered as being statistically significant.

Results

Genetic identification

The G1246T mutation in the antithrombin gene (A384S; antithrombin Cambridge II) is clearly identified by PCR-ASRA (Figure 1A). Because antithrombin Cambridge I (G1246C; A384P)[16] also gives the same restriction pattern, it is necessary to confirm the mutation by sequence analysis (Figure 1B). No subject was a carrier of the antithrombin Cambridge I.

Case-control study

Demographic, clinical, and genetic data of cases and controls are presented in Table 1. These features did not significantly differ in the samples from the 4 hospitals (data not shown).

The median age of patients at the time of the first thrombotic event was 45 years (interquartile range, 33-58 years), and the ratio of men to women was 1:1. Approximately two thirds of the patients had a single thrombotic episode (280 of 1018 had recurrent episodes of venous thrombosis), 218 patients reported risk factors such as oral contraceptive use, the presence of antiphospholipid antibodies, hormone replacement therapy, deficiency of antithrombin, protein C or protein S, and 177 patients (17.4%) reported a familial history of thrombosis. Antithrombin deficiency was diagnosed in 6 patients (0.6%). This diagnosis was performed by functional evaluation of anti-Xa activity. This method is routinely used in clinical practice, because it is the most reliable test to identify congenital antithrombin deficiency.[17] The prevalence of the classic prothrombotic polymorphisms was similar to that described in other series. Thus, the FV Leiden was present in 145 patients (3 in homozygous state) (14.2%), and the prothrombin 20210A allele was identified in 101 patients (7 in homozygous state) (9.9%) (Table 1).

Controls had a significant lower prevalence of these prothrombotic polymorphisms: 31 (3.0%) were heterozygous for the FV

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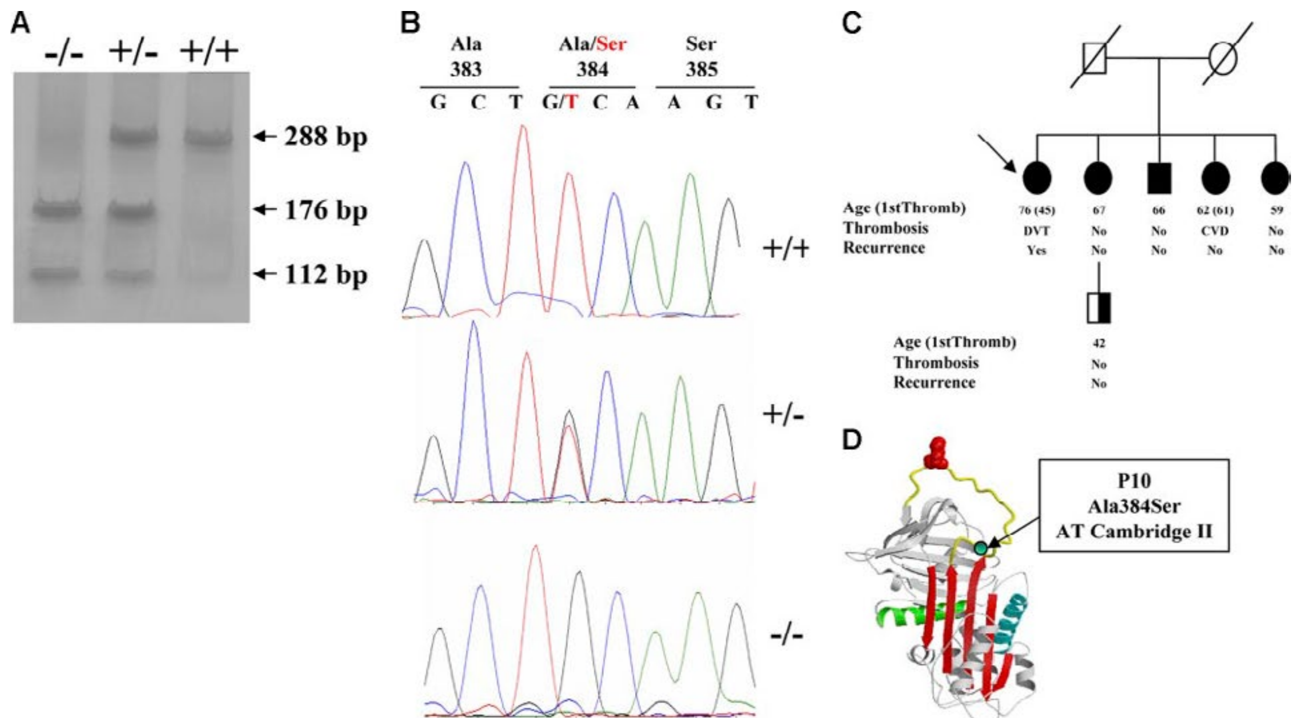

Figure 1. Identification of antithrombin Cambridge II (A384S). (A) PCR-ASRA pattern. (B) Sequence analysis. (C) Pedigree of the family with 5 antithrombin Cambridge II homozygous subjects, with proband identified with an arrow. Type of thrombotic event, age, and recurrence are indicated (NA indicates not available). Filled symbols represent the genetic anomalies identified in this family. (D) Localization of the residue affected by the mutation in the reactive loop (A384S; P10).

Leiden and 26 (2.6%) for the prothrombin 20210A allele (Table 1). Accordingly, these polymorphisms increased 5.29-fold (FV Leiden) and 4.20-fold (prothrombin 20210A) the risk of venous thrombosis ($P < .001$; 95% CI, 3.5-8.0 and $P < .001$; 95% CI, 2.7-6.7, respectively). In this case-control study, we recently identified a new prothrombotic nonsense polymorphism affecting the *ZPI* gene (ZPI R67X).[14] Carriers of the ZPI 67X allele had a 3.40-fold risk of developing venous thrombosis (Table 1). The clinical features associated with these prothrombotic polymorphisms were described elsewhere.[14]

The antithrombin Cambridge II mutation was only identified in 2 controls (0.2%). Both controls were young women aged 31 and 42 years from distant regions of Spain (Barcelona and Murcia). In contrast, this mutation was present in 17 patients with venous thrombosis (1.7%). Thus, this mutation increased 8.63-fold the risk of venous thrombosis ($P < .001$; 95% CI, 1.9-54.2) (Table 1). In all cases, the mutation was identified in a heterozygous state.

The presence of this mutation did not significantly modify the age at the first thrombotic event (49 years in carriers and 45 years in noncarriers). Moreover, the antithrombin Cambridge II mutation did not increase the rate of recurrence (23.5% in carriers and 27.6% in noncarriers, $P = .718$) and was not associated with an increased familial history of venous thrombosis (11.8% in carriers and 17.5% in noncarriers, $P = .544$). Finally, the percentage of patients who displayed an additional risk factor was similar in carriers and noncarriers of this mutation (17.6% versus 21.5%, respectively, $P = .703$).

Table 1. Characteristics of patients with venous thrombosis and control subjects

Characteristic	Patients	Controls	Univariate analysis		Multivariate analysis	
			P	OR (95% CI)	*P*	OR (95% CI)
Median age, y (interquartile range)	49 (36-62)	47 (35-63)	.618	—	—	—
Male sex, no.	509	509	> .999	—	—	—
FV Leiden, no.						
−/−	873	987	—	—	—	—
+/− and +/+	142, 3	31, 0	< .001	5.29 (3.5-8.0)	< .001	5.34 (3.6-8.0)
PT 20210, no.						
G/G*	917	992	—	—	—	—
G/A and A/A	94,7	26, 0	< .001	4.20 (2.7-6.7)	< .001	4.31 (2.8-6.7)
ZPI 728 (R67X), no.						
C/C	988	1009	—	—	—	—
C/T and T/T	29, 1	9, 0	< .001	3.40 (1.5-7.8)	.002	3.37 (1.6-7.3)
AT 1246 (A384S), no.						
G/G	1001	1016	—	—	—	—
G/T	17	2	< .001	8.63 (1.9-54.2)	.002	9.75 (2.2-42.5)

N = 1018 for both the patient and the control group.
— indicates not applicable.

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No patient carrying the antithrombin Cambridge II mutation had the prothrombin 20210A variant or the ZPI nonsense allele. However, 2 patients simultaneously displayed the FV Leiden and antithrombin Cambridge II mutation. Both were male patients who had their first episode of deep venous thrombosis at 46 and 59 years without additional risk factors, and they reported neither recurrence nor familial history of thrombosis. No control had such a combination.

Multivariate analysis, including age, prothrombotic polymorphisms (FV Leiden, PT G20210A, and ZPI R67X), and the antithrombin A384S mutation, confirmed that the antithrombin Cambridge II is an independent and strong risk factor for venous thrombosis (P = .002; OR = 9.75; 95% CI, 2.2-42.5) (Table 1).

In addition, we have analyzed the role of the antithrombin Cambridge II limited to persons with idiopathic first thrombosis, without additional genetic or acquired risk factors. Four hundred thirty-four cases fulfilled these features (median age, 52 years; 56.2% men). The subgroup of 952 controls selected for the analysis of cases with idiopathic venous thrombosis were chosen by excluding controls carrying the genetic risk factors (FV Leiden, PT 20210, and ZPI R67X) (median age, 47 years; 50.3% men). The antithrombin Cambridge II mutation was identified in 9 patients but 2 controls, supporting that this mutation is a genetic risk factor for idiopathic venous thrombosis ($P < .001$; OR, 10.06; 95% CI, 2.03-67.63).

Finally, we identified a family with 5 members carrying the antithrombin Cambridge II in homozygous state. A 7-year-old woman reported recurrent deep venous thrombosis, with the first episode at the age of 45 years. Moreover, one of her sisters also had ischemic cerebrovascular disease. However, 3 other homozygotes relatives did not report thrombotic episodes (Figure 1C).

Antithrombin values

We have evaluated the functional consequences of this mutation in 37 subjects, 17 controls, and 20 carriers of the antithrombin A384S mutation (16 heterozygous and 4 homozygous). Both antigen and anti-Xa activity (in the presence of unfractionated heparin and low molecular weight heparin) of carriers and noncarriers are within the normal range. We did not observe significant differences in these parameters between carriers and noncarriers or between heterozygous and homozygous states (Table 2). Statistical differences were only achieved when comparing the anti-Xa activity in the presence of unfractionated heparin from noncarriers and homozygous (100.2% ± 4.3% versus 91.7% ± 10.5%, respectively; P = .016). However, the anti-IIa activity in the presence of unfractionated heparin was mild but significantly impaired by the Cambridge II mutation (100.0% ± 7.9%; range, 87.5%-118.0% for control versus 79.3% ± 7.8%; range, 66.9%-90.8% for carriers; $P < .001$) (Table 2). In addition, we observed a significant gene-dosage effect on this parameter. Thus, the anti-IIa activity of heterozygous carriers in the presence of unfractionated heparin was 81.8% ± 6.6% (range, 70.6%-90.8%), whereas homozygous subjects displayed 69.5% ± 2.4% (range, 66.9%-72.6%); P = .002 (Table 2). The functional consequences of the antithrombin Cambridge II mutation on anti-IIa activity were reduced when using low molecular weight heparin, but differences between carriers and noncarriers and between heterozygous and homozygous state still reached statistical significance (Table 2).

Discussion

A major challenge for biomedicine in the genomic era is to identify DNA modifications responsible for variation in complex diseases (eg, physiologic risk factors related to thrombotic disease). Identifying the loci that affect susceptibility of those complex phenotypes among the overall polymorphisms that exist in the human genome should bring about improved understanding of such diseases and provide new targets for clinical intervention. To date, most candidate gene studies have directly analyzed the association between disease status and a number of candidate SNPs that have known or predicted functional consequences.[18] This design can easily determine whether a given variant confers significant risk of disease. For venous thrombosis, genetic risk factors have been identified in different candidate genes (antithrombin, protein C, protein S, *FV, PT*).

Herein, we present the results of a classic case-control association study in unrelated Spanish subjects designed to estimate the contribution of genetic and environmental factors to the risk of thrombotic disease. The results of this study show an adjusted OR of 9.75 of developing venous thromboembolism associated with the G1246T mutation in the antithrombin gene (A384S, antithrombin Cambridge II). The statistical power of our study is high because of the large sample size (92.5% and 79.5% with a confidence level of 95% and 99%, respectively). These results suggest that antithrombin Cambridge II itself is an independent risk factor for venous thromboembolism. These results, the wide CIs for the ORs in the case-control study and the identification of a family with 5 carriers of this mutation in homozygous state with moderate thrombotic clinic support that this variant lies between a mild prothrombotic polymorphism (such as FV Leiden) and a rare but severe mutation (such as an antithrombin type I deficiency).

Congenital antithrombin deficiency is an autosomal hereditary disorder with variable penetrance. Type I deficiencies usually associate with a severe risk for venous thrombosis. In contrast, overall type II deficiencies usually have milder thrombotic risk.[19] Thus, type II deficiencies account for approximately 40% of patients with venous thrombosis and antithrombin deficiency, but 88% of identified antithrombin-deficient persons.[13] Our study has important implications because it reveals another possible kind of antithrombin deficiency that is featured by no functional or antigenic deficiency, at least by using classic screening methods for antithrombin deficiency, which however have impaired functional

Table 2. Functional studies in carriers and noncarriers of the antithrombin Cambridge II (A384S) variant

	Noncarriers	All carriers	*P*	Heterozygous	Homozygous	*P*
No. of controls	17	20		16	4	
AT antigen levels (range)	97.9 ± 14.2 (83.6-136.0)	96.1 ± 12.6 (64.2-131.2)	.693	96.3 ± 13.8 (64.2-131.2)	95.4 ± 7.2 (88.3-105.2)	.895
Anti-Xa + UFH (range)	100.2 ± 4.3 (92.3-106.1)	98.5 ± 10.7 (75.7-108.5)	.555	100.2 ± 10.3 (75.7-108.5)	91.7 ± 10.5 (82.3-106.8)	.159
Anti-Xa + LMWH (range)	100.1 ± 8.0 (85.0-1116.9)	97.6 ± 11.8 (70.3-111.3)	.463	98.8 ± 11.2 (70.3-111.3)	92.6 ± 14.5 (75.3-108.9)	.355
Anti-IIa + UFH (range)	100.0 ± 7.9 (87.5-118.0)	79.3 ± 7.8 (66.9-90.8)	< .001	81.8 ± 6.6 (70.6-90.8)	69.5 ± 2.4 (66.9-72.6)	.002
Anti-IIa + LMWH (range)	100.6 ± 7.5 (84.9-113.2)	90.1 ± 9.5 (73.4-107.6)	< .001	92.4 ± 9.1 (73.4-107.6)	81.0 ± 3.6 (76.5-85.1)	.027

Values (mean ± SD) represent percentages of the result obtained in a pool of 100 healthy controls, except where otherwise indicated.

AT indicates antithrombin; UFH, unfractionated heparin; LMWH, low-molecular-weight heparin.

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activity under thrombotic situations. The missense substitution of A384S, discovered in 1991 and called antithrombin Cambridge II, is the result of a G1246T point mutation in exon 6 of the antithrombin gene.[20] This mutation has been classically included in the group of type II deficiencies, affecting the P10 proximal hinge region of the reactive center loop of antithrombin (Figure 1D).[10] However, the replacement of this residue by serine does not cause a complete loss of activity. Certainly, all available data from 41 carriers (from the study of Perry et al[21] and this study) support that this mutation, even in the homozygous state, does not affect the anti-Xa activity (98.6% ± 15.4%). Moreover, 62 carriers, including 4 homozygous subjects (from the studies of Tait et al,[13] Perry et al,[21] and this study) have normal plasma levels of antithrombin antigen (99.5% ± 13.1%). Because these are the methods commonly used to identify antithrombin deficiency,[17,22] we can understand why this mutation has not been identified in other populations, and, probably, its prevalence had been underestimated. Moreover, this variant only slightly impaired the anti-IIa activity (74.3% ± 8.5%) of 60 carriers (from the studies of Tait et al,[13] Perry et al,[21] and this study). Accordingly, plasma methods for detection of antithrombin deficiency might be complemented with the genetic test for G1246T point mutation in the antithrombin gene to avoid the underestimation and misdiagnosing antithrombin-deficient persons. This is particularly important, because antithrombin Cambridge II is the most frequent cause of deficiency of this serpin. Indeed, it is present in 0.2% of the normal population of at least 2 different white populations. Note that this percentage is similar to the antithrombin deficiency caused by the rest of the mutations identified so far. Moreover, this mutation was found in 1.7% of patients with venous thrombosis, more than twice the prevalence of all other antithrombin deficiencies in our study (0.6%). The relevance of this finding is not only restricted to patients with thrombosis but also to asymptomatic relatives and offspring that may carry the same genetic factor. At present, the most reasonable course of action is to carry out thromboprophylaxis under risk situations.[23,24] Although the thrombotic risk associated with this mutation is milder than that associated with other antithrombin deficiencies, asymptomatic relatives carrying the G1246T mutation in the antithrombin gene could benefit from antithrombotic prophylaxis in risk situations.

Finally, an accurate diagnosis of antithrombin Cambridge II could have potential therapeutic relevance. The conservative A384S mutation at the proximal hinge (P10 position) of the reactive center loop of antithrombin slows the initial step of loop insertion because of steric effects and to the buckling of the strand that tip the balance from inhibitory to substrate in the presence of heparin.[25] Thus, in the presence of full-length heparin the Cambridge II mutation displays a 3- and 7-fold increase in the stoichimetry of inhibition of FXa and thrombin, respectively.[25] Our functional studies in blood samples from carriers confirm that the deleterious effect of this mutation is particularly evident with unfractionated heparin. Accordingly, further studies must be performed to clarify whether anticoagulant therapy of carriers with this mutation should avoid unfractionated heparin, as suggested elsewhere.[25]

Potential limitations might have influenced our findings. First, the relatively low prevalence of this mutation explains the very wide confidence intervals of our study. Moreover, although the size of our sample is large, the selection of patients and controls from different hospitals is a methodologic weakness of our study. In addition, there are slight variations in the anti-IIa activity observed in our study compared with that from previous studies which may be attributable to different factors, such as the concentration and type of unfractionated heparin, the chromogenic substrate, the source of FIIa, or slight methodologic variations. Therefore, confirmatory studies from other large case-control studies are critical to define relative risks, likelihood of recurrence, and prevalence of this mutation in other populations to encourage the inclusion of this mutation in routine thrombophilic studies to improve diagnosis and prevention strategies. In addition, it should be profitable to investigate the possible joint effects of the G1246T variant with other risk factors, such as FV Leiden, prothrombin G20210A, or ZPI R67X polymorphisms, and the effectiveness of anticoagulant treatment with heparins in carriers of this common mutation.

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Authorship

Contribution: J.C., I.A., J.F., R.L., and V.V. designed the research; D.H.-E., R.G.-C., A.O., J.M., A.M., M.G., V.R., J.R.G.-P., J.M., E.P.-C, I.S., and M.G. analyzed the data; and J.C. and J.M.S. wrote the paper.

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ORIGINAL ARTICLE

Homozygosity for the C46T polymorphism of the F12 gene is a risk factor for venous thrombosis during the first pregnancy

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Summary. *Background:* A first thromboembolic event during pregnancy and puerperium is predisposed to by polymorphisms G1691A in the factor V gene (F5) (F5G1691A) and G20210A in the prothrombin gene (F2) (F2G20210A). *Objectives:* To study another potentially frequent thrombogenic polymorphism, C46T in the factor XII gene (F12) (F12C46T). *Patients and methods:* The 32 463 previously asymptomatic women included in the NOHA First cohort in their first pregnancy were investigated for these three polymorphisms. No other constitutional or acquired thrombophilic risk factor was studied. *Results:* The overall incidence – absolute risk – of venous thromboembolic events (VTE) was 127 per 100 000 woman-years and was reduced to 22 per 100 000 women-years in women negative for the three polymorphisms ($P < 0.0001$). Homozygosity for F12C46T was associated with a significant relative risk (RR) of VTE [RR: 5.99, 95% confidence interval (95% CI): 2.1–17.3, $P = 0.001$], as was heterozygosity for F5G1691A (RR: 18.7, 95% CI: 8.3–42, $P < 0.0001$), heterozygosity for F2G20210A (RR: 14.3, 95% CI: 6.2–33.2, $P < 0.0001$), maternal age (RR: 1.18, 95% CI: 1.07–1.29, $P = 0.0006$), maternal body mass index (RR: 1.31, 95% CI: 1.11–1.55, $P = 0.002$), conceptus weight (percentiles adjusted for term of delivery; RR: 0.90, 95% CI: 0.88–0.93, $P < 0.0001$) and pre-eclampsia (RR: 3.03, 95% CI: 1.06–8.69, $P = 0.039$). *Conclusions:* Homozygosity for the C46T polymorphism of the F12 gene is associated with venous thrombosis during the first pregnancy/puerperium in previously asymptomatic women.

Keywords: F12 gene, F2 gene, F5 gene, pregnancy, venous thrombosis.

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Introduction

A comprehensive understanding of the components of the relative and absolute risks of venous thromboembolism (VTE) among pregnant and postpartum women may help to assess the theoretical benefits of thromboprophylaxis in clearly defined subgroups of women, until adequate trials, if any, are conducted.

Venous thromboembolism is predisposed to by inherited hypercoagulable disorders that promote thrombosis, collectively termed 'thrombophilia'; the two most common being polymorphisms G1691A of the factor V (FV) gene (F5) (F5G1691A: 'Leiden') and G20210A of the prothrombin gene (F2) (F2G20210A); the apparently common homozygosity for the C46T polymorphism of the factor XII (FXII) F12 gene (F12C46T) [1,2] was more recently described in a Mediterranean population.

The F5G1691A and F2G20210A polymorphisms have been reported to be associated with an increased risk of thrombosis during pregnancy [3]: a recent meta-analysis of the literature focused on the F5G1691A polymorphism and showed discrepancies between data obtained from cohorts and from case–control studies [4], and emphasized the limited available data. No data are currently available on the risk associated with the F12C46T polymorphism.

This led us to prospectively re-examine symptomatic VTE, paying particular attention to F12C46T, F2G20210A and F5G1691A polymorphisms in a cohort of women (NOHA First cohort [5]) followed during their first intended pregnancy and puerperium (6 weeks postpartum).

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Methods

The 'NOHA First' cohort

The Nimes Obstetricians and Haematologist cohort of first intended pregnancies, named the 'NOHA First' cohort, has previously been described in detail [5]. The study began on January 1, 1999 and took place in the geographic region of southern France, the inhabitants of which come under the University Hospital of Nimes (Nimes catchment area). This study was approved by the institutional review board. This included approximately 1 million people principally living in the administrative subdivision of the Gard (625 000 inhabitants) or living close to the boundaries of this subdivision in the neighboring regions of Hérault, Aveyron, Lozère, Ardèche, Drôme, Vaucluse and Bouches-du-Rhône. The annual number of births for this population is approximately 11 000.

A total of 33 033 women in their intended first pregnancy (inclusion criteria) were followed-up by a network of 37 gynecologists and obstetricians selected throughout the whole region (Fig. 1). This network included representatives of gynecologists and obstetricians working in general hospitals and clinics of the Nimes area, and at the University Hospital of Nimes. Members of this network worked with their usual general practitioner contact, which enabled them to be kept informed of all women having their first pregnancy for the ongoing study.

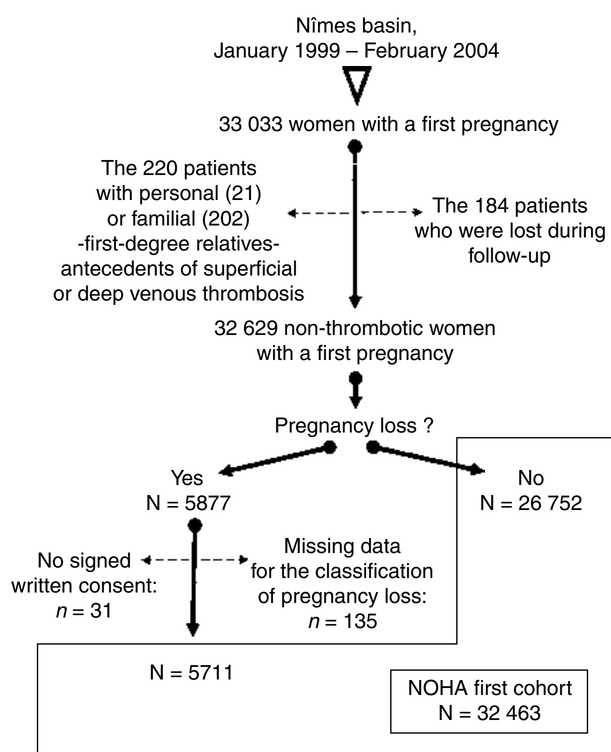

Fig. 1. Nimes Obstetricians and Haematologist (NOHA) First: the recruitment of the women and reasons for exclusion. Dashed lines: exclusion. Predictors of venous thromboembolism-free survival during pregnancy were studied in the whole cohort.

All medical data were collected after the pregnancy started by the corresponding general practitioner from self-reporting by the women and their partners, using a specific diary that women were asked to keep, which followed a standard explanatory document distributed at the time of diagnosis of the pregnancy, and which also contained any external medical reports. Help could be obtained from the related obstetrician (1537 cases), and from members of the steering committee (G.L.-L., I.Q., P.M., J.C.G.) (679 cases).

Thrombophilic conditions such as clotting inhibitor deficiencies or antiphospholipid/cofactor antibodies were not tested for prior to selection and were not used as inclusion or exclusion criteria.

The exclusion criteria were first, any previous occurrence of superficial or deep vein thrombosis (DVT), in the person or in any first degree relative; second, any chronic treatment during pregnancy interfering with the hemostatic system, including low-dose or high-dose aspirin. These treatments were permitted if given for acute medical reasons.

All pregnancy-related clinical events were recorded. Pre-eclampsia (PE), severe PE, eclampsia and placental abruption were defined according to the current international definitions. Fetal growth was evaluated after delivery by weighing the embryo/fetus and results were presented as values adjusted for the term of delivery (percentiles; 'adjusted conceptus weight'): these data were not available for 12.3% of the women with pregnancy loss, mostly before the 10th week of gestation. The number of corresponding cases observed in the cohort is given in Table 1.

Pregnancy loss was defined as a spontaneous interruption of a pregnancy, documented by an objective method (positive home pregnancy test, blood beta-HCG analysis or ultrasonography). Each case of pregnancy loss was systematically examined by the members of the network for any known underlying risk factors according to a fixed protocol that has been previously described [5]. Plasma lupus anticoagulant activity, anticardiolipin and anti-β2-glycoprotein I IgG and IgM antibodies were assayed by methods described previously [6]. Any data missing from this protocol led to the patient being excluded from the study (n = 135 patients; 2.3% of all the patients with pregnancy loss: Fig. 1): the 5711 remaining women underwent all the tests included in the protocol. Any abnormality identified resulted in the pregnancy loss being defined as explained (and unexplained if otherwise) (Table 1).

The 32 463 women who satisfied the inclusion criteria, and gave their signed informed consent to participate in the study and for the collection of blood and subsequent DNA analysis were included in the cohort. Of these, 26 323 were European Caucasians (81.1%; 73.8% from the Mediterranean region and 7.3% not from the Mediterranean region), 4447 were Caucasians from Northern Africa (13.7%); 1006 were Africans (3.1%) and 683 were Asians (2.1%).

Medications used during pregnancy included folic acid (0.4 g day^{-1} during the first trimester in 93.2%) and iron supplementation (80 mg day^{-1} in 9.3%). Acute aspirin intake for transient hyperthermia was recorded in 2.1% of women.

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Table 1 Main characteristics of the women included in the Nimes Obstetricians and Haematologist (NOHA) First cohort

	n (%)
Number of cases	32 463
Mean age (range), years	29.2 (21–36)
Mean BMI (range), kg m^{-2}	24.3 (16.1–33.7)
Outcome of the pregnancies	
Multiple pregnancies	340 (1.0)
Delivery by cesarean section	6957 (21.4)
Spontaneous pregnancy loss	5711 (17.6)
Explained	2162 (6.7)
Unexplained	3549 (10.9)
Pre-eclampsia	1307 (4.0)
Severe form	309 (0.95)
With fetal loss	264 (0.8)
Eclampsia	18 (0.06)
Placental abruption	170 (0.5)
Intrauterine growth restriction	3029/31 450 (9.6)

BMI, body mass index at the time of diagnosis of the pregnancy.

Short-term prophylactic low-molecular heparin treatment (enoxaparin 4000 IU day^{-1}) was given to 7015 patients (21.6%), mostly to almost all of the women who delivered by cesarean section (n = 6,787: 97.6%; duration of treatment: 7 days) but to only 60.5% of the women with severe PE (n = 187, duration of treatment: 7 days). The remaining most common indication was prolonged bed rest (more than 3 days) for premature uterine contractions (n = 114: 28.6%; women with antepartum bed rest lasting 2 weeks or more: n = 77, 93.9%; treatment given during the entire time of bed rest). As a result of information and advice given to the pregnant patients by hospital healthcare professionals around the time of delivery, above-knee graduated elastic compression stockings were also accepted and used by most of the women (78%), until they returned home.

Venous thrombotic disease-related procedures

All of the women were given specific medical information about clinical signs suggestive of venous thromboembolic disease at the time of diagnosis of their pregnancy: signs in the lower limbs (localized pain, swelling, heat, skin tension, flushing or any other abnormal skin coloration, dilation of superficial veins); chest signs (pain, 'stitch' in their side, coughing up blood, shortness of breath); general signs (sweating, anxiety, rapid pulse, unexplained fever, fainting).

If any of these signs were reported, a medical examination was undertaken to authenticate the findings and obtain an objective confirmed diagnosis. An exhaustive, bilateral, comparative color duplex ultrasound investigation was performed in the case of first-line suspected venous thrombosis. A perfusion scintigraphy protocol with 6 mCi of Technetium-labeled albumin aggregates in order to minimize embryo/foetal radiation exposure, and to avoid the induction of foetal hypothyroidism by iodinated contrast medium [7,8] was used in cases of first-line suspected pulmonary embolism, and when a DVT had been identified. All the vascular physicians who performed color duplex ultrasound examinations belong to a group which is regularly assessed by one of the authors (M.D.), who is closely involved in university programs on this diagnostic procedure [9,10].

Laboratory investigation

We detected F5G1691A and F2G20210A polymorphisms using a one-step multiplex allele-specific amplification polymerase chain reaction (PCR) [11] and the F12C46T polymorphism using restriction fragment length polymorphism (RFLP)-PCR as previously described [2]. A reference DNA containing each mutation verified by direct sequence analysis was included in each run.

Other constitutional or acquired thrombophilic conditions were not assessed in this initial work, mainly for cost reasons. Antithrombin deficiency, protein C deficiency, protein S deficiency, high levels of factor VIII (FVIII), FIX or FXI, hyperhomocysteinemia, blood groups and antiphospholipid antibodies were not considered in the analyses. Results for antiphospholipid antibodies were only known in the subgroup of women with pregnancy loss, in order to help classify the women as explained/unexplained pregnancy loss.

Statistical analysis

Analyses were performed on a VAX computer (Digital equipment Corporation, Boston, MA, USA) using SAS software (version 6.08; SAS Institute Inc., Carry, NC, USA) and the SPSS software (version 11; SPSS Inc., Chicago, IL, USA).

Cox proportional-hazards regression analysis was used to estimate univariate and then multivariate hazard ratios for venous thromboembolism (DVT ± pulmonary embolism: VTE) during pregnancy and puerperium. The following variables were analysed: age, body mass index, multiple pregnancy, cesarean section, PE, severe PE, eclampsia, placental abruption, pregnancy loss, type of pregnancy loss (explained/unexplained), conceptus weight, use of prophylactic low molecular weight heparin, F5G1691A genotype, F2G20210A genotype and F12C46T genotype. The multivariate analysis was performed with a step-wise selection process and a stringency level (P-value) of 0.05 was used to both include and exclude variables in the analysis.

Results

Thromboembolic events

One hundred and forty-two pregnant women underwent a total of 169 medical examinations after a suggestive subjective sign. The final diagnoses are given in Table 2. An alternative diagnosis (manifestations of anxiety, shortness of the breath as a result of high weight gain, bronchitis, pneumonia) was ultimately made in 23 women with a first-line suspicion of PE: these 23 cases were followed-up until the end of pregnancy and for an additional 1-year period without any VTE. A colour

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Table 2 Final diagnosis in the 142 pregnant women in the Nimes Obstetricians and Haematologist (NOHA) First cohort who underwent a medical examination after reporting a subjective symptom suggestive of thromboembolic disease

First-line pulmonary embolism suspected	23
Ruled out by perfusion scintigraphy	23
First-line deep vein thrombosis of the lower limb suspected	146
Ruled out by color duplex ultrasound investigation	111
Ruled in by color duplex ultrasound investigation	35
Right side	9
Left side	25
Bilateral	1
Proximal	17
Distal	18
With secondary pulmonary embolism	10
On proximal thrombosis	7
On distal thrombosis	3
First trimester	7
With secondary pulmonary embolism	3
Second trimester	10
With secondary pulmonary embolism	3
Third trimester	7
With secondary pulmonary embolism	2
Puerperium (6 weeks)	11
With secondary pulmonary embolism	2

duplex ultrasound performed 1 year after the end of pregnancy was normal in all these cases. The 111 unconfirmed suspected DVT were similarly followed-up; two symptomatic distal DVT developed (6 months postpartum after local trauma and 8.5 months postpartum after oral contraceptive use); colour duplex ultrasound performed 1 year after the end of pregnancy found residual signs of asymptomatic calf DVT in six cases. The overall incidence of thrombosis (35 events) was computed to 127 per 100 000 woman-years: 99 per 100 000 for pregnant women but 294 per 100 000 woman-years for puerperium ($P < 0.0001$). The mean number of women needed to observe one DVT during pregnancy and puerperium was 928: 1353 during pregnancy and 2951 during puerperium.

Polymorphisms and associated thrombotic events

The incidence of three polymorphisms in the cohort is given in Table 3, together with the corresponding thromboembolic events. Homozygosity for the F12C46T polymorphism was consistently found in our population (3.05%), and was even more frequently detected than the commonly found heterozygosity for F5G1691A or FII20210A polymorphisms ($P < 0.01$), the incidence of which was lower than in other European populations, as previously described (5; control population). Homozygosity for the F12C46T polymorphism was globally associated with a lower mean incidence of VTE (0.5%) than in those heterozygous for F5G1691A or FII20210A polymorphisms (1.33% and 1.34% respectively, $P < 0.01$). Clinical VTE was as common in the group of women heterozygous for the F12C46T polymorphism as in the whole cohort (mean incidences: 0.12% and 0.11%, respectively). Homozygosity for the F5G1691A or FII20210A

Table 3 Incidence of the C46T polymorphism of the F12 gene (F12C46T), the G20210A polymorphism of the prothrombin gene (F2) (F2G20210A) and the Factor V gene (F5) Leiden polymorphism found in the cohort of women with a first intended pregnancy, together with the corresponding cases of deep vein thrombosis (DVT)

	n (%)	DVT	NNDVT P + Puer	P	Puer
F12C46T					
Homozygous	991 (3.05)	5	198	496	330
Isolated	939	4	235	939	313
Associated	52	1	52	52	–
Heterozygous	12 297 (37.9)	15	820	1118	3074
Isolated (A)	11 792	3	3930	3930	–
Associated	505	12	42	63	126
F2G20210A					
Homozygous	3 (0.009)	2	1.5	3	3
Isolated	1	0	–	–	–
Associated	2	2	1	2	2
Heterozygous	597 (1.84)	8	75	119	199
Isolated	324	3	108	108	–
Associated	273	5	55	137	91
F5G1691A					
Homozygous	12 (0.037)	4	3	4	12
Isolated	3	1	3	3	–
Associated	9	3	3	4.5	9
Heterozygous	679 (2.09)	9	75	97	340
Isolated	388	4	97	129	388
Associated	291	5	58	73	291
Whole cohort	32 463	35	928	1353	2951
Negative* (B)	18 443 (56.8)	6	3074	4611	9222
Positive†	14 020 (43.2)	29	483	701	1558
(B) or (A)	30 235 (93.1)	9	3360	4320	15 118
Positive, non-(A)	2228 (6.9)	26	86	131	248

N, number women; NNDVT, number of women needed for one DVT (P, during pregnancy; Puer: during 6 weeks postpartum). Isolated: only positive for the corresponding polymorphism; associated: positive for more than the corresponding polymorphism. *Negative: women negative for the three polymorphisms. †Positive: women positive for at least one of the polymorphisms.

polymorphisms was rare but highly symptomatic. Women carrying more than one polymorphism were more frequently symptomatic than women with isolated positivity for one of the polymorphisms ($P < 0.001$). VTE was three to four times less frequent in women not homozygous for the F12C46T polymorphism and negative for both F5G1691A and FII20210A polymorphisms, than in the whole cohort. The relative distribution between DVT occurring during pregnancy and DVT occurring during puerperium was not changed by the presence of a given positive polymorphism.

Predictors of thrombosis in pregnancy and the puerperium

Women who developed VTE were more often homozygous for the F12C46T polymorphism, or were carriers of the F5G1691A or F2G20210A polymorphism than those who did not. They were also older and of higher body mass index, had a lower conceptus weight and had more often suffered PE or pregnancy loss (mainly unexplained). All these parameters were associated with significant relative risks (RR) of VTE according to proportional-hazard regression models

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Table 4 Relative risks of venous thromboembolism (deep vein thrombosis and pulmonary embolism) in the cohort of 32 463 first pregnancies, according to the proportional-hazards regression models. (A) univariate analysis; (B) step-wise multivariate analysis

	RR	95% CI	*P*
(A)			
F12C46T			
Homozygous	6.46	2.4–17.8	0.0003
Heterozygous	1.56	0.76–3.19	0.22
F2G20210A			
Homozygous	983	232–4160	< 0.0001
Heterozygous	17.2	7.7–38.0	< 0.0001
F5G1691A			
Homozygous	604	208–1753	< 0.0001
Heterozygous	19.3	8.9–41.9	< 0.0001
Maternal age (years)	1.146	1.054–1.247	0.0015
Maternal body mass index (kg m^{-2})	1.234	1.054–1.445	0.009
Multiple pregnancy	2.78	0.380–20.3	0.32
Caesarean section	0.76	0.32–1.83	0.54
Pre-eclampsia	3.08	1.09–8.74	0.034
Placental abruption	0.03	0-∞	0.98
Pregnancy loss	2.77	1.396–5.5	0.0036
Type of pregnancy loss			
Explained	1.65	0.50–5.52	0.42
Unexplained	3.48	1.65–7.35	0.0011
Conceptus weight (adjusted percentile)	0.91	0.886–0.934	< 0.0001
Low molecular weight heparin use	0.75	0.31–1.81	0.52
(B)			
F5G1691A			
Homozygous	135	32–574	< 0.0001
Heterozygous	18.7	8.3–42	< 0.0001
F2G20210A			
Homozygous	310	47–2049	< 0.0001
Homozygous	135	32–574	< 0.0001
Conceptus weight (adjusted percentiles)	0.90	0.88–0.93	< 0.0001
Maternal age (years)	1.18	1.072–1.292	0.0006
Maternal body mass index (kg m^{-2})	1.31	1.105–1.552	0.0018
F12C46T			
Homozygous	5.99	2.1–17.3	0.001
Heterozygous	1.45	0.69–3.1	0.33
Type of pregnancy loss			
Explained	1.23	0.31–4.72	0.62
Unexplained	1.99	1.34–7.35	0.003
Pre-eclampsia	3.03	1.06–8.69	0.039

RR, relative risk of venous thromboembolism; 95% CI, 95% confidence interval.
F5G1691A: factor V gene (F5) Leiden polymorphism; F2G20210A: G20210A polymorphism of the prothrombin gene (F2); F12C46T: C46T polymorphism of the F12 gene.

(Table 4A), whereas multiple pregnancy, cesarean section and placental abruption were not. Cesarean section was associated with a non-significant trend for a protective effect against VTE and none of the women with placental abruption suffered any venous thrombosis. The multivariate analysis showed that homozygosity or heterozygosity for F5G1691A or FII20210A, conceptus weight, maternal age, maternal body mass index, unexplained pregnancy loss, homozygosity for F12C46T and PE were associated with significant RRs of VTE (Table 4B).

Discussion

We have studied thromboembolic events in a cohort of previously asymptomatic women with a first pregnancy, with particular attention to a recently described predisposing polymorphism: C46T in the F12 gene and to two classic thrombogenic polymorphisms: G1691A in the F5 gene and G20210A in the F2 gene.

Other classic thrombotic risk factors, some rare (antithrombin deficiency, etc.) but others more common (high levels of coagulation factors, antiphospholipid antibodies, etc.), were not considered. The possible consequence being an overestimation of the risk estimate computed in women carrying the homozygous C46T polymorphism in the F12 gene. The significance of our results may therefore be limited. There is no available work, to date, linking the F12 gene polymorphism under focus to other classic thrombotic risk factors or showing a correlation between this polymorphism and the quantitative levels of other thrombotic risk factors. However, the initial work by Tirado *et al.* [2], also performed in a Caucasian population from the Mediterranean region, found homozygosity for C46T in the F12 gene to be an thrombotic risk factor independent of protein C deficiency, protein S deficiency, high levels of factor VIII, antiphospholipid antibodies and hyperhomocysteinemia.

Another limitation of the study is that women with any previous symptomatic superficial or DVT in their personal or first-degree family history were excluded from the study. It is possible that by excluding these patients, a significant number of whom may be positive for classic rare constitutional thrombophilias, the C46T polymorphism of the F12 gene emerges as a significant risk factor, which would not be found to be independent if studied in the whole cohort of women. Our results are thus strictly limited to previously clinically asymptomatic young women in their first pregnancy.

Homozygosity for C46T polymorphism of the F12 gene is a frequent finding in our population of women: the observed incidence of the marker may be slightly higher than described in male and female Spanish controls (3.05% vs. 2% [2]). Its frequency is however strikingly lower than has been reported in different Caucasian populations from central and Western Europe: 7% in 100 Austrian newborns [12], 8.3% in 990 Scottish men [13], 6.4% in 471 Dutch people [14] and 6.4% in 342 Austrian adults [15]. Together with the results obtained in the Spanish population, our relative low incidence of homozygosity for the C46T polymorphism of the F12 gene may reflect the genotypic distribution in the Caucasian population from the Mediterranean region.

We show that homozygosity, but not heterozygosity, is a risk factor for a first DVT of the lower limb during the first pregnancy even in the absence of a family history of thrombosis. Homozygosity for the C46T polymorphism of the F12 gene is, however, a weak absolute risk factor in the situation in which we have studied it, isolated positivity being associated with one thrombotic event out of 235 exposed patients (upper limit of the 95% confidence interval: 1/119). This is half of that

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associated with isolated heterozygosity for F2G20210A or F5G1691A polymorphisms (respective upper limits: 1 out of 51 and 1 out of 49 exposed women). Even although this mean absolute risk is thirteen times higher than in patients with none of the three polymorphisms considered (1/3074), its isolated positivity and even isolated positivity of heterozygosity for the F2G20210A or F5G1691A polymorphisms cannot, *per se*, justify giving thromboprophylactic drug therapy throughout pregnancy and the purperium to previously asymptomatic women with no other predisposing clinical factors. Assuming the mean frequency of pregnancy-related symptomatic venous thromboembolism to be approximately 1/1000, we can calculate from published data [3] that the absolute risks of symptomatic pregnancy-related venous thromboembolism are 0.9% (0.5–1.7%) and 1.5% (0.4–5.3%) respectively in asymptomatic carriers of the FV gene (F5) Leiden or prothrombin G20210A polymorphisms. These intervals overlap with our results.

The combination of homozygosity for F12C46T with heterozygosity for F5G1691A was found in 32 women and was associated with one DVT during pregnancy: it approximately tripled the absolute risk associated with the isolated Leiden polymorphism; mean absolute estimates giving 3.1 DVT for 100 women followed during pregnancy and puerperium [upper limit of the 95% confidence interval (CI): 9.2 DVT for 100 women, i.e. 1 in 11 women]. The benefit of thromboprophylaxis should probably be assessed in these women. No association of homozygosity for the F12C46T with heterozygosity for F2G20210A was found in our population.

Homozygosity for the F12C46T was more prevalent than heterozygosity for F5G1691A or F2G20210A in our Mediterranean Caucasian population. The probability of being positive for this homozygous polymorphism, when another risk factor for venous thrombosis is present, is consequently one of the highest. It would appear justifiable to recommend screening for the C46T polymorphism of the F12 gene in pregnant women with familial thrombophilia: a positive result may significantly increase the clinical risk associated with a known familial thrombophilia, as described above for women who are heterozygous for F5G191A. Future prospective studies are needed to analyse the impact of homozygosity for the F12C46T polymorphism on the clinical phenotype of classic familial thrombophilias: demonstration of an adverse influence may help in the decision to offer primary prevention during pregnancy, with, for example, prophylactic low-molecular-weight heparins (LMWH) to asymptomatic women.

The incidence of DVT was strikingly high in homozygous F2G20210A or F5G1691A women. Despite the small number of these women in our population (3 and 12, respectively), this should prompt us to give thromboprophylaxis throughout pregnancy and the puerperium. However, studies in F5G1691A homozygous women have shown conflicting results about pregnancy-related VTE [3,16–22] and various approaches have been proposed, from heparin prophylaxis throughout and after pregnancy to anticoagulant prophylaxis restricted to the postpartum period.

Apart from RRs, a symptomatic VTE occurred during the first pregnancy and puerperium in 1 out of 697 to 1387 women. This is consistent with available published data using objectively confirmed diagnoses [23–32]. We confirm that DVT occurs during pregnancy at a similar incidence in each of the three trimesters, mainly affects the left side [33,34] and that symptomatic antenatal DVT is more common than symptomatic postpartum DVT [34,35]. The daily risk of VTE during the 6-week postpartum period is three times that in the antenatal period. This estimate is also consistent with published figures [34]. Unlike our own findings, data recently published on the risk of first lifetime DVT or PE, between 1965 and 1995 in the cohort of Olmsted County residents, USA [32] reported a higher absolute number of cases of DVT or PE during the postpartum period than during pregnancy itself. In this case, the postpartum period was defined as delivery of a newborn within 3 months before VTE, compared with only 6 weeks in our study: however, this difference is unlikely to explain the discrepancy, most of the thrombotic events having occurred during the first 6 postpartum weeks. Despite the absence of real knowledge of the corresponding benefit/risk ratio, our local widely accepted practise of using prophylactic low-molecular heparin treatments in clinical situations known to increase the VTE risk, and use of above-knee graduated compression stockings, may partly explain our low relative incidence of VTE among postpartum women. Similar numbers of proximal and distal symptomatic DVT were found, whereas most cases during pregnancy are usually described as being iliofemoral rather than calf vein thrombosis [35]. This may have been because of prewarning the women about the clinical signs of DVT: many distal thrombi are not necessarily symptomatic and might otherwise have been ignored or assigned as transient pregnancy-related cramps or neuralgia.

Maternal age and body mass index were risk factors: a body mass index value over 25 kg m^{-1} [2] has previously been identified as a risk factor during pregnancy in a large British cohort and case–control study [31]. Women aged 35 and older appeared to be at increased risk in a recent American healthcare research study [36]. Cesarean section is classically associated with venous thrombosis [37], although this was not found in our study, probably because of prophylaxis with LMWH and graduated compression stockings during the postoperative period.

The C46T homozygous polymorphism of the F12 gene is common in Caucasian populations from the Mediterranean region and acts as a risk factor for a first thromboembolic event during pregnancy.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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ORIGINAL ARTICLE

ABO blood groups and risk of venous thromboembolism during pregnancy and the puerperium. A population-based, nested case–control study

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Summary. *Objectives:* To examine possible associations of ABO blood types with the risk of venous thromboembolism (VTE) in pregnancy and the puerperium. *Patients and methods:* We conducted a nested case–control study within a cohort of 71 729 women who gave birth to 126 783 children in the North Jutland County, Denmark, from 1980 to 2001. We identified 129 cases with VTE in pregnancy ($n = 61$) or the puerperium ($n = 68$), and 258 controls with no VTE. We collected information on ABO blood groups and possible maternal confounding factors and estimated the relative risk [odds ratio (OR)]. *Results:* Women with an A or AB blood group had elevated risk estimates of VTE in pregnancy or the puerperium compared with women with a O blood group [adjusted ORs 2.4, 95% confidence interval (CI) 1.3, 4.3, and 2.0, 95% CI 0.7, 5.8, respectively]. No increased risk estimate was found for group B (adjusted OR 1.2, 95% CI 0.5, 3.0). The increased risk estimates of VTE for blood groups A and AB appeared present in both pregnancy (adjusted ORs of 3.9, 95% CI 1.5, 9.7, and 2.2, 95% CI 0.4, 12.5) and in the puerperium (adjusted ORs of 2.4, 95% CI 1.0, 4.9 and 2.7, 95% CI 0.8, 9.3). Furthermore, blood groups A and AB appeared to be associated with increased risk estimates for both DVT and pulmonary embolism. *Conclusion:* Keeping the modest statistical precision of our study in mind, blood groups A and AB may be associated with increased risk estimates for VTE in pregnancy and the puerperium.

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Introduction

Venous thromboembolism (VTE), is a rare but serious complication of pregnancy or the puerperium, and pulmonary embolism (PE) is the most common cause of maternal death in the Western world [1,2].

Risk factors for VTE during pregnancy and the puerperium include a personal or family history of VTE [3], obesity, diabetes mellitus [4,5], and hereditary or acquired hypercoagulable disorders [1,4,6–8].

In 1969 Jick and colleagues reported an association between ABO blood groups and VTE [9]. This study was a cooperative case–control study with patients from the UK, Sweden and the USA. The study groups were characterized by marked heterogeneity regarding gender, pregnancy, and age. The data were assembled from drug-surveillance programs, retrospective record searches, questionnaires, and information from the Committee on Safety of Drugs in United Kingdom. The relative risk of VTE was 1.9 [95% confidence interval (CI) 1.1, 3.2] when comparing subjects with non-O blood groups with subjects with blood group O. The strongest association, however, was found in a subgroup ($N = 166$) of pregnant women, among whom subjects with non-O blood groups had a relative risk of 2.7 (95% CI 1.5, 3.1) compared with those with blood group O. A similar British study conducted in the early 1970s [10] found in a subgroup of pregnant women a relative risk of 1.7 (95% CI 1.1, 2.6). Given the limitations of the existing studies, the extent to which ABO blood groups are associated with the risk of VTE in pregnancy and the puerperium is still uncertain. Therefore, we conducted a population-based nested case–control study among pregnant women in the North Jutland County, Denmark. Our specific purpose was to examine ABO blood groups as risk factors for VTE, not

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only in pregnancy but also during the puerperium, a high-risk period for VTE [3].

Materials and methods

Study design and population

This study is a nested case–control study within a cohort of 71 729 women and 124 833 deliveries, of which 123 007 (98.5%) were singleton deliveries. In total, the women had given birth to 126 783 children (including 102 still births) in North Jutland County (about half a million residents), Denmark during the period from 1 January 1980 to 31 December 2001.

Identification of VTE cases

We identified the VTE cases through a two-step approach. First, all possible cases, i.e. women registered with a VTE discharge diagnosis during pregnancy or puerperium, were drawn from the population-based regional Hospital Discharge Registry of North Jutland County. Second, we retrieved the medical records of the possible cases in order to verify the diagnosis.

The Hospital Discharge Registry holds information on all patients discharged from non-psychiatric hospitals in the county since 1977. We did not include VTE patients from the period 1 January 1977 to 31 December 1979 in order to sort out prevalent VTE cases, i.e. patients who may have suffered a previous VTE. The International Classification of Diseases 8th revision (ICD-8) was used during the period from 1977 to 1993, and the 10th revision (IDC-10) was used thereafter. ICD-9 has never been used in Denmark. All discharge diagnoses were exclusively assigned by the physicians who discharged the patients. The data include the civil registry number, unique to every Danish citizen, the dates of admission and discharge, the surgical procedures performed, and up to 20 discharge diagnoses [11]. Using the personal registration number, a complete hospital discharge history can be established for each individual, and linkage between population-based registries can be performed [12].

We identified all births of which the women during the study period were residents in North Jutland County and registered with an incident diagnosis of DVT or PE (ICD-8: 450.00–451.99, 650–666; ICD-10: I26.0–I26.9, I80.0–I80.9, O80–O84) during pregnancy or the puerperium, defined as a period of 42 weeks preceding the index birth and 8 weeks postpartum. We included the ICD codes of superficial phlebitis to screen for possible miscoded events.

Data collection and hospital record review criteria for VTE diagnoses

Medical records and hospital discharge summaries were retrieved and reviewed for the possible VTE cases using a standardized form. The records could be retrieved for 302/311 (97.1%) of the cases. The records were reviewed by three physicians who were experienced with VTE and pregnancy (T.B.L., C.I.M., and H.L.).

We collected detailed information about the pregnancy and the puerperium for each case including baseline characteristics at the initial birth control, data on pregnancy and birth complications, and data for verifying the VTE diagnosis. Data on the mother were mainly collected from antenatal records used by midwives and general practitioners.

A diagnosis of deep venous thrombosis (DVT) was considered verified when both typical clinical symptoms and the venography or ultrasonography findings (stated in the medical records) were in agreement with DVT. PE was defined as clinical symptoms of PE supported by findings from a pulmonary angiography, a ventilation-perfusion lung scan, a computed tomography, or an autopsy [13,14]. Finally, a diagnosis of superficial thrombophlebitis was considered confirmed when a physician clearly observed and stated this finding in writing in the medical record. The actual imaging films were not re-interpreted. All cases with an uncertain diagnosis based on the available information were discussed until a consensus was reached. All records were checked for any notion of a previous VTE event during pregnancy or puerperium. If so, the patients were excluded. Only verified cases of DVT or PE were included as cases in the study, i.e. patients with a verified diagnosis of superficial thrombophlebitis were not considered as cases.

Identification of controls

We selected two controls for each case, using the Hospital Discharge Registry. The controls were residents from North Jutland County, matched on the date of delivery for the case, who had not been registered with a discharge diagnosis of a first episode of VTE during pregnancy or the puerperium. Medical records for the controls were retrieved and reviewed in the same way as the medical records of the cases.

ABO blood groups and possible confounding factors

Data about blood groups were obtained from the blood transfusion sheets included in the medical records. Information regarding possible confounding factors was obtained from the medical records, using the standardized review form.

The possible confounding factors to be examined in this study included: age (< 25, 25–35, and > 35 years), smoking (yes, no), parity (primi- or multiparity), *in vitro* fertilization (IVF) pregnancy or preceding hormone stimulation with clomiphene citrate, history of diabetes mellitus, and body mass index (BMI) (< 25, 26–30, and > 30). A number of other possible confounding factors were also considered, including preeclampsia and sectio; however, none of these had any influence on the risk estimates. Information on clomiphene citrate use from the medical records was supplemented with data from the population-based Pharmaco-Epidemiologic Database of North Jutland in order to maximize the sensitivity

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Ref. 6

of the variable. This database, which was initiated in 1989, has complete coverage from 1 January 1991. It retains key information on prescriptions dispensed from all pharmacies outside hospitals in the county. The information includes the unique personal identification number of the customer, type of drug prescribed, date of prescription, tablet size, number of tablets in the package and number of packages. Using the personal identification number, a complete prescription history can be established for each individual, and unambiguous linkages with other registers can be performed. The study was approved by the Danish Registry Board (J. no. 2002-41-1993).

Statistical analysis

We first constructed contingency tables for case or control status, ABO blood groups and possible confounding factors. The absolute rate of VTE according to blood group was estimated based upon the assumption that the distribution of blood groups in the control group was representative for the entire study population. Risk estimates [odds ratios (ORs)] adjusted for possible confounding factors were estimated by logistic regression. Separate analyses were carried out for VTE events during pregnancy and puerperium, respectively, and for DVT and PE, respectively. For all analyses, 95% CIs were estimated. Data were analyzed using SAS Version 8.02 (SAS Institute, Cary, NC, USA).

Results

We identified 129 verified cases of VTE and 258 controls during the study period. Of the VTE events, 61 (47.3%) were observed during pregnancy and 68 (52.7%) were postpartum events (Table 1). Data on all variables were available for 327/387 (84.5%) of the cases and controls.

Data on blood groups were missing for three cases and three controls. Ninety-two of 126 cases (73.0%) with a hospital discharge diagnosis of VTE had a blood group A, B, or AB (non-O), compared with 153 of the 255 controls (60.0%). The absolute rate of first-time VTE events during pregnancy and puerperium per 1000 births for blood groups A, AB, B and O were 1.4 (95% CI 1.1, 1.8), 1.2 (95% CI 0.5, 2.3), 0.8 (95% CI 0.4, 1.3), and 0.7 (95% CI 0.5, 1.0), respectively.

Indications of a higher risk of VTE in pregnancy or the puerperium was seen when women with blood group A or AB were compared with women with blood group O, whereas no increased risk was found for women with blood group B (Table 2). This pattern remained after adjustment for possible confounding factors. Hence, the adjusted ORs for blood groups A and AB were 2.4 (95% CI 1.3, 4.3) and 2.0 (95% CI 0.7, 5.8), respectively, whereas the adjusted OR for group B was 1.2 (95% CI 0.5, 3.0).

Adjusted ORs of VTE according to blood group are shown separately in Table 3 for events occurring during pregnancy and the puerperium, respectively. No substantial differences were found.

Finally, blood groups A and AB appeared to be associated with an increased risk for both DVT (adjusted ORs 2.4, 95% CI 1.4, 4.2, and 2.2, 95% CI 0.8, 6.3) and PE (adjusted ORs 2.3, 95% CI 0.6, 8.7, and 2.5, 95% CI 0.2, 27.2), respectively.

Table 1 Descriptive characteristics of pregnancy-related venous thromboembolism cases and controls from North Jutland County, Denmark, 1980–2001

Characteristics	Cases ($n = 129$)	Controls ($n = 258$)
Blood groups (%)		
O	26.4	39.5
A	54.3	39.9
B	10.9	14.0
AB	6.2	5.4
Missing	2.3	1.2
Age, mean (min.–max.)	27.7 (16–40)	27.8 (18–40)
Body mass index	24.8 (17–57)	22.8 (15–40)
Active smokers (%)	50.5	28.9
Primipara (%)	45.1	33.9
Diabetes (%)	2.3	1.6
IVF pregnancy (%)	7.0	1.2

Table 2 Crude and adjusted odds ratios (OR) of venous thromboembolism according to ABO blood groups

Blood group	Cases ($n = 129$)	Controls ($n = 258$)	Crude OR (95% CI)	Adjusted OR* (95% CI)
O (reference)	34	102	1.0	1.0
A	70	103	1.9 (1.2, 3.1)	2.1 (1.1, 4.0)
B	14	36	1.1 (0.5, 2.3)	1.0 (0.4, 2.6)
AB	8	14	1.6 (0.6, 4.2)	2.5 (0.8, 7.8)

*Adjusted for age of mother, smoking, parity, clomiphene citrate stimulation, diabetes mellitus and body mass index.

Table 3 Adjusted odds ratios (OR) of venous thromboembolism according to A and AB blood groups during pregnancy and the puerperium

	Adjusted OR* (95% CI)	
Blood group	Pregnancy	Puerperium
O (reference)	1.0	1.0
A	3.9 (1.5, 9.7)	2.4 (1.0, 4.9)
B	1.5 (0.4, 5.5)	1.0 (0.3, 3.3)
AB	2.2 (0.4, 12.5)	2.7 (0.8, 9.3)

*Adjusted for age of mother, smoking, parity, clomiphene citrate stimulation, diabetes mellitus and body mass index.

Discussion

We found indications of increased risk estimates for VTE among pregnant or puerperal women with an A or AB blood group compared with women with an O blood group. No association was found with blood group B. The apparent increased risk of VTE associated with blood groups A and AB appeared to be present in both pregnancy and in the puerperium and included both DVT and PE, although the

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CIs around several of the risk estimates were wide and included unity. Previous studies have also indicated a difference in VTE risk between non-O blood groups and O blood group [9,10,15,16], but have not provided data on specific blood groups in relation to VTE in pregnancy.

The strengths of our study include the population-based design and the use of prospectively recorded data. This reduces the risk of selection and information biases in the study. All medical records were retrieved and reviewed, but only cases fulfilling objective criteria for VTE were included in the study. Limitations include the fact that we relied on routine registered hospital discharge codes for the identification of possible cases. Thus, we had no possibility of identifying patients who had been insufficiently examined or recognizing errors in the diagnostic process or the coding of the discharge diagnosis. However, we have no reason to believe that these errors should be associated with blood group and therefore do not expect them to have substantially biased our findings. Efforts were made to ensure that only incident VTE cases were included by checking the hospital discharge history and medical records; however, we cannot exclude the possibility that some of the cases may have had a previous VTE event, in particular, if the event did not lead to hospitalization. Furthermore, we had no biological specimens for an investigation of possible interactions from other genetic risk factors. This would be of special interest in relation to the factor (F)V Leiden mutation, which is seen among almost 7% of Danes [17] and has been associated with the risk of VTE in pregnancy and the puerperium [3,18,19]. Thus, a higher prevalence of non-O blood groups has been reported in non-pregnant symptomatic carriers of FV Leiden, compared with non-pregnant asymptomatic carriers (OR 3.9, 95% CI 1.7, 8.8) [20]. Furthermore, recent findings from the Leiden Thrombophilia Study indicate that FV Leiden increases substantially the risk of thrombosis associated with a non-O blood group [16]. This calls for more studies on the effect of ABO blood groups on common genetic risk factors, also in relation to pregnancy and the puerperium. Finally, it should be noted that although a relatively large number of cases were included compared with previous case–control studies [9,10,15], our CIs for several of the estimates were wide and included unity.

A causal relationship between non-O blood groups and VTE among pregnant women is supported by a number of biochemical and genetic observations in patients with VTE [16,20,21]. In the Leiden Thrombophilia Study, the significance of ABO blood groups, von Willebrand factor (VWF), and clotting factor (F)VIII in relation to thrombosis was studied in 301 consecutive patients with VTE compared with 301 healthy age- and sex-matched controls [20]. Non-O blood groups, high concentrations of VWF, and FVIII were all related to DVT. The findings pointed to an effect on thrombosis risk from higher concentrations of VWF and presence of a non-O blood group, the former fully, and the latter partly, mediated through high FVIII levels. Others have found that ABO genotyping could distinguish A alleles from B alleles, in terms of higher VWF antigen concentration in the A group [22]. O alleles had a significantly lower VWF antigen concentration, but the exact mechanisms involved remain unknown. Independently of which mechanisms may be responsible for the increased VTE risk associated with blood groups A and AB, it is interesting to note that the relative risk among women in pregnancy and in puerperium in our study was not higher than the relative risk reported among non-pregnant populations, despite the potential interaction of non-O blood groups with acquired activated protein C resistance, as well as FVIIIc/VWF levels or FV Leiden in pregnancy [22].

In conclusion, women with a blood group A or AB appeared to have a 2-fold increased risk for VTE during pregnancy and the puerperium compared with women with blood group O, whereas no increased risk was found for women with blood group B. These findings are in accordance with the risk estimates found in non-pregnant populations. The precise mechanism behind the association is as yet unknown, but since blood group A occurs in more than 40% of caucasians, information on ABO blood groups may prove useful for a combined risk assessment in high-risk pregnancies.

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Contribution of authors

T.B.L.: Study conception and design, data collection and hospital record review, data analysis and interpretation, paper writing. S.P.J.: Study conception and design, data analysis and interpretation, paper writing. M.G.: Study design, data interpretation, and critical revision. C.A.M., H.L.: Study design, data collection and hospital record review. H.T.S.: Study design, data interpretation, and critical revision.

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Ref. 7

Functional characterization of recombinant FV Hong Kong and FV Cambridge

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In factor V (FV) Cambridge (Arg306Thr) and Hong Kong (Arg306Gly), a cleavage site for anticoagulant activated protein C (APC), which is crucial for the inactivation of FVa, is lost. Although patients carrying FV Hong Kong have a normal APC response, those with FV Cambridge were reported to be APC resistant. To elucidate the molecular characteristics of the 2 FV mutants, we recreated them in a recombinant system and evaluated their functional properties. The 2 FV variants yielded identical APC resistance patterns, with APC responses being intermediate to those of wild-type FV and FV Leiden (Arg506Gln), which is known to be associated with the APC resistance phenotype. In the absence of protein S, APC mediated FVa inactivation curves obtained with the 2 variants were identical, resulting in partial FVa inactivation. In the presence of protein S, both FVa variants were almost completely inactivated because of protein S stimulation of the cleavage at Arg679. In a FVIIIa degradation system, both FV variants demonstrated slightly impaired APC cofactor activity. The ability of APC to cleave at Arg506 and at Arg679 in FVa Cambridge and Hong Kong and the slight decrease in APC cofactor activity of the 2 FV variants may explain the low thrombotic risk associated with these Arg306 mutations. In conclusion, we demonstrate that recombinant FV Cambridge and Hong Kong behave identically in in vitro assays and provide a mechanism for the low thrombotic risk associated with these FV mutations. (Blood. 2002;100:524-530)

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Introduction

Activated factor V (FVa) functions as a cofactor to factor Xa (FXa) in the inactivation of prothrombin, enhancing the rate of thrombin generation several thousand times. The procoagulant function of FVa is down-regulated by the anticoagulant serine protease activated protein C (APC). APC cleaves FVa at 3 sites—Arg306, Arg506, and Arg679—which results in the loss of FVa activity.[1,2] Cleavage at Arg506 is kinetically favored[1,3,4] but results in an inactivation intermediate with approximately 40% remaining procoagulant activity.[3] The slower Arg306 cleavage is required for full inactivation of FVa,[2] and this cleavage is enhanced by protein S.[4] Mutations at the position 306 site have been created in recombinant systems,[5,6] and studies of the recombinant FV variants demonstrate the importance of the Arg306 cleavage for full inactivation of FVa. Cleavage at Arg679 has not been studied in detail, and it is thought that this cleavage site is of minor physiological importance.

In addition to its role as a precursor of procoagulant FVa, circulating FV has an anticoagulant role functioning as a synergistic APC cofactor to protein S in the inactivation of FVIIIa.[7,8] The anticoagulant APC cofactor function of FV is lost on the procoagulant activation of FV, which is the result of FXa- or thrombin-mediated cleavages at Arg709, Arg1018, and Arg1545.[9] Cleavage at Arg1545, resulting in the dissociation of the B domain from the light chain, is responsible for the complete loss of anticoagulant activity, whereas the other 2 cleavages do not affect this activity. Proteolysis of Arg506 by APC in intact FV is associated with an increase in anticoagulant activity of FV.[10] Thus, proteolysis of FV is crucial for the modulation of FV activity in procoagulant and anticoagulant direction, and the relative ratio between the generation of thrombin and APC determines the direction.

APC resistance, which is characterized by a poor anticoagulant response to APC in plasma, is a major risk factor for venous thrombosis.[11,12] In 90% of patients, it is caused by a point mutation in FV that results in the replacement of Arg506 with Gln (FV Leiden).[12,13] This inhibits the efficiency by which APC degrades FVa; in addition, it impairs the APC cofactor function of FV in the degradation of FVIIIa because 506-cleavage is needed for the generation of full anticoagulant FV function.[10,14] These dual functions of the FV Leiden mutation result in the generation of a hypercoagulable state, which constitutes a lifelong risk factor for thrombosis.[12]

In 1998, 2 new mutations in FV, FV Hong Kong and FV Cambridge, were reported among patients with thrombosis.[15,16] Both mutations result in the replacement of Arg306, in FV Hong Kong with a Gly and in FV Cambridge with a Thr. FV Cambridge was identified in a thrombosis patient with unexplained APC resistance. In total, the study involved 602 patients with venous thrombosis from which 17 patients were selected because of low APC resistance ratios unrelated to FV Leiden. The FV Cambridge mutation was found in a patient and his mother, both of whom were heterozygous for the mutation and had subnormal APC resistance ratios. In contrast, FV Hong Kong has not been associated with APC resistance. The FV Hong Kong mutation was found in 2 of 43 Chinese patients with venous thrombosis and in 1 of 40 controls. Subsequent studies have confirmed a high prevalence (4.7%) of FV

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Hong Kong among Hong Kong Chinese but failed to demonstrate it as a risk factor for thrombosis.[17] Several other studies report low prevalence of the 2 mutations and have failed to associate them with increased risk for thrombosis.[17-22]

The present investigation aims at elucidating whether there are any functional differences between FV Cambridge and FV Hong Kong. In addition, we wanted to determine the biochemical background for the differences in risk for thrombosis associated with FV Leiden on the one hand and FV Cambridge and FV Hong Kong on the other. A series of recombinant FV variants with mutations in APC cleavage sites were created and studied in a panel of functional assays. In all assays, FV Cambridge and FV Hong Kong behaved indistinguishably, demonstrating subnormal APC ratios when tested in an APC resistance test using FV-deficient plasma. The APC-mediated FVa inactivation of the two 306 mutants was impaired but was stimulated by the APC cofactor protein S to such an extent that almost complete FVa inactivation was obtained. The APC cofactor activities of FV Cambridge and FV Hong Kong were subnormal but were considerably higher than those of FV Leiden.

Materials and methods

Materials

Restriction enzymes *Bsu*36I, *Bsp*EI, and *Xcm*I were from New England Biolabs (Beverly, MA), and *Bln*I was from Boehringer (Mannheim, Germany). All oligonucleotides were from DNA Technologies (Aarhus, Denmark). Big Dye Terminator sequencing kit was from Perkin Elmer (Warrington, United Kingdom). BioTrace polyvinylidene fluoride (PVDF) membrane was from Pall Corporation (Ann Arbor, MI). Chromogenic substrates S2366, S2222, and S2238 and Coatest APC resistance kits were kindly provided by Chromogenix (Milan, Italy). Russel viper venom (RVV-V) was purified as described.[23] Human FXa, human prothrombin, human protein S, bovine FX, bovine FIXa (beta) and Pefabloc were from Kordia (Leiden, The Netherlands). α-Thrombin was from Haematologics (Essex Junction, VT). Human FVIII (Octanative) was from Pharmacia (Uppsala, Sweden), and hirudin was from Sigma (St Louis, MO). Human FV was essentially purified from plasma as described[24] with minor modifications.[25] Recombinant human APC was prepared as described,[26] and its concentration was determined by chromogenic substrate S2366. A mouse monoclonal antibody against human factor V (HV-1), ovalbumin, and bovine serum albumin were obtained from Sigma. A polyclonal antibody (A299) against FV was from DAKO (Glostrup, Denmark). Monoclonal antibody AHV-5146 against the heavy chain of FV was from Haematologics. Monoclonal antibody Mk30 against the B-domain of FV was raised as previously described.[7] Phosphatidylserine (PS, brain extract), phosphatidylethanolamine (PE, egg extract) and phosphatidylcholine (PC, egg extract) were purchased from Avanti Polar Lipids (Alabaster, AL).

Phospholipid vesicle preparation

Phospholipid stocks were dissolved in 10/90 methanol–chloroform solution, and the concentrations were determined by phosphate analysis.[27] Mixtures of the lipids were prepared in 10/90 methanol–chloroform and kept at −20°C. Aliquots were drawn from the stocks and dried under N_2 and were then resuspended in HEPES buffer at room temperature. Phospholipids were sonicated in an XL 2020 sonicator (Misonix, Farmingdale, NY) at amplitude 3 for 10 minutes. Fresh phospholipid vesicles were prepared every day.

Mutagenesis

Full-length cDNA of human FV was located in the expression vector, pMT2.[28] Two FV variants, 306Q and 506Q, have been described.[10] To construct 306Q/679Q and 506Q/679Q, the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used with cDNA for 306Q and 506Q, respectively, as templates. Two complementary oligonucleotides were used as mutagenesis primers. The sense oligo was 5'GTCATGGCTACACAGAAAATGCATGATCGT-3' (underlined nucleotides resulting in the R679Q mutation). To ascertain that no other mutation had occurred during polymerase chain reaction, a 1.3-kb fragment containing the mutation was isolated after cleavage with restriction enzymes *Bsp*EI and *Bsu*36I. This fragment was inserted into either 306Q or 506Q FV cDNA that had been cleaved with the same enzymes, and the whole 1.3-kb fragment was sequenced. To construct 306Q/506Q/679Q, 306Q was combined with 506Q/679Q by cleaving both vectors with *Bsp*EI and *Bsu*36I and then ligating the 506Q/679Q fragment into the cleaved 306Q vector. DNA was sequenced at the 3 mutated sites to ascertain their presence.

The 306G and 306T were constructed using wild-type FV cDNA as template. Sense oligos for 306G and 306T were 5'-CCA AAG AAA ACC GGG AAT CTT AAG AAA ATA-3' and 5'-CCA AAG AAA ACC ACG AAT CTT AAG AAA ATA-3', respectively. DNA fragments encoding the 306G and 306T mutations were isolated after *Bsu*36I–*Bln*I and *Xcm*I–*Bsu*36I digestions, respectively, and introduced into wild-type cDNA cleaved with the same enzyme combination. Whole mutant inserts were sequenced.

Expression and quantification of recombinant factor V variants

Recombinant proteins were transiently expressed in Cos-1 cells using the diethyl aminoethyl–dextran transfection method, as described.[28] The proteins were harvested in serum-free medium (Optimem; Gibco, Paisley, Scotland) and concentrated in Macrocep having a molecular weight cutoff of 100 000 (Pall Gelman). Aliquots were frozen at −80°C. Concentrations of the recombinant proteins were determined with enzyme-linked immunosorbent assay (ELISA) and prothrombinase assay. ELISA was performed essentially as described,[10] but samples were diluted in Tris-buffered saline–bovine serum albumin (TBS-BSA) with 10 mM benzamidine and 2 mM $CaCl_2$. Pooled normal citrated plasma was used to create the standard curve (diluted in TBS-BSA-benzamidine). The standard and the samples were incubated overnight at 4°C. As secondary antibody, 0.075 μg/mL biotinylated monoclonal against the light chain (HV-1) was used.

In the prothrombinase-based FVa assay, a prothrombinase (PTase) mixture containing 0.5 μM prothrombin and 50 μM phospholipid vesicles (10/90 wt/wt PS/PC) was prepared in 25 mM HEPES (4-(2-Hydroxyethyl-1)-1-piperazineethanesulfonic acid), 150 mM NaCl, 2 mM $CaCl_2$, pH 7.7, containing 0.5 mg/mL ovalbumin (HEPES,NaCl,ovalbumin [HNO]–buffer). FV was activated by thrombin (final concentration, 0.5 U/mL) at 37°C for 10 minutes. FXa (final concentration, 5 nM) and the FVa samples were added to the PTase mix, and after 2 minutes the prothrombin activation was stopped by 40-fold dilution in ice-cold EDTA buffer. The EDTA buffer contained 50 mM Tris, 100 mM NaCl, 20 mM EDTA, 1% polyethylene glycol6000 (PEG 6000), pH 7.9. The amount of thrombin formed was measured kinetically with a chromogenic substrate, S2238. Normal plasma diluted between 1/50 and 1/1600 was used as standard in the assay.

Western blot analysis of recombinant protein

Recombinant and plasma-derived human factor V (hFV) (2 μg/mL) were incubated with 0.05 to 2.25 U/mL thrombin for 30 minutes at 37°C. Intact and thrombin-cleaved FV were reduced, run in 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE),[29] and transferred to PVDF membranes. Two different antibodies were used, a monoclonal antibody (Mk30) against the B-domain and a polyclonal antibody (A299). To develop the Western blots, Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used according to the manufacturer's instructions.

To investigate the APC cleavage pattern of the recombinant proteins, FVa was incubated for 30 minutes at 37°C with 0.25 nM APC and 100 nM Protein S in the presence of 25 μM phospholipids (PS/PE/PC wt/wt/wt 20/20/60). Samples were run in 12% SDS-PAGE, transferred to PVDF membranes, and analyzed with AHV5146 (Haematologics), a monoclonal against the heavy chain.

Ref. 7

Table 1. Activity/antigen levels of the recombinant proteins

Recombinant protein	ELISA (μg/mL)	PTase assay (μg/mL)	Ratio Ptase/ELISA
WT	0.992	1.97	1.99
306G	2.68	4.5	1.95
306T	4.39	9.3	2.2
306Q	2.7	4.6	1.88
506Q	2.9	4.9	1.78
506Q/679Q	3.2	6.7	2
306Q/679Q	0.8	1.2	1.5
306Q/506Q/679Q	6.8	10.86	1.6

PTase assay and ELISA were performed as described in "Materials and methods," and a ratio between the results from the 2 assays was calculated.

Activation of recombinant FV by thrombin

Recombinant FV variants (final concentration, 0.4 nM) were incubated with increasing amounts of thrombin (0-0.3 U/mL) at 37°C for 10 minutes, and the FVa-activities were measured in the PTase assay. In this case, Pefabloc (1 μM), an inhibitor of thrombin, was included to avoid the activation of FV during the assay, and thrombin generation was only followed for 1 minute.

Inactivation of FVa by activated protein C

To study the inactivation of FVa, FV was incubated with thrombin (0.5 U/mL) for 10 minutes at 37°C in 25 mM HEPES, 150 mM NaCl, pH 7.7, with 5 mg/mL BSA and 5 mM $CaCl_2$ (HNBSACa). After the activation of FV (0.8 nM, corresponding to 0.04 U/mL, as final concentrations in the experiments) phospholipid vesicles (PS/PE/PC wt/wt/wt 10/20/70, a final concentration of 25 μM) was added, and a subsample was drawn from the mixture and diluted one fifth in ice-cold HNBSACa buffer. APC was subsequently added to a final concentration of 0.2 nM. At different time-points, samples were drawn from the inactivation mixture and diluted one fifth in ice-cold HNBSACa to stop the reaction. FVa activities in the diluted samples were then measured in the PTase assay for the remaining FVa activity.

APC resistance testing of plasma containing recombinant FV variants

APC sensitivity ratios were measured using Coatest APC Resistance kit (Chromogenix) in a KC-10 coagulation instrument (Amelung). Recombinant FV variants were added to FV-depleted plasma (Biopool, CA) to a final concentration of 2.8 nM. FV-deficient plasma was supplemented with 160 nM human protein S because we observed that some FV-deficient plasmas were low in protein S activity. The samples were run according to manufacturer's instructions. Results were expressed as ratios between clotting times measured in the presence and absence of APC. Recombinant FV variants were also activated with the FV activator from RVV-V before the APC resistance test. RVV-V is efficient in cleaving at Arg1545, which results in a total loss of anticoagulant APC cofactor activity. Mutants were incubated with RVV-V (final concentration, 0.5 μg/mL) at 37°C for 45 minutes and diluted to a concentration of 1.25 nM. The APC resistance test was run as above.

Ability of recombinant FV variants to correct APC resistance

Plasma from a patient with homozygous FV Leiden (506Q) (12 μL) was mixed with FV-deficient plasma (36 μL) and the recombinant FV variants (12 μL at a concentration of 14 nM). As a negative control, 12 μL TBS with 0.2% BSA was used instead of the recombinant FV. The APC resistance test was performed as above, and APC ratios were calculated.

APC cofactor activity of recombinant FV variants

APC cofactor activities of the recombinant FV variants were measured in a FVIIIa-degradation assay. APC (final concentration, 2 nM) and protein S (final concentration, 2.5 nM) were mixed with increasing concentrations of FV (0.1-0.9 nM) in total volumes of 45 μL. A FVIIIa–phospholipid–FIXa solution (R1) was prepared by mixing 50 mU/mL FVIII with 100 mU/mL bovine FIXa and 10 μg/mL phospholipid vesicles (PS/PE/PC wt/wt/wt 10/40/50) in a buffer containing 50 mM Tris, 150 mM NaCl (TBS), 10 mM $CaCl_2$, and 0.2% BSA, pH 7.5. FVIII was activated by the addition of thrombin (3 mU/mL), and the reaction was stopped by the addition of hirudin (final concentration, 8 mU/mL). R1 was prepared immediately before use because of the instability of FVIIIa. Sixty microliters R1 was added to the mixture of FV, APC, and protein S and incubated for 2.5 minutes at 37°C before the addition of 20 μL bFX (1.2 U/mL). After 6 minutes, 50 μL chromogenic substrate S2222 (2.5 mM) was added to measure the amount of FXa formed. The reaction was stopped after 6.5 minutes by the addition of 50 μL of 50% acetic acid. Absorbance was read at 405 nm.

Results

Expression of recombinant FV Cambridge and FV Hong Kong

FV cDNAs corresponding to FV Cambridge (306T) and FV Hong Kong (306G) were created by site-directed mutagenesis of wild-type (WT) FV cDNA, and the recombinant FV variants were expressed in Cos-1 cells. In addition, several other FV variants, such as 306Q, 506Q, 306Q/679Q, 506Q/679Q, and 306Q/506Q/679Q, were created and expressed. Expression levels, as measured by the PTase assay, were approximately 200 ng/mL for all FV variants (data not shown). FV antigen levels were determined using ELISA, and the ratios between activity and antigen levels were found to be approximately 2 for all the recombinant FV variants (Table 1). This is consistent with results on record.[30] In contrast, plasma-derived FV yielded a ratio of 1. To exclude that the cell culture medium interfered in the assays, plasma-purified FV was diluted in mock medium or in buffer. In both cases, the PTase/ELISA ratio for plasma-derived FV was approximately 1, indicating that the difference observed in analysis of recombinant protein was not caused by interference by the medium. Differences in glycosylation of recombinant and plasma-derived FV, which might affect the binding of FV to the antibodies in the ELISA, could be the explanation to the results obtained. In subsequent studies of the recombinant proteins, the concentrations obtained by the functional PTase assay were used. To ensure that activation of the FV variants was unaffected by the introduced mutations, FV variants were incubated with increasing amounts of thrombin for 10 minutes before they were tested in the PTase assay (data not shown). All the FV variants demonstrated similar sensitivity to thrombin, and they were activated to the same extent as WT FV.

To further characterize the recombinant FV variants, they were analyzed by Western blotting before and after activation by thrombin (Figure 1). In all the blots of nonactivated FV, a 300-kd

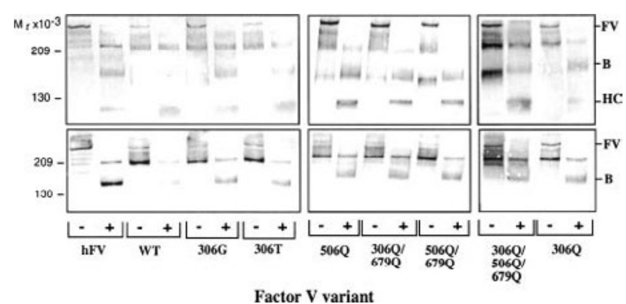

Figure 1. Western blot analysis of the recombinant FV variants. Concentrated conditioned medium from transfected Cos-1 cells (approximately 2 μg/mL FV) was incubated with 4.5 U/mL thrombin for 30 minutes at 37°C to activate the FV. Intact FV and thrombin-cleaved FV were analyzed on the Western blots (7.5% SDS-PAGE under reducing conditions). FV was detected using (top) a polyclonal antibody (A299) and (bottom) a monoclonal antibody (MK30) against the B-domain. Vectastain Elite ABC kit was used to develop the Western blots. (−) indicates intact FV; (+), thrombin-cleaved FV; HC, heavy chain; and B, B-domain.

Ref. 7

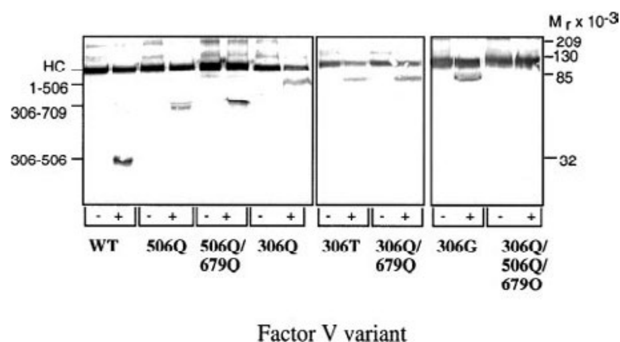

Figure 2. Western blot analysis of APC-cleaved recombinant FV variants. Concentrated conditioned medium from transfected Cos-1 cells (approximately 2 μg/mL FV) was incubated with thrombin for 30 minutes at 37°C to activate the FV. Phospholipids (PS/PE/PC wt/wt/wt 20/20/60) 25 μM was added together with 0.25 nM APC and 100 nM protein S (final concentrations) and incubated for 30 minutes. at 37°C. FVa variants were analyzed before and after APC cleavage on the Western blots (12% SDS-PAGE under reducing conditions) using a monoclonal antibody, AHV-5146, against the heavy chain. Vectastain Elite ABC kit was used to develop the Western blots. (−) indicates FVa without APC; (+), APC-cleaved FVa; HC, heavy chain.

band was detected that corresponded to the intact form of FV. In addition, a 200-kd band was observed that corresponded to the C-terminal half of FV that had been cleaved in the B domain.[31] In addition, the polyclonal FV antibody recognized a 150-kd band that corresponded to the N-terminal part of FV. On activation by thrombin, the 300-kd band disappeared and a 150-kd band appeared that corresponded to the B-domain fragment that was released by cleavages at R1018 and R1545. Furthermore, a band of approximately 100 kd, corresponding to the heavy chain, appeared.

Recombinant proteins were also incubated with APC and protein S and analyzed by Western blotting using a monoclonal antibody, AHV5146, against the heavy chain (Figure 2). APC cleavage of WT FVa yielded a 30-kd fragment that corresponded to the fragment generated by cleavages at R306 and R506. All FV variants mutated at position 306—that is, 306G, 306Q, 306T, and 306Q/679Q—yielded a 75-kd band. This fragment represented the N-terminal half of the heavy chain that had been cleaved at the R506 site. APC cleavage of the 506Q/679Q FVa variant gave rise to a 60-kd band that corresponded to the C-terminal part of the heavy chain, cleaved at position 306. After cleavage of 506Q FVa, this band was also observed together with a slightly smaller band that represented the 306-679 fragment. The 306Q/506Q/679Q variant, which is mutated at all the 3 cleavage sites, was unaffected by the incubation with APC.

APC resistance testing of FV Cambridge and FV Hong Kong

To elucidate whether recombinant FV Cambridge and FV Hong Kong behaved differently in the APC resistance test, recombinant FV variants were added to FV-deficient plasma, which was then used in the APC resistance test (Figure 3A). In this test, the activated partial thromboplastin time (APTT) was measured in the presence and absence of exogenous APC, and the APC ratio was calculated from the 2 clotting times. The APC ratio obtained in the presence of WT FV was approximately 1.9, whereas the recombinant FV Leiden (506Q) yielded an APC ratio of less than 1.1. A similarly low value was obtained with the 506Q/679Q variant. Three FV 306 variants (306G, 306T, and 306Q) yielded essentially identical APC ratios, which were intermediate to those obtained with WT and 506Q FV.

A poor APC response associated with mutations in FV may be caused by 2 different molecular mechanisms. One involves impaired APC-mediated inactivation of FVa, and the other is caused by deficient APC cofactor activity of FV in the degradation of FVIII.[10] To specifically investigate the effects on the degradation of FVa, the FV variants were activated before the APC resistance test with RVV-V (Figure 3B). The activation process results in loss of APC cofactor function of FV. When activated, all FV variants yielded APC ratios that were lower than those obtained with WT FV, and there were no significant differences between APC ratios of 506Q (FV Leiden) and the 3 FV 306 mutations.

In the first report on the anticoagulant function of FV, it was shown that the addition of normal FV to APC-resistant plasma resulted in a dose-dependent correction of the APC resistance.[7] This was thought to be at least partly due to the APC cofactor function of FV. To elucidate whether FV Cambridge and FV Hong Kong could also correct the APC resistance, recombinant FV variants were added to plasma from a person with homozygous FV Leiden (FV 506Q) (Figure 3C). WT FV was able to partially correct the APC resistance, whereas recombinant 506Q did not. All 3 recombinant FV 306 variants (306Q, 306G, and 306T) yielded partial corrections that were slightly less than those obtained with WT FV. These results suggested the FV 306 variants have partial APC cofactor activity.

APC cofactor activities of FV Cambridge and FV Hong Kong

To further elucidate whether the various FV variants were able to function as APC cofactors, they were tested in a FVIIIa degradation assay that specifically measured the APC cofactor activity of FV (Figure 4). In this assay, a preformed tenase complex—FVIIIa in complex with FIXa on the surfaces of phospholipid

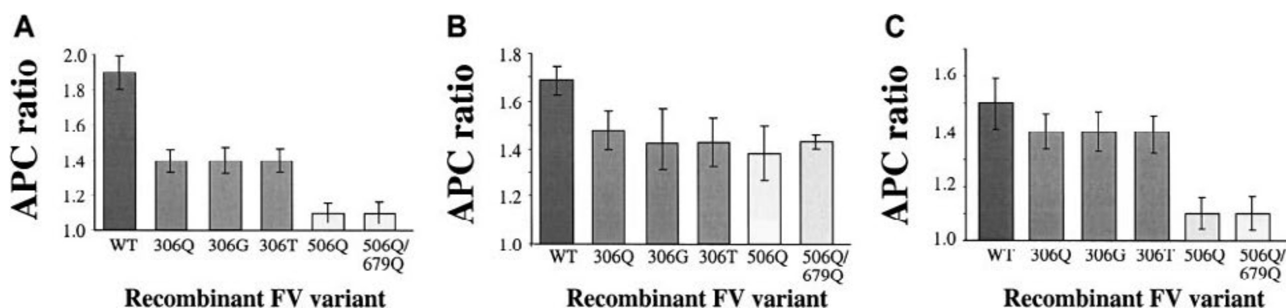

Figure 3. APC resistance testing of recombinant FV variants. (A) 14 nM FV was diluted 1:5 in FV-depleted plasma. Equal amounts of plasma and APTT reagent were mixed and incubated for 5 minutes. Clotting was started by addition of $CaCl_2$ with or without APC. A ratio was calculated between the clotting time in the presence and absence of APC. (B) FV was activated with RVV-V (final concentration, 0.5 μg/mL) at 37°C for 45 minutes and diluted in FV-deficient plasma to a concentration of 1.25 nM. APC resistance test was performed as described above. (C) Correction of APC resistance by recombinant FV. Plasma (12 μL) from a person with homozygosity for FV Leiden was mixed with 36 μL FV-deficient plasma and 12 μL recombinant FV (14 nM). As a negative control, 12 μL TBS with 0.2% BSA was used instead of the FV. APC resistance tests were performed as described above.

Ref. 7

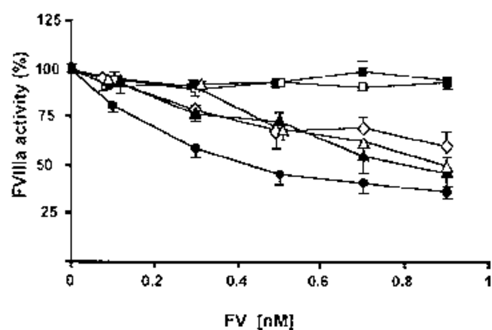

Figure 4. APC cofactor activities of FV variants as elucidated by FVIIIa degradation. Increasing amounts of FV (final concentration, 0.1-0.9 nM) were mixed with APC (final concentration, 2.0 nM) and protein S (final concentration, 2.5 nM). Preformed tenase complexes containing phospholipid-bound FIXa and FVIIIa were added, and the FVIIIa degradation was followed. (closed circle) WT FV; (closed triangle) FV 306G; (open triangle) FV 306T; (open diamond) FV 306Q; (open square) FV 506Q; (closed square) FV506Q/679Q.

membranes—was incubated with APC, protein S, and increasing amounts of FV for approximately 2 minutes. During this incubation, FVIIIa was degraded by APC, and the remaining FVIIIa activity was measured by the ability of the remaining tenase complexes to catalyze the activation of FX. The FXa generation was linearly related to the remaining FVIIIa activity. Although WT FV worked well as an APC cofactor, 506Q demonstrated no APC cofactor activity. The 3 FV 306 variants were able to support APC-mediated degradation of FVIIIa, but their APC cofactor activities were lower than those of WT FV. There were no significant differences among the 3 FV 306 variants.

Inactivation of FVa Cambridge and FVa Hong Kong by APC

APC-mediated inactivations of the activated FV variants were followed over time in the presence and the absence of protein S. Because the recombinant proteins were not purified before analysis, control experiments were performed to ensure that the medium did not interfere in the FVa degradation assay. Plasma-purified human FVa was diluted either in buffer or in the concentrated control medium under conditions that mimicked those used for the recombinant proteins and was subjected to APC-mediated inactivation. Inactivation curves obtained in buffer and medium conditions were indistinguishable (not shown), demonstrating that the cell expression medium did not interfere in the FVa degradation. The final FVa concentration in the experiment was approximately 0.8 nM (0.04 U/mL); exact values are detailed in Figures 5 and 6 legends. Under the experimental conditions used, the rate of FVa inactivation should be independent of the FVa concentration.[3] This was also found to be the case in control experiments using half the concentration of FVa.

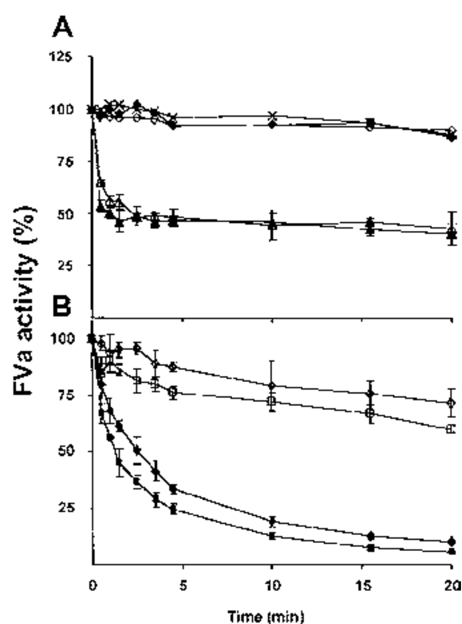

Figure 6. APC-mediated inactivation of FVa 306Q/679Q, FVa 506Q, and FVa 506Q/679Q. Inactivation assays were performed as described in legend to Figure 4, both in the absence (open symbols) and the presence (closed symbols) of 100 nM protein S. (A) (triangle) FVa 306Q/679Q; (diamond) FVa 306Q/506Q/679Q; (cross) control without APC. (B) (square) FVa 506Q; (diamond) FVa 506Q/679Q. The following final FVa concentrations in U/mL were used: 306Q/679Q, 0.040 ± 0.005; 306Q/506Q/679Q, 0.049 ± 0.003; WT, 0.040 ± 0.005; 506Q, 0.048 ± 0.003; and 506Q/679Q, 0.042 ± 0.0005. Plotted values represent the mean of 3 individual experiments; error bars represent SD.

Inactivation of WT FVa by APC alone demonstrated a biphasic reaction, with an initial rapid phase resulting in approximately 60% loss of FVa activity. This was followed by a slower inactivation phase that was not completed at the last time-point of 20 minutes (Figure 5A). In the presence of protein S, full inactivation of WT FVa was obtained within 5 minutes. In the APC-mediated inactivations of the 3 FVa 306 variants, the rapid phase was present, but the slow inactivation phases were absent (Figure 5B-D). Inactivation curves were essentially identical for the 3 FVa variants. In the presence of protein S, FVa-inactivation was more pronounced (Figure 5B-D), suggesting that protein S under these conditions functioned as an APC cofactor. Because APC could not cleave at the 306 site in these mutants, the protein S–dependent stimulation of FVa inactivation must have been caused by enhanced cleavage at R506 or R679. To examine whether the increased cleavage was caused by enhanced R506 cleavage, the FV variant 306Q/679Q, which can only be cleaved at R506, was inactivated under the same conditions (Figure 6A). There was no difference in the inactivation

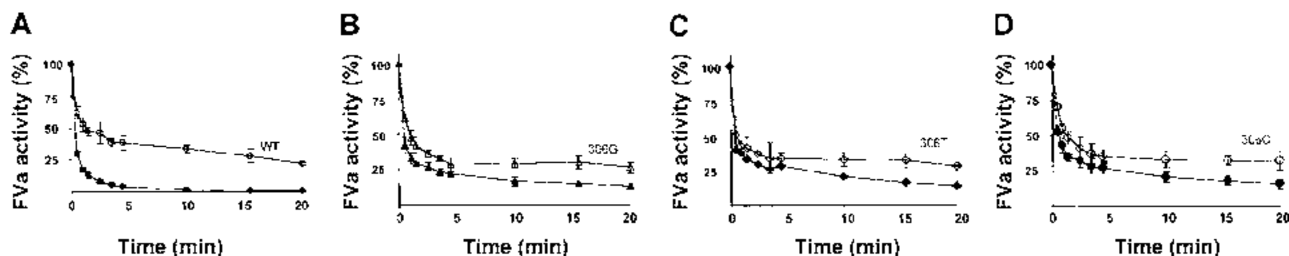

Figure 5. APC-mediated inactivation of FV 306 variants. FV (final concentration, 0.8 nM) was incubated with 0.5 U/mL thrombin for 10 minutes at 37°C. APC (final concentration, 0.2 nM) was added to the reaction mixtures, which also contained phospholipids (PS/PE/PC wt/wt/wt 10/20/70) at a final concentration of 25 μM. Experiments were performed in the absence (open symbols) and presence (closed symbols) of 100 nM protein S. At intervals, samples were drawn, and the FVa degradation was stopped by one fifth dilution in ice-cold HNBSACa. FVa activity was measured with the PTase assay. FVa activity was related to the activity observed before the addition of APC. (A) WT; (B) 306G; (C) 306T; (D) 306Q. The following final FVa concentrations in U/mL (mean ± SD) were used: WT, 0.04 ± 0.005; 306G, 0.042 ± 0.004; 306T, 0.033 ± 0.002; and 306Q, 0.039 ± 0.01. Plotted values represent the mean of 3 individual experiments; error bars represent SD.

Ref. 7

of the FVa variant 306Q/679Q in the presence and absence of protein S, indicating that the difference observed for the 3 FV 306 variants was not caused by increased cleavage at R506. APC-mediated inactivation of the FVa variant 506Q in the presence of protein S was significantly faster than the inactivation of 506Q/679Q ($P < .05$; time, 1-20 minutes), indicating cleavage at R679 to contribute to the APC-mediated inactivation of the 3 FV 306 variants (Figure 6B). In the absence of protein S, the inactivation of 506Q and 506Q/679Q did not significantly differ. To exclude that an as of yet unidentified cleavage site was the cause for the efficient cleavage of 306G and 306T, we also performed inactivation of 306Q/506Q/679Q (Figure 6A). No APC-mediated inactivation of this FVa variant was observed in the presence or absence of protein S.

Discussion

A single point mutation in the *FV* gene, resulting in the replacement of Arg506 with a Gln (FV Leiden), constitutes the most common risk factor for thrombosis. Loss of the APC cleavage site at position 506, which results in impaired FVa degradation and loss of APC cofactor activity of FV, is the molecular background.[2,10,12,14,32] APC-mediated cleavage at Arg506 only partially inactivates FVa, whereas cleavage at the Arg306 site is required for complete FV inactivation.[1,3] Therefore, it is easy to suspect that mutations resulting in the loss of the Arg306 site would cause a more severe thrombotic state than mutations affecting the Arg506 cleavage site. However, the contrary seems to be the case. Neither FV Cambridge (Thr replacing the Arg306) nor FV Hong Kong (Gly replacing Arg 306) appears to be associated with increased risk for thrombosis.[17,19] The FV Hong Kong mutation has been found with high prevalence among Hong Kong Chinese, whereas FV Cambridge seems to be rare. Clinical studies have suggested that the 2 phenotypes give different APC resistance ratios. Although persons with FV Hong Kong have normal APC ratios, those few reported with FV Cambridge have demonstrated APC resistance. That persons with FV Hong Kong have normal APC response has been confirmed in several studies, whereas few reports have evaluated the APC resistance pattern of FV Cambridge, which is explained by the low prevalence of this mutation.[17-22] So far, only heterozygous patients with FV Hong Kong or FV Cambridge have been found, which complicates the evaluation of the APC resistance pattern of the mutations.[15-22]

To characterize the FV Hong Kong and FV Cambridge variants, we created them in a recombinant system. Proteins were transiently expressed using a characterized eukaryotic system.[28] Recombinant FV variants were collected in serum-free medium and concentrated before they were used in the various experimental setups. To ensure that the conditioned medium did not influence the results of any assay, control experiments were performed in which plasma-derived FV was added to medium from mock-transfected cells. In none of the experiments did we find any influence of the medium on the behavior of plasma-derived FV/FVa. Recombinant proteins were characterized by Western blotting before and after activation by thrombin and yielded the expected cleavage patterns. Already at the time of collection of the medium, the recombinant FV variants were all partially cleaved at a site, which is close to Arg1018 in the B domain. This cleavage generated 2 fragments of 220 and 150 kd, which are noncovalently associated. Similar partially cleaved FV has been shown to be present in plasma-purified FV. This FV variant can be activated to FVa and is then fully active. Moreover, it also expresses full anticoagulant APC cofactor activity because it is not cleaved as Arg1545.[9] The APC cleavage pattern was also analyzed by Western blotting, and for all the mutants the expected patterns were yielded.

We found no difference in the APC resistance patterns of FV Cambridge and FV Hong Kong, and the APC resistance ratios obtained using these 2 FV variants were intermediate to those observed for WT FV and FV Leiden (506Q). So far only patients with heterozygosity for FV Cambridge or FV Hong Kong have been described, and they are expected to have approximately 50% of the plasma FV pool deriving from the normal FV allele. It is likely that the slightly decreased APC ratios associated with heterozygosity for one of the 2 mutations potentiates a poor APC response primarily caused by another factor. Using the original APC resistance test, it has been observed that approximately 5% of all patients with APC resistance do not have FV Leiden.[33] The presence of FV Cambridge or FV Hong Kong could contribute to the APC resistance phenotype even if they would not be the primary cause of it. The explanation for the apparent connection between FV Cambridge and APC resistance may be found in the design of the original study describing this mutation.[16] In a large cohort of thrombosis patients, a group of patients with unexplained APC resistance was further investigated. Among those, the FV Cambridge mutation was found. Thus, the patients were selected for unexplained APC resistance, and it was not proven that the FV Cambridge mutation was the cause of the low APC response. From our present results, one would suspect that persons with FV Cambridge as a group have slightly lower APC ratios than persons without any FV mutation. The mean APC ratio could be close to the cut-off limit for APC resistance.[16,34] Studies presented so far suggest that FV Cambridge and FV Hong Kong are in themselves not risk factors for thrombosis. However, the 2 mutations may increase the risk for thrombosis if combined with other genetic or acquired risk factors.[34]

In APC-mediated FVa-degradation, FVa Cambridge and FVa Hong Kong yielded essentially identical patterns. The rapid partial degradation resulting from the APC-mediated cleavage at Arg506 was unaffected by the mutations at position 306 and resulted in a partially active FVa, which in the absence of protein S was stable over the time course of the experiment. In contrast, in the presence of protein S an additional slow phase of APC mediated degradation was observed. This slow phase was absent in the FVa 306Q/679Q variant, indicating that the Arg679 cleavage was responsible for the slow phase of APC-mediated degradation of FVa Cambridge and FVa Hong Kong. Thus, it can be concluded that protein S is not only a cofactor for the cleavage of Arg306, but also for APC-mediated cleavage of the Arg679. The protein S stimulation of the Arg679 cleavage site was also apparent in the degradation of FVa Leiden (506Q), which in the presence of protein S was significantly faster cleaved than that of FVa 506Q/679Q. FVa, which was mutated at all 3 APC cleavage sites, was unaffected by APC both in the presence and absence of protein S, excluding additional cleavage sites to effect the results of the FVa-degradation experiment. The cleavage at Arg679 may significantly contribute to the inactivation of FVa Hong Kong and FVa Cambridge in vivo, which may be part of the explanation for why the 2 FV variants are not associated with increased risk of thrombosis.

A major difference between FV Leiden and FV Cambridge and FV Hong Kong was observed in the FVIIIa degradation experiment, which tests the anticoagulant cofactor function of FV.[8] FV Leiden was inefficient as an APC cofactor, whereas FV Cambridge

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and FV Hong Kong mutations only yielded slightly decreased APC cofactor activities. The results support the concept that the Arg506 cleavage is important for the expression of APC cofactor activity,[10] whereas cleavage at position 306 seems to be of minor importance. When fully activated, FV loses its anticoagulant activity concomitant with the cleavage at Arg1545, which results in the release of the B-domain from the light chain.[9] When the FV variants were activated before APC resistance testing, the difference between the 506Q and the 306 variants in the APC resistance test disappeared. The anticoagulant response in the APTT-based APC resistance test is dependent on the APC cofactor activity of FV, and the pronounced APC resistance associated with FV Leiden is at least partly caused by lost APC cofactor activity. The dose-dependent correction of APC resistance obtained when normal FV is added to APC resistant plasma from a homozygous person is most likely due to the substitution of APC cofactor activity. The ability of the FV 306 variants to express APC cofactor activity in a FVIIIa degradation assay is consistent with the capacity of the mutants to correct APC resistance when added to APC-resistant plasma.

In conclusion, no differences were observed between FV Cambridge and FV Hong Kong in any of the functional assays performed in this study. APC resistance ratios obtained with FV Cambridge and FV Hong Kong were intermediate to those obtained with FV Leiden and WT FV. Furthermore, the anticoagulant activities of FV Cambridge and FV Hong were not impaired to the extent they were for FV Leiden. In the presence of protein S, the APC-mediated inactivation of FVa Cambridge and FVa Hong Kong was efficient, indicating a role for the Arg679 cleavage in the FVa inactivation. Taken together with the abilities of FV Cambridge and FV Hong Kong to express anticoagulant APC cofactor function, this may explain why mutations at position 306 in FV do not result in prothrombotic and APC-resistant phenotypes.

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Ref. 8

Predicting the risk of thromboembolic disease by analyzing multiple genetic variants: incorporating risk profiles into clinical practice

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INTRODUCTION

Venous thromboembolism (VTE), i.e. Deep Vein Thrombosis (DVT) or pulmonary embolism (PE), is an important cause of mortality and morbidity in the developed world and the main preventable cause of mortality in hospitals[1]. Each year around 300.000 people in the United States die from acute PE, many cases of them being only diagnosed at autopsy[1]. Similarly, an European study estimates in 370.012 VTE-related deaths in 2004 in a group of 6 European countries[2]. DVT affects approximately two million Americans annually, while PE is the most common cause of preventable hospital death accounting for 60.000 deaths in the United States every year[3].

VTE has an important genetic component. In addition to the classic variants FV Leiden (FVL) and prothrombin G20210A (PT), currently determined in routine, there are new genetic variants associated with thrombosis but not yet added to the assessment of the genetic risk of thrombophilia[4,5,6]. Moreover, there are no interpretation protocols combining all thrombophilia genetic factors with the classic prothrombotic factors and there is not any commercial service suggesting therapeutic measures taking into account all these factors.

Given the high incidence and recurrence rates of VTE, a better identification of patients at risk of thrombosis might improve the preventive strategy and decrease the incidence of DVT and associated complications.

OBJECTIVES

The objective of this study was to determine whether the genetic variants included in THROMBOINCODE (genetic profile) improve the capacity of FVL and PT to predict the development of thrombosis.

METHODS

Two case-control studies of VTE were included:

- MARTHA[7]: 1,150 cases (347 males, 803 females; 38.0±13.9 years old) / 801 controls (383 males, 418 females; 47.4±14.0 years old) designed to assess the association of FV Leiden and Prothrombin with other risk factors.

- A study with Spanish population (SP): 249 cases (111 males, 138 females; 47.1±14.0 years old) / 248 controls (109 males, 139 females; 49.0±14.9 years old).

Genetic profile analyzed

- Genetic variants analyzed in this study are located in the following genes: FXII, ABO Group (A1 carriers), Serpin A10, Serpin C1, Factor V and PT.

Statistical Analysis

The association between variants and thrombosis was calculated using the OR adjusted for age and sex: [OR (95%)].

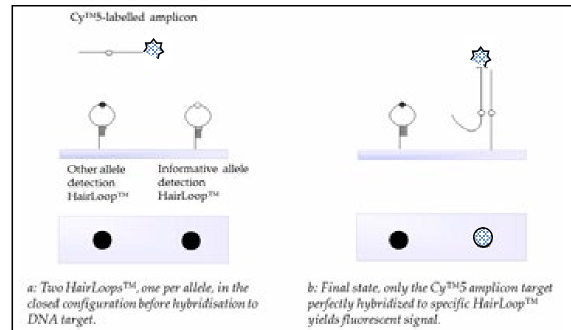
The predictive capacity was calculated using the c-statistic (AUC-ROC) observed when using the FVL, PT or the genetic profile.

The reclassification capacity was calculated using NRI (*net reclassification improvement*) and IDI (*integrated discrimination improvement*) observed when using the FVL, PT or the genetic profile.

Thrombo inCode

Thrombo inCode test kit is and IVD-CE marked kit for the simultaneous allele determination of 12 variants in 7 genes (PT, FV, FXII, FXIII, ABO, Serpin A10, and Serpin C1) associated with thrombosis in genomic DNA, extracted from either saliva or blood samples.

The calculated sensibility and specificity of the kif is ≥98% per SNP. The allele detection is performed by using HairLoop™ Technology.



a: Two HairLoops™, one per allele, in the closed configuration before hybridisation to DNA target.

b: Final state, only the Cy™5 amplicon target perfectly hybridized to specific HairLoop™ yields fluorescent signal.

RESULTS

Table 1: Association between variants and thrombosis [OR (95%)] and the proportion of FVL and PT carriers compared with carriers of the genetic profile assessed by THROMBOINCODE.

		FV Leiden	Prothrombin	ABO (A1)	FXII	Serpin C1	Serpin A10
MARTHA	OR	2.3	0.9	1.8	0.9	0.9	2.3
Cases	Carriers	50.4 %					
		87.5 % ⟵ THROMBOINCODE					
SP	OR	7.2	2.8	2.62	3.1	4.1	2.5
Cases	Carriers	19.7 %					
		71.5 % ⟵ THROMBOINCODE					

Table 2: c-statistic and reclassification [NRI (net reclassification improvement) and IDI (integrated discrimination improvement)] comparing the use of the genetic profile assessed by THROMBOINCODE to FVL and PT.

	c-statistic		NRI		IDI	
	MARTHA	SP	MARTHA	SP	MARTHA	SP
FV Leiden + Prothrombin	0.54	0.58	ref	ref	ref	ref
THROMBOINCODE	0.58	0.69	5.3	23.4	1	5.9
P-value	<0.001	<0.001	>0.05	<0.001	<0.05	<0.001

CONCLUSIONS

- The selected genetic profile included in THROMBOINCODE improves significantly the discrimination of VTE risk by identifying a genetic thrombophilia in **37.1-51.8%** of the subjects who developed VTE, but who had not showed genetic thrombophilia when analysing only FV Leiden and PT (Table 1).

- The predictive capacity of the FV Leiden and PT was significantly improved by the inclusion of the genetic panel (assessed using the c-statistic) both in MARTHA and the Spanish Population studies. Moreover, in the Spanish Population study, the use of the genetic profile improves in a very significant way the risk assessment done by the use of only FV Leiden and PT as evidenced by the very high values of both NRI and IDI. In the case of the MARTHA study, only IDI achieves statistical significance.

- THROMBOINCODE is the first IVD-CE commercial tool that integrates and automates in a single kit the detection of the most relevant genetic risk variants that significantly enhances our predictive capacity and will improve the prevention strategy for patients (and their relatives) who are at risk of developing thromboembolic events and also the diagnosis and the treatment of the thromboembolic disease.

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REVIEW ARTICLE

New developments in the area of factor XIII

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Summary. Coagulation factor (F)XIII is best known for its role in fibrin stabilization and cross-linking of antifibrinolytic proteins to the fibrin clot. From patients with congenital FXIII deficiency, it is known that FXIII also has important functions in wound healing and maintaining pregnancy. Over the last decade more and more research groups with different backgrounds have studied FXIII and have unveiled putative novel functions for FXIII. FXIII, with its unique role as a transglutaminase among the other serine protease coagulation factors, is now recognized as a multifunctional protein involved in regulatory mechanisms and construction and repair processes beyond hemostasis with possible implications in many areas of medicine. The aim of this review was to give an overview of exciting novel findings and to highlight the remarkable diversity of functions attributed to FXIII. Of course, more research into the underlying mechanisms and (patho-)physiological relevance of the many described functions of FXIII is needed. It will be exciting to observe future developments in this area and to see if and how these interesting findings may be translated into clinical practice in the future.

Keywords: bone metabolism, coagulation, factor XIII, inflammation, transglutaminase, wound healing.

Introduction

For a long time, coagulation factor (F)XIII has been known as a neglected coagulation factor at the 'less interesting' end of the clotting cascade beyond the diagnostically more relevant thrombin generation and fibrin polymerization. FXIII research underwent a revival when a role in cardio- and cerebrovascular diseases was first suggested [1–4]. Since then more and more research groups with different backgrounds have studied FXIII and thanks to their great work over the last decade, FXIII is now recognized as a multifunctional protein which is involved in many regulatory and construction and repair processes with possible implications in many areas of medicine. In this article, we aim to review many exciting new functions of a protein belonging to the family of transglutaminases, which exhibit fundamental biological reactions in most organisms and are therefore thought to have appeared early in the evolutionary history [5].

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Plasma FXIII (pFXIII) is a heterotetramer of two A and two B subunits (FXIII-A_2B_2). The A subunit (FXIII-A) contains the catalytic domain and the B subunit (FXIII-B) serves as carrier and regulatory protein. Figure 1 [6] shows the structure of the FXIII-A_2 homodimer. In pFXIII, the B subunits are thought to be wrapped around FXIII-A_2. pFXIII circulates in plasma at an average concentration of 21.6 μg mL^{-1} [8] and is non-covalently bound to fibrinogen. In plasma, all FXIII-A exists in complexed form, whereas there are free FXIII-B_2 homodimers present. In platelets and monocytes/macrophages, cellular FXIII (cFXIII) is present as FXIII-A_2. In plasma, thrombin initiates the physiological conversion of the zymogen into the active enzyme by cleavage of the activation peptide (AP-FXIII), consisting of amino acids 1–37, of FXIII-A resulting in FXIII-$A_2'B_2$. This reaction is greatly enhanced by polymerized fibrin. The dissociation of the A′ and B subunits is induced by conformational changes as a result of binding of Ca^{2+} and is again enhanced by fibrin. The free thiol group of the active site Cys314 is now exposed for the transglutaminase reaction to form a covalent bond between a peptide-bound glutamine residue and a peptide-bound lysine residue. Fig. 2 schematically shows the activation and action of pFXIII [9]. The best known function of pFXIII is clot stabilization during the hemostatic process: FXIII provides a mechanically stronger clot by cross-linking fibrin chains [10], and by incorporating antifibrinolytic proteins FXIII prevents the clot from premature degradation by the fibrinolytic system [11]. Many other established and emerging functions of FXIII and underlying mechanisms are discussed below and summarized in Figs. 3 and 4. An excellent review article by Muszbek *et al.* [12] gives a very detailed description of FXIII biochemistry and functions.

FXIII deficiency

Since the description of the first case of congenital FXIII deficiency in 1960 until the 1990s, congenital FXIII deficiency

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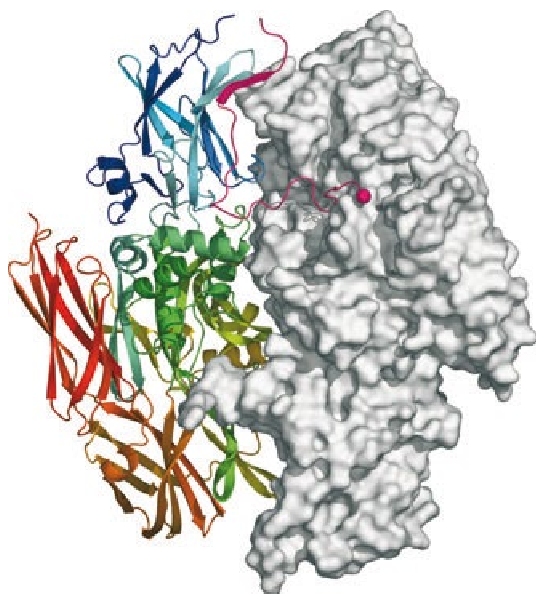

Fig. 1. Structure of the factor FXIII A_2 homodimer (Reproduced from [6] with permission). One monomer is shown in ribbons with the activation peptide colored in pink and the N-terminus indicated as a ball. The β-sandwich domain is colored in blue, the catalytic core domain in green, and the barrel 1 and barrel 2 domains in orange and red, respectively. The other monomer is represented as a surface. The coordinates from the protein database (PDB) originate from the crystal structure [7].

was the main area of research on FXIII. With more than 100 mutations described so far, occurring in all exons of the FXIII-A gene [13], we have learned that almost every affected family has their individual mutation. Contrary to hemophilia A, mutations in the FXIII-A gene usually lead to a complete absence of the FXIII-A protein, preventing clear genotype–phenotype correlations. However, the very first functional mutation was recently discovered at position 37, the thrombin cleavage site, leading to expression of a FXIII-A protein that cannot be cleaved by thrombin [14]. In spite of a raised awareness and availability of specific and sensitive assays, the diagnosis of FXIII deficiency is still often insufficient and the use of inappropriate assays may lead to missed diagnoses with fatal consequences even in developed countries [15]. Diagnosis should therefore follow the guidelines of the FXIII and Fibrinogen SSC Subcommittee of the ISTH [16]. The treatment of FXIII-A deficiency is undergoing important changes. The first recombinant product has proven to be safe and effective and will be available soon [17,18]. Although the old-fashioned dogma that 5% of FXIII plasma levels is enough for efficient hemostasis is unfortunately still believed by some clinicians, there is increasing evidence that patients with 'mild' FXIII deficiency as a result of congenital heterozygous deficiency, acquired deficiency owing to consumption or acquired deficiency because of autoantibodies experience bleeding

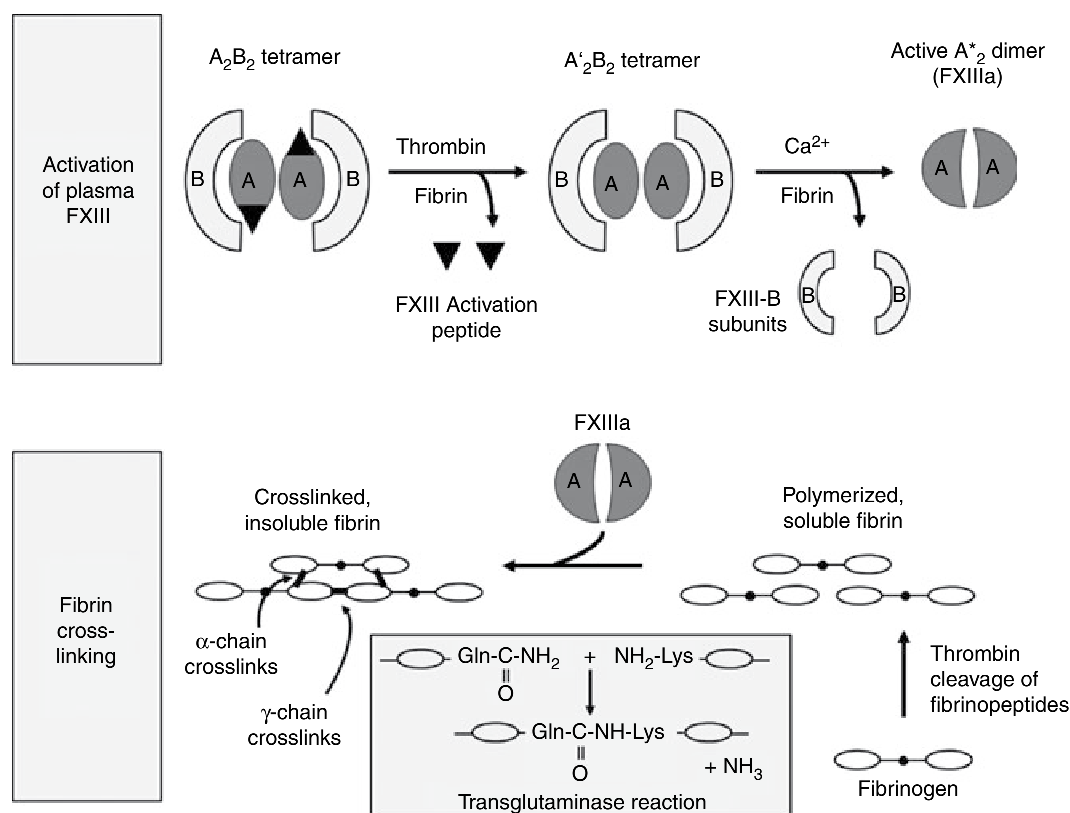

Fig. 2. Activation and action of plasma factor FXIII. Thrombin initiates FXIII activation by cleavage of the FXIII activation peptide. Then A- and B-subunits dissociate in the presence of Ca^{2+}. Both FXIII activation steps are enhanced by fibrinogen/fibrin. Thrombin also initiates conversion of fibrinogen into soluble fibrin by cleaving off fibrinopeptides A and B. Activated FXIII (FXIIIa) cross-links lysine (Lys) and glutamine (Gln) residues of fibrin α- and γ-chains in a transglutaminase reaction leading to a three-dimensional, insoluble fibrin network.

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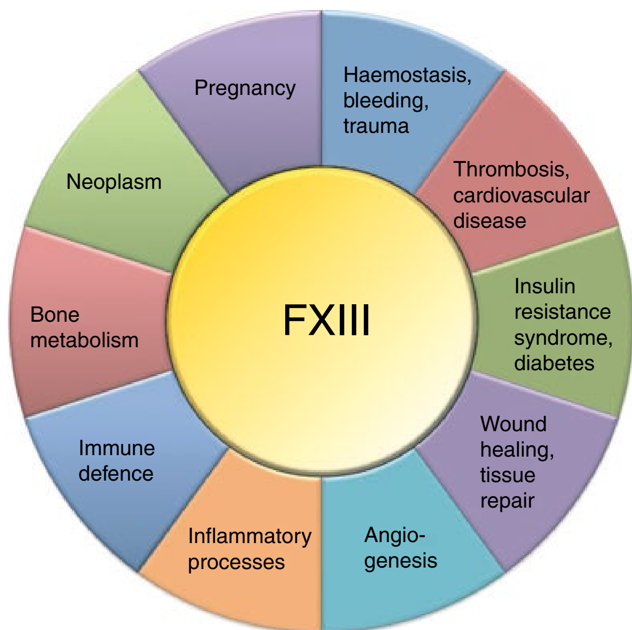

Fig. 3. Diversity of factor FXIII functions. As a result of its multiple functions, FXIII is involved in many different physiological and patho-physiological processes and hence FXIII is of interest in different areas of biology and medicine.

complications in situations such as trauma or surgery [13,19–21]. Data are collected from such cases in ongoing studies with the aim to improve diagnosis, treatment and ultimately the outcome in those patients.

FXIII and pregnancy

Women with congenital FXIII deficiency usually suffer pregnancy loss within the first trimester, if not treated with prophylactic FXIII substitution therapy [22,23]. In these patients, poor development of the cytotrophoblastic shell and fibrinoid layers, as a result of impaired protein cross-linking, at the interface of maternal and fetal tissue leads to premature detachment of the placenta [24]. Therefore, FXIII has an important function in maintaining pregnancy. However, it remained unknown whether FXIII plasma levels are altered in FXIII-competent women with unexplained recurrent pregnancy loss. In a first study in women with recurrent pregnancy loss, low FXIII levels did not predict subsequent miscarriages [25]. However, this study did not compare absolute FXIII levels between patients with and without a subsequent miscarriage, nor were FXIII levels compared with a control group of women without a history of recurrent miscarriage. We therefore measured FXIII-A and -B levels in women with

FXIII				
Fibrin formation & structure	Extracellular matrix formation	Intracellular functions	Interactions with inflammatory cells	Interactions with complement system
• Crosslinking of fibrin γ- and α-chains • Covalent binding of anti-fibrinolytic proteins (α_2-anti-plasmin, TAFI, PAI-2) to fibrin → Influence on fibrin fiber thickness and branching → Viscoelastic properties → Resistance against fibrinolysis	• Crosslinking of ECM proteins, e.g. fibronectin, collagen type-1, vitronectin, thrombospondin, osteopontin • Crosslinked ECM modulates cell ad-hesion & function → Development of cytrotrophoblastic shell and fibrinoid layers in pregnancy → Angiogenesis → Tissue remode-ling & wound healing → Endothelial barrier function	• Interactions with integrins & VEGFR • Up-regulation of several endothelial transcription factors • Down-regulation of TSP-1 → Increased endothelial cell proliferation, de-creased apoptosis → Angiogenesis • Enhanced microtubule dynamics → Osteoblast differentiation and protein secretion	• Recruitment of macrophages and resolution of neutrophil response → Improved myo-cardial healing • Dimerization of monocyte AT1 receptor → Monocyte activa-tion & entry into arte-rial wall promotes atherosclerosis • Neutrophil elasta-se from PMN leuko-cytes activates FXIII • Enhanced prolife-ration & migration of monocytes	• Covalent binding of complement C3 to fibrin • MASP-1 activates FXIII → Influences on fibrin structure and fibrinolysis → Interactions may contribute to prothrombotic state in inflammatory conditions

Fig. 4. Mechanisms and substrates of FXIII. As a transglutaminase, activated FXIII cross-links many different proteins of the coagulation cascade, extracellular matrix, complement system and intracellular proteins. FXIII also interacts, directly or indirectly, with various cell types (TAFI, thrombin-activatable fibrinolysis inhibitor; PAI-2, plasminogen-activator inhibitor-2; ECM, extracellular matrix; VEGFR, vascular endothelial growth factor receptor; TSP-1, thrombospondin-1; AT1, angiotensin-1; PMN, polymorphonuclear; MASP-1, mannan-binding lectin-associated serine protease-1).

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two or more unexplained consecutive miscarriages and women without a history of a miscarriage and at least one successful pregnancy [26]. As FXIII levels did not differ between these groups, we conclude that recurrent pregnancy loss in the general population is not associated with reduced FXIII plasma levels. Whether locally reduced FXIII-A levels or impaired FXIII function in the placenta may contribute to an increased risk of an abortion, remains to be investigated.

FXIII in wound healing, angiogenesis and atherosclerosis

Role of FXIII in wound healing after myocardial infarction and atherosclerosis

An interesting observation was made about the role of FXIII in myocardial wound healing after a myocardial infarction (MI). In a murine model of MI, transglutaminase activity during an acute infarction predicted healing outcome and left ventricular remodeling [27]. FXIII treatment induced a faster resolution of the neutrophil response, enhanced macrophage recruitment, increased collagen content and augmented angiogenesis in the healing infarct. The study also showed decreased FXIII tissue levels in patients with insufficient healing after MI. A small clinical study by the same authors confirmed their experimental study: in three consecutive patients presenting with acute myocardial rupture after MI, FXIII levels were consistently reduced to 50% [28]. To determine whether a truly causative relationship existed between FXIII activity and myocardial healing, myocardial repair after left coronary artery ligation was studied in FXIII-deficient mice. The results showed that mice lacking FXIII suffered from impaired wound healing and fatal rupture of the left ventricle after MI [29].

Transglutaminases in general play an important role in cardiovascular disease in numerous ways [30]. FXIII may act as signal transduction protein and maintains endothelial barrier function by modifying paracellular transport in endothelial cell monolayers [31]. Therefore FXIII administration may prevent capillary leakage syndrome in certain clinical scenarios [32].

Direct evidence for a role of FXIII in atherosclerosis comes from findings that activated FXIII (FXIIIa) cross-links angiotensin-1 (AT1) receptor dimers of monocytes at the onset of atherosclerosis. Expression of a FXIIIa-inhibiting peptide reduced AT1-stimulated monocyte activation and monocyte entry into the artery wall and inhibited the development of atherosclerosis in hypercholesterolemic Apo E−/− mice [33]. In general, atherosclerosis is a disease in which inflammation plays a significant role and the modulation of inflammatory processes by transglutaminases may be a new approach to further investigate the role of FXIII and other transglutaminases in cardiovascular disease.

Proangiogenic properties of FXIII

Proangiogenic properties of FXIII have been discovered recently showing another until then unknown specific role of this unique coagulation factor. FXIII exerts a direct proangiogenic effect on endothelial cells *in vitro* and promotes angiogenesis in several *in vivo* animal models. Dardik *et al.* [34] showed for the first time that FXIIIa increased endothelial cell migration and proliferation and inhibited apoptosis. The observed proangiogenic effects of FXIII were dependent on its transglutaminase activity since the proangiogenic capacity of FXIIIa was completely abolished by blockade of its active site. The proangiogenic effect of FXIIIa on endothelial cells was accompanied by downregulation of the anti-angiogenic factor thrombospondin-1 (TSP-1) [34,35]. TSP-1, an extracellular matrix protein, acts as a modulator of various cell processes such as migration, adhesion, proliferation, but also apoptosis [36]. In a rabbit cornea model, FXIIIa enhanced neovascularization which was associated with an almost complete loss of TSP-1 [34].

Substantial *in vivo* evidence for the proangiogenic activity of FXIII was given by two murine models [37]. In a neonatal cardiac allograft transplant model, the number of new vessels was higher in FXIII-injected animals than in controls. In a Matrigel plug model, FXIII-deficient mice showed a lower number of new vessels compared with control mice. Furthermore, the number of vessels almost reached normal levels after administration of FXIII. Using a different animal model Kilian *et al.* [38] confirmed that FXIII stimulated neovascularization in bone defects filled with hydroxyapatite paste.

The molecular mechanisms underlying the proangiogenic effects of FXIII are complex. Binding of FXIII-A to endothelial cells requires integrins [39] and FXIIIa induces up-regulation of several transcription factors affecting cell proliferation and differentiation, vasculogenesis and angiogenesis [40]. Dardik *et al.* [41] showed that the proangiogenic effect of FXIIIa is mediated by (i) enhancement of cross-linked and non-covalent $\alpha v\beta 3$/VEGFR-2 complex formation ($\alpha v\beta 3$: integrins involved in angiogenesis and vasculogenesis; VEGFR-2: vascular endothelial growth factor receptor 2); (ii) tyrosine phosphorylation and activation of VEGFR-2; (iii) upregulation of transcription factors c-Jun and Egr-1; and (iv) downregulation of TSP-1 induced indirectly by c-Jun through WT-1 (Wilm's tumor-1). These findings shed light on the mechanisms by which FXIII is involved in angiogenesis and tissue repair.

The role of FXIII in bone metabolism and bone disease

Transglutaminases, bone metabolism and extracellular matrix stabilization

Bone represents a dynamic tissue that is under constant remodeling throughout life [42]. The two major cells involved in these processes are osteoclasts that resorb bone and osteoblasts that form new bone tissue. The extracellular matrix (ECM) represents the biological substratum that supports these cells by facilitating cell attachment, cell differentiation, but also regulates bone mineralization. Secretion and assembly of bone ECM is conducted by osteoblasts and is regulated by cytokines, hormones and by their ionic microenvironment and the ECM

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itself. Stimulation of osteoblasts leads to ECM production and finally matrix mineralization [43]. Fully differentiated osteoblasts deposit bone matrix of which approximately 90% is collagen type-1. The remaining part is composed of proteoglycans and various proteins. Disturbed osteoblast activity contributes to defective bone deposition.

Transglutaminases are well known to be involved in ECM stabilization in different tissues. From the transglutaminase family only transglutaminase 2 (TG2) and FXIII are involved in cartilage and endochondral ossification [44–46]. It is interesting to note that TG2-knockout mice have no overt skeletal phenotype, suggesting that besides TG2 another transglutaminase with at least partially overlapping functions must be involved [47]. Nakano *et al.* [48] confirmed by immunohistochemistry, *in situ* hybridization and biochemical methods that FXIII-A was expressed *in vivo* by osteoblasts and osteocytes in bones formed by both intramembranous and endochondral ossification. FXIII-A was present in bone tissue and in osteoblast cultures mostly as a small 37-kDa form, presumably resulting from posttranslational proteolytic processing of the parent enzyme. This 37-kDa form of FXIII-A was found to be associated with the osteoblast plasma membrane as part of the osteoblast differentiation process.

Al-Jallad *et al.* [49] presented new functions of FXIII in osteoblast matrix secretion and deposition. They showed that FXIII-A and its cross-linking activity were colocalized with plasma membrane-associated tubulin. Thus FXIII-A cross-linking activity appeared to be directed towards stabilizing the interaction of microtubules with the plasma membrane. These results provide strong evidence how transglutaminase activity could affect protein secretion and matrix deposition in osteoblasts and suggest a novel function for plasma membrane FXIII-A in microtubule dynamics. How FXIII-A activation occurs remains elusive; possible candidates include membrane-bound proteases matrix metalloproteinase-2 (MMP-2) and PHEX (phosphate-regulating gene with homology to endopeptidases on the X-chromosome) [50]. Newer results from the same group [51] showed that osteoblasts secreted a latent, inactive dimeric ECM form of FXIII-A (ecmFXIII-A) which was activated upon binding to the matrix by a so far unknown mechanism. Cross-linking activity was detected at sites where fibronectin colocalized with collagen type-1, indicating that ecmFXIII-A secretion could function to stabilize newly deposited matrix. Thus, FXIII-A may be an integral part of the collagen type-1 deposition machinery and of the ECM-feedback loop, both of which regulate matrix deposition and osteoblast differentiation.

In summary, these data show another important function of FXIII besides its role in coagulation. In bone metabolism, FXIII seems to play a synergistic role to TG2 in ECM deposition and osteoblast differentiation.

The role of FXIII in bone disease

Osteoarthritis is a common cause of disability in the elderly that is characterized by cartilage degradation, synovium and tendon inflammation, osteophyte formation accompanied by subchondral bone remodeling by osteoid substance accumulation, and decreased mineralization. Sanchez *et al.* [52] investigated gene expression in human osteoblasts isolated from sclerotic or non-sclerotic areas of subchondral bone. FXIII-A expression was significantly up-regulated in sclerotic osteoblasts compared with non-sclerotic osteoblasts confirming a role of FXIII in bone remodeling [52].

Significance of FXIII antigen levels in severe trauma and surgery

Hemorrhagic shock represents a dangerous complication of severe trauma and is associated with high mortality and morbidity owing to inadequate capillary perfusion in vital organs and tissues. The condition is called trauma-hemorrhagic shock (THS) which leads to a reduction in the circulatory blood volume. This results in insufficient organ microcirculation, tissue hypoxia and finally organ damage. Most THS patients develop severe coagulopathy owing to loss of coagulation and fibrinolytic proteins by bleeding and/or consumption [53,54]. Loss of these proteins cause prolongation of routine coagulation tests such as activated partial thromboplastin time (APTT) and prothrombin time (PT). Thrombelastography (TEG) and rotation thrombelastometry (ROTEM) are ideal bedside tests to provide fast and reliably information on the existence or development of coagulopathies [55–57]. Immediate identification of patients at risk is of critical importance in order to goal-direct transfusion therapy with specific coagulation proteins, platelets and/or antifibrinolytic agents.

FXIII has a significant impact on thrombelastographic parameters suggesting a major role of FXIII in patients with these conditions [58]. However, its benefit in trauma as well as surgical patients is still under debate, especially the question what level of FXIII antigen is required to maintain hemostasis. Clinical studies in surgical patients suggested an increased bleeding tendency at FXIII activity levels below 60% [59–62].

Still, there is insufficient evidence to judge the extent of blood loss or acute coagulopathy that lead to a critical decrease in FXIII antigen levels which could be potentially dangerous during and after a surgical procedure. However, it is well known that trauma-induced shock and coagulopathy lead to disseminated intravascular coagulation including significant consumption of FXIII [63]. A recent animal study investigated the role of FXIII in shock-induced organ dysfunction: rats were subjected to THS or trauma sham shock and were treated with either recombinant cellular FXIII-A_2 (rcFXIII) or a placebo. Administration of rcFXIII diminished THS-induced multiple organ dysfunction, presumably by preservation of the gut barrier function, limitation of polymorphonuclear leukocyte activation and modulation of the cytokine response [64].

FXIII as part of the insulin resistance syndrome

Insulin resistance represents a common metabolic abnormality increasing the risk of type 2 diabetes mellitus (T2DM) and

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cardiovascular disease, the major cause of morbidity and mortality in most parts of the world. Insulin resistance is not simply a problem of deficient glucose uptake in response to insulin but represents a multifaceted syndrome called insulin resistance syndrome which is associated with atheromatous risk factors such as dyslipidemia, hyperinsulinemia, obesity and hypertension, affecting around 25% of the Western population [65,66]. It became more and more evident that not only the clustering of atheromatous risk factors belongs to the syndrome but also atherothrombotic risk factors such as increased plasma levels and/or certain genetic variants of fibrinogen, FVII and most notably plasminogen activator inhibitor-1 (PAI-1) [67]. In addition, there is also evidence that FXIII levels cluster with these risk factors contributing to the prothrombotic state which may in turn enhance the cardiovascular risk. FXIII-A and -B antigen levels are elevated in patients with T2DM and FXIII-A antigen levels are increased in relatives of subjects with T2DM [68]. The specific role and the underlying mechanisms of FXIII in this complex syndrome need further investigation. As inflammation is another feature of the insulin resistance syndrome and FXIII is also involved in inflammatory processes as outlined below, these may represent a link between FXIII and the insulin resistance syndrome.

FXIII and immune defense and inflammation

The simultaneous activation of coagulation and inflammatory processes after injury is a phylogenetically ancient adaptive response that can be traced back to early eukaryotic evolution [69]. The aim of co-activation and interactions between coagulation and inflammatory processes is to protect the host from blood loss and infection. FXIII plays a role in infection control and interacts with complement factors and inflammatory cells (recently reviewed by [70]).

Induction of coagulation leads to immobilization and killing of bacteria inside the clot. This entrapment is mediated via cross-linking of bacteria to fibrin fibers by FXIIIa. FXIII knock-out mice developed severe signs of inflammation at the site of infection, whereas FXIII treatment of wild-type animals reduced bacterial dissemination during early infection [71]. In sepsis, FXIII protected mucosal capillary perfusion against endotoxin-induced impairment in a rat model [72]. Administration of FXIII also reduced hemorrhagic shock-induced organ dysfunction in rats by preserving lung and gut endothelial barrier function and limiting leukocyte activation [64]. While activation of FXIII is beneficial in fighting infection and improving endothelial barrier function (first shown by Noll *et al.* [31]), it also has negative effects in sepsis by increasing the risk of intravascular thrombosis, as depletion of FXIII was shown to prevent disseminated intravascular coagulation-induced organ damage in rabbits [73].

The complement system is an important part of innate immunity and FXIII interacts with proteins of the complement system. Its central component complement C3 is incorporated into fibrin clots and prolongs fibrinolysis [74,75]. Incorporation occurs by non-covalent binding to fibrinogen/fibrin and covalent cross-linking by FXIIIa. Thus, complement C3 is a novel substrate for FXIIIa [76,77]. Mannan-binding lectin-associated serine protease-1 (MASP-1) of the complement lectin pathway has a similar substrate specificity to thrombin, and we and others have shown that MASP-1 also activates FXIII [78]. These interactions may contribute to the prothrombotic state accompanying many inflammatory diseases.

FXIII also interacts with cells of the immune system (recently reviewed by Bagoly *et al.* [79]). Interactions include activation of FXIII by human neutrophil elastase, down-regulation of FXIIIa within the clot by granulocyte proteases, and enhancing effects of FXIII on monocyte proliferation and migration and inhibition of monocyte apoptosis [79]. Monocytes/macrophages have been discussed as a source of FXIII-A in plasma, and in spite of some evidence for a non-classic secretion pathway of cFXIII from these cells [79,80] the origin of plasma FXIII-A is not yet proven. An association between the FXIIIVal34Leu polymorphism and monocyte and neutrophil cell counts after lipolysaccharide infusions in humans has been suggested [81]; however, as a result of the small sample size in this study larger studies are needed to confirm this finding and investigate possible underlying mechanisms.

Inflammatory bowel diseases (IBD) have long been associated with decreased FXIII levels. A recent study in Crohn's disease has shown, however, that FXIII levels cannot be recommended as a marker for disease activity [82]. The mechanisms leading to decreased FXIII levels in IBD are controversially discussed. While one study suggested FXIII consumption due to coagulation activation based on findings of elevated D-dimer and prothrombin fragment 1 + 2 in patients with active ulcerative colitis or Crohn's disease [83], another study did not find increased thrombin–antithrombin complex levels in patients with active Crohn's disease and suggested that FXIII was not consumed as a result of coagulation activation but because of repair of injured tissue [84]. This was supported by a histological study which detected tissue transglutaminase and FXIII-A in damaged areas of the colon underpinning the important role of transglutaminases in mucosal healing [85]. However, it is not yet clear whether patients with IBD benefit from administration of pFXIII as clinical studies have yielded contradictory results [86,87]. Severe graft-versus-host disease (GvHD) of the gut is a relatively frequent complication of hematopoietic stem cell transplantation and manifests with similar symptoms as IBD. There is also a significant decrease in FXIII plasma levels in patients with GvHD [88] and these patients may benefit from FXIII replacement therapy [89].

FXIII in neoplasm

With regard to neoplasm FXIII has come to attention (i) as a marker for certain types of leukemia and carcinoma and (ii) with decreased plasma levels owing to coagulation activation and consumption.

The cellular form of FXIII (FXIII-A_2) is present in platelets, megakaryocytes, monocytes and macrophages and thus has

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Ref. 9

been detected in mono- and megakaryocytic leukemias [90]. In patients with acute myeloblastic leukemia (AML) M4 and M5, FXIII-A was a sensitive marker for blast cells [90,91] in which expression levels were markedly increased compared with normal cells. Recently, FXIII-A has also been detected as a marker in acute promyelocytic leukemia (APL) M3 [92]. Surprisingly, FXIII-A expression was found in 19 out of 47 cases of newly diagnosed B cell acute lymphoblastic leukemia (ALL) [93]. Expression of FXIII-A can be considered as a leukemia-associated immunophenotype which may be of value for diagnosis and disease monitoring [90,92].

Leukemia is also associated with consumption of pFXIII. In a child presented in a case report, FXIII plasma levels of only 56% and increased D-dimer levels preceded a diagnosis of ALL by 6 weeks and FXIII levels normalized when the child was in remission [94]. In a young woman, retro-bulbar hematoma associated with FXIII-A antigen levels as low as 7.6% preceded a diagnosis of APL by 2.5 weeks [95].

FXIII has also been studied in regard to solid tumors. FXIII is related to certain types of neoplasms of the skin. In normal skin, FXIII-A is expressed in specific dermal dendrocytes (DD) derived from the monocyte/macrophage lineage or from a mesenchymal origin [96]. In tumor pathology, expression of FXIII-A is used for example to distinguish between dermatofibroma and dermatofibrosarcoma protuberans [97,98]. In addition, FXIII-A+ DD are found in fibrovascular lesions including fibrous papules of the nose, acquired digital fibrokeratomas, angiofibromas, oral fibromas [99] or desmoplastic neoplasms where FXIII-A is possibly acting as a growth factor. FXIII-A+ DD may also be involved in the progression and regression of some malignancies including cutaneous melanoma and basal cell carcinoma [96]. A study in 130 patients with oral squamous cell carcinoma and 135 healthy controls suggested that the Leu allele of the FXIIIVal34Leu polymorphism was associated with an increased risk for this type of cancer. As a possible mechanism it was poposed that a less porous fibrin network composed of thinner fibers may facilitate tumor stroma formation and tumor cell proliferation [100]. In 110 patients with breast cancer, significantly lower expression levels of FXIII were found in tumor tissues compared with normal mammary tissues ($n = 27$) [101].

FXIII may also play a role in metastasis. Wild-type and FXIII-deficient mice were injected with Lewis lung carcinoma and B16–BL6 melanoma cells. The metastatic potential was significantly diminished in FXIII-A-deficient mice relative to control animals. FXIII was shown to support metastasis primarily by limiting natural killer cell-mediated clearance of micrometastatic tumor cells [102]. Human data, however, are lacking so far.

Novel functions of FXIII

FXIII in tears

FXIII-A and -B subunits and FXIII tetramer have been detected in human tears at a low concentration [103,104]. The source of FXIII in tears remains unknown, but possible sources include leakage from plasma or production in conjunctival macrophages or corneal epithelial cells. As most of the FXIII in tears exists as FXIII-A_2, non-proteolytic activation in the presence of Ca^{2+} is likely to occur. Alternatively, proteolytic cleavage by thrombin that has leaked from plasma or by granulocyte elastase is possible. In patients undergoing corneal transplantation, FXIII concentrations (normalized for protein concentration) increased up to 25-fold on the first post-operative day, followed by a gradual decrease over the next 7 days. Patients who later developed the complication of neovascularisation of the donor cornea showed the highest FXIII levels. It was suggested that FXIII in tears may be involved in corneal wound healing, whereas high FXIII levels may represent a risk factor for neovascularisation by promoting angiogenesis [104].

Optic nerve regeneration

A novel function for cellular FXIII-A in neuronal regeneration has been proposed in fish [105]. In fish, unlike in mammals, neurons of the central nervous system are capable of self-repair and regeneration, and research is ongoing to identify factors inducing/involved in repair processes. Upon optic nerve injury in goldfish, *in situ* FXIII activity increased accompanied by expression of FXIII-A mRNA. The cells producing FXIII-A were identified as astrocytes/microglial cells in the optic nerve. In retinal cell culture, overexpression of FXIII-A promoted neurite sprouting and elongation [105]. Further studies are needed to elucidate the underlying mechanisms. Whether this may have future implications in human medicine is so far unknown.

Liver remodeling

In a murine model of acute liver injury, FXIII-A deficiency led to increased hepatocyte apoptosis and a delay in hepatocyte regeneration. It was concluded that the effects of FXIIIa on ECM protein cross-linking and matrix formation could promote survival of hepatocytes in liver remodeling [106].

FXIII-B subunit is not only a carrier for the FXIII-A subunit

The gene encoding FXIII-B is located on chromosome 1 and belongs to the regulator of the complement activation (RCA) gene cluster [107]. FXIII-B with its 10 short consensus repeats, also called Sushi domains, resembles other binding and regulatory proteins such as complement factor H or C4b-binding protein. Furthermore, the FXIII-B plasma concentration is twice as high as the FXIII-A concentration, this means that 50% of FXIII-B is free and in excess over FXIII-A. Therefore it would be plausible if the FXIII-B subunit had other functions in addition to its carrier function for FXIII-A. One novel function has been recently described by the group of L. Muszbek [108]. They have shown that FXIII-B binds *Staphylococcus aureus* protein A (SpA) with high affinity. SpA

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Ref. 9

on the bacterial surface binds human IgG in incorrect orientation preventing recognition and phagocytosis by macrophages. FXIII-B saturates SpA and inhibits incorrect binding of IgG and may thus promote opsonization and subsequent phagocytosis of the bacteria. This may represent a novel role for FXIII-B in immune defense.

AP-FXIII as marker of thrombosis and regulator of coagulation

It has long been known that thrombin initiates the physiological conversion of FXIII-A_2B_2 zymogen into the active enzyme by cleavage of the N-terminal activation peptide (AP-FXIII). Until recently, however, it was unclear whether the AP-FXIII is indeed released into plasma under physiological conditions. Neither had it been explored whether free AP-FXIII, in case it was indeed released into circulation, might have any physiological functions.

We therefore developed an ELISA method with two sensitive and specific monoclonal antibodies against free AP-FXIII and we showed that AP-FXIII is released into plasma upon FXIII activation [6,109]. We then performed a pilot study to provide proof-of-principle, that *in vivo* generated AP-FXIII can be detected in patients with an acute thrombotic event [110]: we investigated FXIII activation in the early phase of acute ischemic stroke by repeated measurements of free AP-FXIII, FXIII-A and FXIII-B subunit antigen levels in plasma samples from patients within 48 h of acute ischemic stroke. Free AP-FXIII could be detected in 34 out of 66 patients upon hospital admission (range 0.2–26.3 ng mL^{-1}), on day 1 in 15 patients (0.2–10.4 ng mL^{-1}) and on day 2 in 11 patients (0.1–15.1 ng mL^{-1}). AP-FXIII was higher in patients with a severe stroke. Lower AP-FXIII levels upon admission were associated with clinical improvement. Larger studies are needed to assess whether AP-FXIII might serve as a diagnostic and/or prognostic marker for acute thrombotic diseases.

We are currently investigating whether free AP-FXIII may affect FXIII function and fibrin formation and structure. Preliminary results show that free AP-FXIII, but not a scrambled peptide of the same amino acid composition but in random order, reduces thrombin-induced FXIII activation and affects the fibrin clot structure, suggesting that free AP-FXIII may interact with thrombin and compete with the thrombin substrates FXIII and fibrinogen [111; and unpublished own data]. Whether free AP-FXIII may act as a negative feedback regulator of thrombin-induced clot formation remains to be confirmed.

Concluding remarks

The aim of this review was to highlight the remarkable diversity of functions attributed to FXIII. This diversity may partly originate from its enzymatic characteristics as a transglutaminase, as transglutaminase reactions represent important post-translational modifications by covalently cross-linking proteins which can change their properties and biological effects. This makes FXIII unique among the hemostatic proteins which are mainly protein-cleaving serine proteases. Indeed, there is increasing evidence that FXIII actually has more functions beyond than within hemostasis. Therefore and as the only transglutaminase circulating in plasma, the name 'plasma transglutaminase' may be more appropriate than 'coagulation FXIII'.

Having discussed FXIII as a protein with so many important functions in different processes beyond hemostasis, it may seem at odds that FXIII deficiency does exist and hence is compatible with life. One conclusion may be that FXIII has a rather modulating than exclusive role and that FXIII and other transglutaminases may have evolved as redundant systems supporting each other in fulfilling certain tasks. The specific contributions and interrelations of the different transglutaminases especially on the cellular level are still not well known. In regard to its function in blood coagulation, however, there is no physiological replacement or compensation for FXIII, and the severity of congenital FXIII deficiency is highlighted by two facts: (i) without medical treatment, most of the affected individuals die at a young age. An extensive Swiss family pedigree going back to the 17th century [9,112] illustrated the devastating effect FXIII deficiency had on that family over the years. (ii) A normal pregnancy is unlikely in affected women which possibly makes FXIII deficiency more difficult to pass on than other hereditary diseases.

Clearly, further research into the underlying mechanisms and (patho-) physiological relevance of the many described functions of FXIII is needed. It will be exciting to observe future developments in this area and to see if and how these interesting findings may be translated into clinical practice in the future.

Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

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A Quantitative-Trait Locus in the Human Factor XII Gene Influences Both Plasma Factor XII Levels and Susceptibility to Thrombotic Disease

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One approach to the identification of genetic loci that influence complex diseases is through the study of quantitative risk factors correlated with disease susceptibility. Factor XII (FXII) plasma levels, a related phenotype correlated with thrombosis, is such a risk factor. We conducted the first genomewide linkage screen to localize genes that influence variation in FXII levels. Two loci were detected: one on chromosome 5 and another on chromosome 10 (LOD scores 4.73 and 3.53, respectively). On chromosome 5, the peak LOD score occurred in the 5q33-5ter region, near the *FXII* gene. Addition of a 46C/T mutation in the *FXII* gene increased the multipoint LOD score to 10.21 ($P = 3.6 \times 10^{-12}$). A bivariate linkage analysis of FXII activity and thrombosis further improved the linkage signal (LOD = 11.73) and provided strong evidence that this quantitative-trait locus (QTL) has a pleiotropic effect on the risk of thrombosis ($P = .004$). Linkage analysis conditional on 46C/T indicated that this mutation alone cannot explain the chromosome 5 signal, implying that other functional sites must exist. These results represent the first direct genetic evidence that a QTL in or near the *FXII* gene influences both FXII activity and susceptibility to thrombosis and suggest the presence of one or more still unknown functional variants in *FXII*.

Introduction

Thrombosis is a common cause of morbidity and mortality in industrialized nations. Both venous and arterial thrombosis can be life-threatening events, and both are of great public health importance. Although there is little direct information on prevalence, retrospective and prospective data (Coon et al. 1973; Nordstrom et al. 1992; Anderson et al. 1991) suggest a minimum lifetime prevalence of 5%–10% for deep-vein thrombosis alone. After the inclusion of arterial thromboses, other venous thromboses, and undiagnosed thrombotic conditions, the true lifetime prevalence of thrombosis must be substantially larger than 10%.

The canonical causes of thrombosis include both environmental and genetic factors. The high prevalence of thrombosis and its known environmental influences (e.g., oral contraceptive use) suggest that multiple genes of varying effects will be involved in determining susceptibility to thrombosis. There are several well-characterized genetic defects that lead to increased thrombotic risk (Lane et al. 1996). However, very little information is available on the relative importance of genetic factors in thrombosis risk in the general population. Moreover, it is unlikely that these known mutations, with their comparatively low frequencies, constitute the primary genetic influences on the risk of common late-onset thrombosis. Recently, as part of the GAIT (Genetic Analysis of Idiopathic Thrombophilia) project, we quantified the genetic component of susceptibility to thrombosis and related phenotypes (Souto et al. 2000*a*, 2000*b*). Among the clotting factors studied, factor XII (FXII) levels exhibited one of the highest heritabilities (67%) and a significant positive genetic correlation (0.351) with thrombotic disease (Souto et al. 2000*a*), indicating that some of the genes that influence variation in this physiological risk factor also influence liability to thrombosis. Therefore, we performed a genome scan to identify genes influencing FXII levels. To our knowledge, our study represents the first genomewide scan undertaken to identify regions containing genes that influence variation in susceptibility to thrombotic disease and its intermediate phenotypes. The identification of such regions and genes may help to elucidate the mecha-

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nism underlying the risk of common thrombosis and therefore may suggest preventive strategies to reduce thrombosis-related morbidity and mortality.

Subjects and Methods

Subjects and Phenotypes

The recruitment, sampling, and phenotyping methods used in the GAIT project have been extensively described elsewhere (Soria et al. 2000; Souto et al. 2000*b;*). In brief, the sample included 21 families (398 individuals) selected primarily for pedigree size, to maximize the power to detect genetic effects. To be included, a family was required to have ⩾10 living individuals in three or more generations. Twelve families were selected through a proband with idiopathic thrombophilia, which was defined as a personal history of multiple thrombotic events (at least one of which spontaneous), a history of a single spontaneous episode of thrombosis plus a first-degree relative who was also affected, or onset of thrombosis at <45 years of age. Of the 12 probands, 10 had onset at age <45 years, 8 experienced multiple thromboses, and only 2 were ascertained because of a single episode of thrombosis with a relative also affected. Diagnoses of the thrombophilic probands were verified by objective methods. A proband's thrombophilia was considered idiopathic if all known (during the recruitment period of 1995–1997) biological causes (e.g., antithrombin deficiency, protein S and C deficiencies, activated protein C resistance, plasminogen deficiency, heparin cofactor II deficiency, Leiden factor V, dysfibrogenemia, lupus anticoagulant, and antiphospholipid antibodies) of thrombophilia were excluded. These thrombophilic factors were also absent in all affected relatives. The remaining 9 of the 21 families were selected without regard to phenotype.

FXII was assayed using deficient plasma from Diagnostica Stago (Asnières) by automated coagulometry as described elsewhere (Souto et al. 2000*b*). To reduce measurement error, assays were performed in duplicate, and the average value calculated for each person. The interassay coefficient of variation was estimated in 5.5%.

All procedures were reviewed by the institutional review board of the Hospital de la Santa Creu i Sant Pau (Barcelona). Adult subjects gave informed consent for themselves and their minor children.

Genotypes

DNA was extracted using a standard protocol (Miller et al. 1988). The present genome scan used a total of 363 highly informative microsatellite markers, spaced at ~9.5 cM. The microsatellites consisted primarily of the ABI-Prism genotyping set MD-10. Linkage mapping was undertaken, using multiplex PCR with the PE LMS II fluorescent marker set; in a few instances, nearby Généthon markers were substituted for LMS II markers, to improve robustness. The PCR products were analyzed on PE 310, PE 377, and PE 3700 automated sequencers and were genotyped using the PE Genotyper software. Information on microsatellite markers can be found in the publicly accessible Genome Database. The average heterozygosity of these markers was 0.79.

Markers in or near several hemostasis-related candidate genes were used to augment this genome scan. The 46T/C polymorphism in the FXII gene was amplified as reported elsewhere (Kanaji et al. 1998), with minor modification. In brief, we used *Sfa*NI, which provided better resolution of the digested bands. The genotypic data were entered into a database and were analysed for discrepancies (i.e., violations of Mendelian inheritance), using the INFER (PEDSYS) program (Dyke 1995). Discrepancies were checked in the laboratory for mistyping, and markers for discrepant individuals were either corrected or excluded from the analysis.

Linkage Analysis

Standard oligogenic multipoint variance-component linkage methods, as implemented in the SOLAR software program, were used for the genome scan (Almasy and Blangero 1998). Previous studies have suggested that such methods may be vulnerable to deviations from multivariate normality and particularly to high levels of kurtosis in the trait distribution (Allison et al 1999). Levels of FXII in the GAIT sample exhibited a kurtosis of 0.05. Recent statistical genetic theory demonstrates that this level of kurtosis will not affect the distribution of LOD scores and that the standard nominal P values for LOD scores are appropriate for the FXII linkage screen (Blangero et al. 2000). Allele frequencies were estimated from the GAIT sample, and marker maps for multipoint analyses were obtained from ABI-Prism and from the Marshfield Center for Medical Genetics. Because 12 of the families were ascertained through thrombophilic probands, all analyses included an ascertainment correction achieved by conditioning the likelihood of these pedigrees on the likelihoods of their respective probands (Boehnke and Lange 1984). genomewide P values were calculated using the method of Feingold et al. 1993. Bivariate linkage analyses using the mixed discrete/continuous trait multivariate model were conducted with a modified version of SOLAR (Williams et al. 1999).

Measured Genotype and Conditional Linkage Analyses

Quantitative-trait association analysis was performed using the measured genotype approach (Hopper and Mathews 1982) by testing for genotype-specific differ-

Table 1

Results from the Initial and Conditional Genomewide Linkage Screens of FXII

Chromosome	Position (cM)	LOD Score for First Pass	LOD Score for Second Pass	LOD Score for Third Pass
5	193	4.73	...	...
10	38	3.53	3.43	...
2	9	2.26	1.83	.59
11	10	1.30	.44	.19
14	63	1.16	.75	.90
15	79	1.03	.41	.35

NOTE.—All loci with LOD scores >1.0 in the initial pass are shown. The second linkage pass was conditional on a locus on chromosome 5q, and the third pass was conditional on loci on 5q and 10p.

ences in the means of traits while allowing for the nonindependence among family members. These analyses were performed using SOLAR (Almasy and Blangero 1998). To assess linkage conditional on the observed association (Amos 1994), an extension of the variance-component–based linkage test was performed by simultaneously incorporating the genotype-specific means of the measured genotype test and estimating a linkage component.

Results

Multipoint variance-component methods were used to assess linkage between highly informative autosomal DNA markers, spaced at ~9.5 cM, and plasma levels of FXII. The results of the initial FXII linkage screen are shown in figure 1 and table 1. The linkage analysis revealed strong evidence that quantitative-trait loci (QTLs) on chromosomes 5q influence FXII levels (LOD = 4.73; nominal $P = 1.5 \times 10^{-6}$; genomewide $P = .00046$), as do QTLs at chromosome 10p (LOD = 3.43, nominal $P = 3.5 \times 10^{-5}$; genomewide $P = .0114$). Additionally, suggestive evidence of linkage (LOD scores of 1.0–2.3) were observed on chromosomes 2p, 11p, 14q, and 15q. Given the strong evidence for multiple QTLs that influence FXII levels, we performed a series of multilocus linkage screens, in which QTLs were sequentially incorporated into the linkage model and the genome

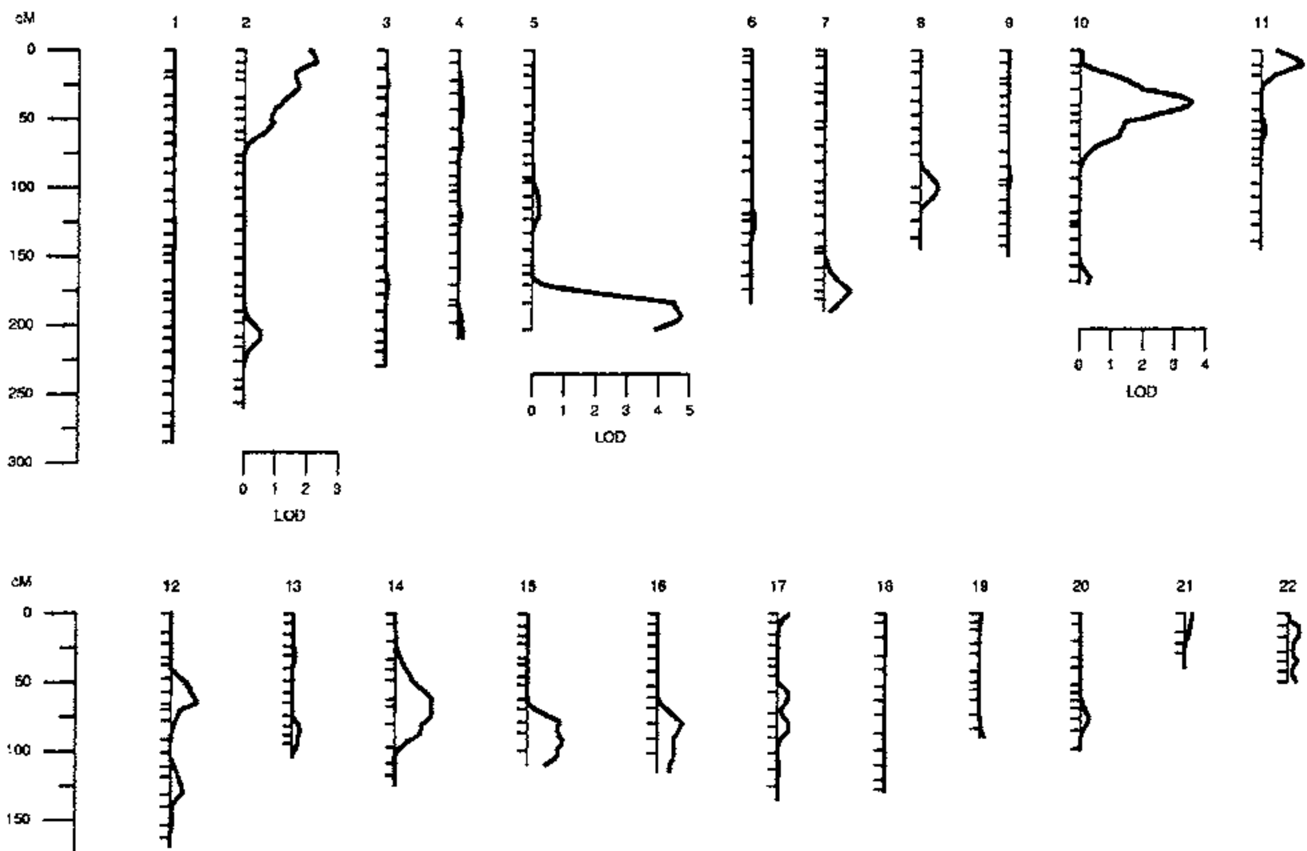

Figure 1 Results of the initial linkage screen

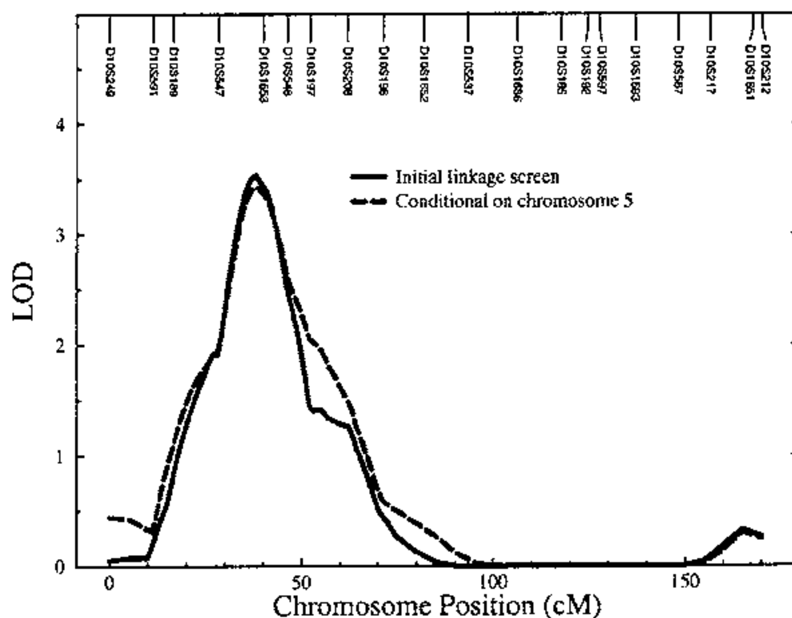

Figure 2 Initial and conditional LOD scores on chromosome 10

was rescreened (table 1). In the second linkage pass, which was conditional on the chromosome 5 QTL that was detected in the initial screen, evidence for a QTL on chromosome 10 remained strong (LOD = 3.43), whereas LOD scores in other regions that previously showed suggestive linkage declined slightly. Conditional on the chromosome 5 and 10 QTLs, little evidence for additional QTLs remained in a third linkage screen (all LOD scores < 1.0).

The peak LOD score on chromosome 10 occurred between markers D10S189 and D10S1653 in the10p13 region (fig. 2). On chromosome 5, the peak LOD score occurred between markers D5S400 and D5S408 in a region that maps to 5q33-5ter (fig. 3). Because the hu-

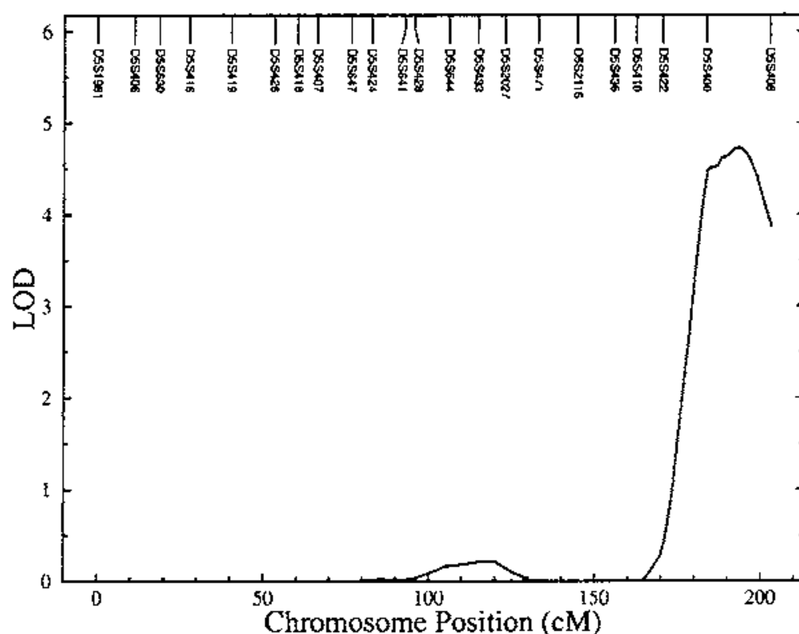

Figure 3 Detailed linkage results for chromosome 5

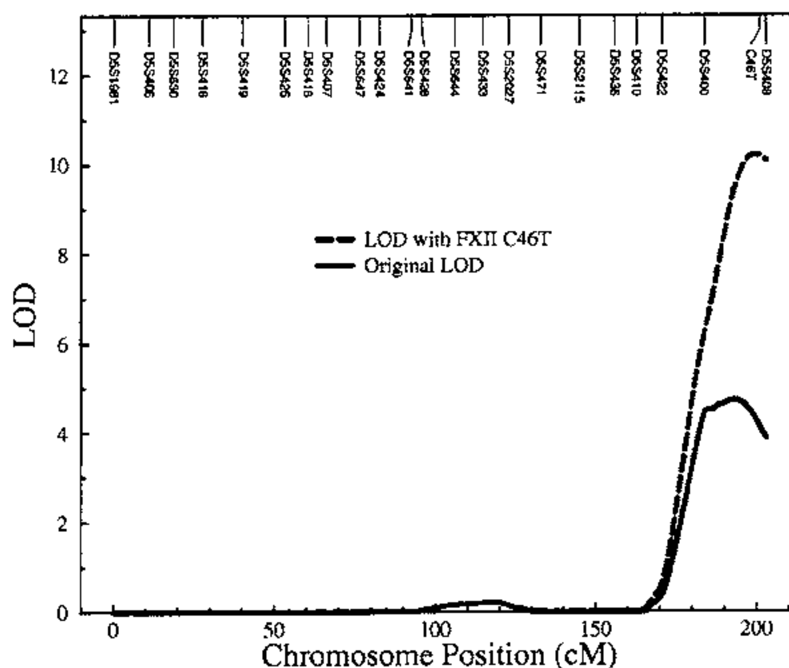

Figure 4 Detailed linkage results for chromosome 5 when the 46C/T variant was added

man *FXII* gene has been mapped to this region (Royle et al. 1988), we have genotyped a known polymorphism in exon 1 of the *FXII* gene (46C/T). In the present study, we found 264 homozygotes for the common variant (*C/C*), 117 heterozygotes (*C/T*), and 11 homozygotes (*T/T*); the maximum-likelihood estimate of the frequency of the T allele was 0.21. If Hardy-Weinberg equilibrium is assumed, this allele frequency predicts a heterozygote frequency of 0.331.

When this variant was added to the chromosome 5 linkage map, the multipoint LOD score increased dramatically to 10.21 ($P = 3.6 \times 10^{-12}$; fig. 4). The results support the presence of a major QTL in the region of the *FXII* gene. Given this unequivocal evidence for linkage, we performed an association analysis to test for linkage disequilibrium between the QTL and the 46C/T variant. This measured-genotype–association analysis revealed significant differences among the three 46C/T genotypes in their plasma FXII values ($CC = 128.9$; $CT = 92.2$; $TT = 55.6$; $P < 1 \times 10^{-7}$). Under the assumption that 46C/T affects the function of the *FXII* locus, we calculated that this mutation accounts for 40% of the variance in FXII activity levels in this population.

Because our previous studies have suggested that FXII levels are correlated with risk of thrombosis and that this relationship is, in part, a result of genes that influence both traits (Souto et al. 2000*a*), we wished to test whether either of the QTLs influencing FXII levels might also contribute to genetic susceptibility to thrombosis. This was accomplished through bivariate linkage analysis of FXII and thrombosis (Williams et al. 1999). Such bivariate analyses with related phenotypes have been shown to increase power to detect linkage (Soria et al. 2000). In the combined analysis of FXII levels and thrombosis, the LOD score on chromosome 10 remained at its previous level, providing no evidence that this QTL influences thrombosis risk, whereas the LOD score on chromosome 5 rose to 11.73, providing strong evidence that this QTL has a pleiotropic effect on the risk of thrombosis ($P = .004$).

To determine whether the 46C/T marker could be the functional QTL underlying our linkage signal, we performed a conditional linkage analysis (Soria et al. 2000) that simultaneously accounted for association with the 46C/T mutation. Conditional on 46C/T, evidence for linkage to 5q still remained (LOD = 0.9; fig. 4), indicating that, although 46C/T may be functional, there must be one or more additional polymorphisms in the same region that influence FXII levels and thrombosis risk.

Discussion

Our results represent the first genomewide scan undertaken to identify regions containing genes that influence variation in susceptibility to thrombotic disease and their

related phenotypes. Initial and subsequent conditional passes of variance-component linkage analyses revealed two regions, one on chromosome 5 and another on chromosome 10, that showed strong evidence of linkage with levels of plasma FXII, an important intermediate phenotype correlated with thrombosis (Souto et al. 2000*a*).

In the region of the linkage signal on chromosome 10, there are no obvious candidate hemostasis-related genes. In contrast, we have documented the close linkage between a QTL influencing FXII levels and the *FXII* gene (specifically, the 46C/T FXII DNA variant). When we added this genetic variant as a marker, a highly significant LOD score of 10.21 was calculated, providing strong evidence for the existence of a QTL that influences FXII activity.

Recent case-control studies demonstrated association between the 46C/T polymorphism in exon 1 of the *FXII* gene and variation in levels of FXII (Kanaji et al. 1998; Ishii et al. 2000). In our study, under the assumption that 46C/T is itself a functional QTL, we calculated that this mutation accounts for 40% of the variance in FXII activity levels in our population. If 46C/T is not itself functional but is only in disequilibrium with another site, this effect size may be an underestimate. There remains substantial residual genetic variation in FXII activity, even after taking into account the effects of 46C/T. This indicates that there are other QTLs that influence FXII activity level, as is suggested by the highly significant linkage signal on chromosome 10. Moreover, the residual linkage signal (LOD = 0.9) in the combined linkage/disequilibrium analysis (Soria et al. 2000), which simultaneously allowed for association between the 46C/T polymorphism and FXII levels, indicated that, although 46C/T may be functional, there must be one or more additional polymorphisms in the region that influences FXII levels. This result supports the observation we reported elsewhere that multiple QTLs of varying effects will be involved in determining variation in hemostasis-related phenotypes (Souto et al. 2000*a*, 2000*b*).

In addition to our analyses, there is biochemical evidence of a functional role of the 46C/T in the phenotypic variability of FXII levels. Kanaji et al. (1998) reported that this mutation decreased the translation efficiency and led to low plasma levels of FXII activity and antigen, probably as a result of the creation of another ATG codon and/or the impairment of the consensus sequence for the translation-initiation scanning model. On the basis of these biochemical results and our linkage analyses, we expect that 46C/T is a functional polymorphisms in *FXII*. To identify the other variant(s), the next step is to catalog, by use of DNA resequencing, the complete menu of DNA sequence variation within the *FXII* gene. Because variation within noncoding regions may influence the regulation of transcription and other genetic functions, DNA resequencing should not be limited to the exons of the gene, but should include 5′ and 3′ regulatory regions, as well as introns. Because *FXII* consists of 13 introns and 14 exons that cover 12 kb (Cool and MacGillivray 1987), this task may be attainable.

It is important to note that this study confirms and extends our previous observation that FXII levels are correlated with risk of thrombosis and that this relationship is due in part to genes that influence both traits (Souto et al. 2000*a*). This was accomplished through bivariate linkage analysis of FXII and thrombosis (Williams et al. 1999). Such bivariate analyses with related phenotypes increase the power to detect linkage (Soria et al. 2000). The combined analysis of FXII levels and thrombosis risk substantially enhanced the linkage signal on chromosome 5, providing strong evidence that this QTL has a pleiotropic effect on the risk of thrombosis. However, the LOD score on chromosome 10 remained at its previous level, providing no evidence that this QTL influences thrombosis risk. Our results confirm the valuable potential of this statistical approach as a basic tool for mapping the genes that affect complex diseases.

Despite the important putative role of FXII—which is a serine protease precursor involved in the initiation of the intrinsic coagulation pathway, in fibrinolysis, in the generation of bradykinin, and in the complement system (Kaplan and Silverberg 1987; Kluft et al. 1987)—its biologic role is not fully understood. Furthermore, there is controversy over the clinical significance of a decreased or increased FXII concentration on venous and arterial thrombosis (Halbmayer et al. 1992; Helft et al. 2000). Further investigation of *FXII* and other genes may enhance our understanding of the factors influencing thrombosis, especially the chromosome 10p13 region, which should be targeted for fine mapping, and gene-identification studies.

In conclusion, our study represents the first direct genetic evidence that at least one QTL in the *FXII* gene influences both FXII levels and susceptibility to thrombosis. Our results also support the conclusion that the 46C/T polymorphism, in addition to at least one other unknown functional variant in the *FXII* gene, is likely to be one of these QTLs. Therefore, variations in the *FXII* gene should be considered as potential genetic risk factors for thrombosis. However, exhaustive enumeration of all potentially functional variants will be required to completely document the allelic architecture of such thrombosis risk–related variation.

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Electronic-Database Information

The URLs for data in this article are as follows:

ABI-Prism, http://www.appliedbiosystems.com/ (for genetic markers)
Centre National de Genotypage, http://www.cng.fr/ (for genetic markers)
Genome Database, http://www.gdb.org (for genetic markers)
Center for Medical Genetics, Marshfield Medical Research Foundation http://research.marshfieldclinic.org/genetics/ (for genetic markers)
Southwest Foundation for Biomedical Research, http://www.sfbr.org/ (for SOLAR program package)

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Economic evaluation of Thrombo inCode, a genetic platform for the assessment of venous thromboembolism (VTE) risk, in patients with a pattern of VTE or a condition that suggests a hereditary component

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OBJECTIVE

- To conduct, from the National Health System perspective, an economic analysis of the risk assessment of VTE with Thrombo inCode, a genetic platform, in patients with a pattern of VTE or a condition that suggests a hereditary component, compared with the standard methods so far used (Factor V Leiden and prothrombin G20210A mutation).

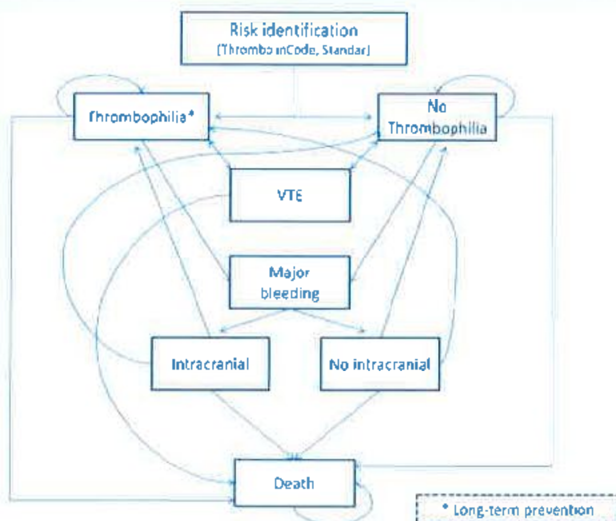
METHODS

- A Markov model was developed with 7 states of health (thrombophilia, no thrombophilia, VTE, major bleeding, intracranial hemorrhage, no intracranial hemorrhage, and death) (Figure 1). The predictive ability of VTE from the identification of thrombophilia with Thrombo inCode and the standard method, was obtained from three studies of the method validation performed in three different populations: FARIVE, MARTHA and St. PAU (3,661 patients in total) (1). It was assumed that patients with thrombophilia positively identified undergo a preventive treatment of VTE, which involves both reducing the number of VTE as the increase in major bleeding. The utilities (quality-adjusted years, QALY) and costs of Markov states were obtained from the literature (2) and Spanish sources (3). The analysis was done from the National Health System perspective, for a time horizon of 5 years and lifetime. An annual discount rate of 3.5% for costs and benefits was applied.

RESULTS

- For a Thrombo inCode price of 290 €, this genetic platform would be the dominant or the cheapest option for any time horizon from 5 years (Table 1). The threshold price of Thrombo inCode to reach the incremental cost-effectiveness ratio (ICER) threshold generally accepted in Spain (30,000 €/QALY) would range between € 1,069 and € 1,284. Probabilistic analyses indicate that Thrombo inCode assessment is dominant in the 97.3, 97.2 and 98.6% of the tests, according to the selected population (FARIVE, MARTHA, St. PAU) (Figure 2).

Figure 1. Markov model for the evaluation of Thrombo inCode, genetic platform used to estimate the risk of venous thromboembolism (VTE) in patients with a VTE pattern or a pathology which suggests a hereditary component, in comparison with conventional methods used until now (Factor V Leiden and prothrombin G20210A).



Abbreviation: VTE: venous thromboembolism.

Table 1. Results of the deterministic cost-effective analysis. Base case. Time horizon: 5 years.

Method	Cost (€)	QALY	ICER (€)
A) Population: FARIVE			
Thrombo inCode*	2436.49	3.3201	Thrombo inCode[1] is dominant
Standard	3137.24	3.3113	
Differences	-700.75	0.0088	
Method	Cost (€)	LY	ICER (€)
Thrombo inCode*	2436.49	4.0698	Savings with Thrombo inCode[2]
Standard	3137.24	4.0700	
Differences	-700.75	-0.0002[1]	
B) Population: MARTHA			
Method	Cost (€)	QALY	ICER (€)
Thrombo inCode*	2220.98	3.3222	Thrombo inCode[1] is dominant
Standard	2772.63	3.3146	
Differences	-551.65	0.0076	
Method	Cost (€)	LY	ICER (€)
Thrombo inCode*	2220.98	4.0695	Savings with Thrombo inCode[2]
Standard	2772.63	4.0692	
Differences	-551.65	0.0003[1]	
C) Population: S. PAU			
Method	Cost (€)	QALY	ICER (€)
Thrombo inCode*	2376.43	3.3211	Thrombo inCode[1] is dominant
Standard	3092.99	3.3118	
Differences	-716.56	0.0093	
Method	Cost (€)	LY	ICER (€)
Thrombo inCode*	2376.43	4.0706	Savings with Thrombo inCode[2]
Standard	3092.99	4.0702	
Differences	-716.56	0.0004	

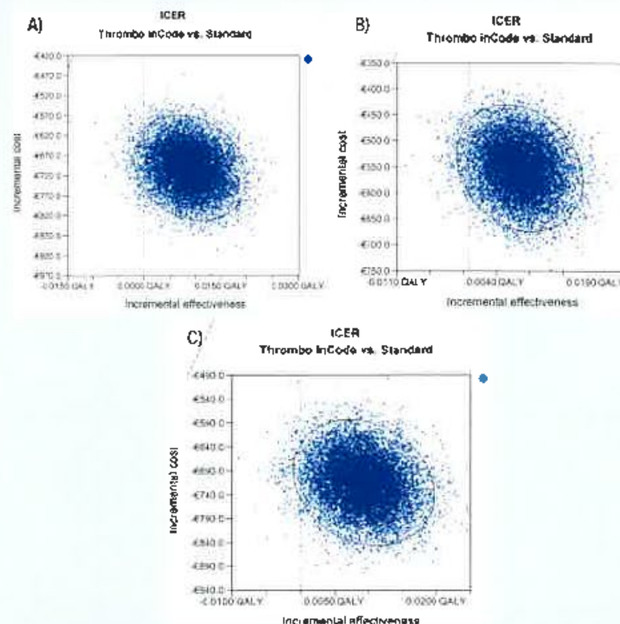
Abbreviations: ICER: incremental cost-effectiveness ratio (cost difference / effectiveness difference); LY: life years; QALY: quality adjusted life years. Cost per QALY gained or cost per life year gained (LYG). 1- Thrombo inCode is more effective, with lower costs: is the dominant choice. 2- For differences well below 0.030 QALYs or LY is consider only the cost differences, without calculate the ICER, which is well above the 30000 €.

Figure 2. Probabilistic analysis of Monte Carlo. A) FARIVE; B) MARTHA; C) St. PAU.

A) Thrombo inCode is dominant in 97.33% of the simulations. The Standard option was more effective with a cost per QALY more than 30,000 € in 2.67% of the remaining simulations.

B) Thrombo inCode is dominant in 97.17% of the simulations. The Standard option was more effective with a cost per QALY more than 30,000 € in 2.83% of the remaining simulations.

C) Thrombo inCode is dominant in 98.59% of the simulations. The Standard option was more effective with a cost per QALY more than 30,000 € in 1.41% of the remaining simulations.



CONCLUSIONS

- **Thrombo inCode is a cost-effective genetic option in VTE risk assessment compared with the standard method.**

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Genetic Susceptibility to Thrombosis and Its Relationship to Physiological Risk Factors: The GAIT Study

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Although there are a number of well-characterized genetic defects that lead to increased risk of thrombosis, little information is available on the relative importance of genetic factors in thrombosis risk in the general population. We performed a family-based study of the genetics of thrombosis in the Spanish population to assess the heritability of thrombosis and to identify the joint actions of genes on thrombosis risk and related quantitative hemostasis phenotypes. We examined 398 individuals in 21 extended pedigrees. Twelve pedigrees were ascertained through a proband with idiopathic thrombosis, and the remaining pedigrees were randomly ascertained. The heritability of thrombosis liability and the genetic correlations between thrombosis and each of the quantitative risk factors were estimated by means of a novel variance component method that used a multivariate threshold model. More than 60% of the variation in susceptibility to common thrombosis is attributable to genetic factors. Several quantitative risk factors exhibited significant genetic correlations with thrombosis, indicating that some of the genes that influence quantitative variation in these physiological correlates also influence the risk of thrombosis. Traits that exhibited significant genetic correlations with thrombosis included levels of several coagulation factors (factors VII, VIII, IX, XI, XII, and von Willebrand), tissue plasminogen activator, homocysteine, and the activated protein C ratio. This is the first study that quantifies the genetic component of susceptibility to common thrombosis. The high heritability of thrombosis risk and the significant genetic correlations between thrombosis and related risk factors suggest that the exploitation of correlated quantitative phenotypes will aid the search for susceptibility genes.

Introduction

Thrombosis is a common cause of morbidity and mortality in industrialized nations. Both venous and arterial forms of thrombosis are of great public-health importance. Although there is little direct information on prevalence, retrospective and prospective data (Coon et al. 1973; Anderson et al. 1991; Nordstrom et al. 1992) suggest a minimum lifetime prevalence of 5%–10% for deep-vein thrombosis. After the inclusion of arterial thromboses, other venous thromboses, and undiagnosed thrombotic conditions, the true lifetime prevalence of thrombosis must be substantially >10%.

The canonical causes of thrombosis include both environmental and genetic factors (Rosendaal 1999). The high prevalence of thrombosis and its known environmental influences, such as smoking and oral contraceptive use, suggest that multiple genes of varying effects will be involved in determining susceptibility to thrombosis. Such complex oligogenic inheritance is also likely to involve gene-gene and gene-environment interactions (Hasstedt et al. 1998). Although there are a number of well-characterized genetic defects that lead to increased thrombotic risk (Lane et al. 1996), it is unlikely that these comparatively infrequent mutations constitute the primary genetic influences on risk of common late-onset thrombosis. In fact, very little information is available on the relative importance of genetic factors in thrombosis risk in the general population. Because of the paucity of family-based studies, there are no extant estimates of the heritability of thrombosis risk.

The physiological cascade that underlies the normal formation of thrombin and the pathological endpoint of thrombosis is complex, with many components involved in the coagulation and fibrinolytic pathways. The identification of quantitative risk factors for thrombosis has accelerated in recent years. Numerous hemostatic factors—including fibrinogen, factor VII, factor VIII, von Willebrand factor, and homocysteine—have been implicated as possible concomitants of both

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venous (Koster et al. 1994, 1995; MacCallum et al. 1995; den Heijer et al. 1996) and arterial thrombosis (Meade et al. 1986; Hamsten et al. 1987; Ernst and Resch 1993; Ridker et al. 1993; Folsom et al. 1997; Nygard et al. 1997). Regardless of their causal relationships with thrombosis, such correlated phenotypes can provide additional information about the genetic basis of thrombosis risk. Recent advances in statistical genetics allow the simultaneous examination of the genetic and environmental sources of correlations between such continuous physiological measures and discrete disease outcomes (Williams et al. 1999*b*) through the examination of data from large families. Such approaches, when coupled with modern molecular genetic technologies, will soon permit the localization and identification of the quantitative trait loci (QTLs) that underlie thrombosis risk. Prior to embarking on the potentially expensive search for the actual loci involved, it is prudent to evaluate the magnitude of genetic effects on thrombosis and to test for the pleiotropic effects of genes on both risk factors and disease.

As a first step toward the ultimate goal of the identification of novel genes involved in thrombosis susceptibility, we performed a family-based study of the genetics of thrombosis in the Spanish population. This study design has allowed us to quantify the heritability of thrombosis and to identify the joint actions of genes on thrombosis risk and a number of related quantitative phenotypes. Previous analyses of these 27 quantitative phenotypes have already demonstrated strong heritabilities for most of these traits (Souto et al. 2000). The majority of the heritabilities ranged between 0.22 and 0.55, with somewhat higher values seen for factor XII (0.67), activated protein C resistance ratio (0.71), and activated partial thromboplastin time (0.83), and somewhat lower values observed for D-dimer (0.11) and tissue factor (0.17).

Subjects and Methods

Study Population and Diagnosis

The Genetic Analysis of Idiopathic Thrombophilia (GAIT) Study is composed of 21 extended families, 12 of which were ascertained through a proband with thrombophilia and 9 of which were obtained randomly. Thrombophilia was defined as multiple thrombotic events (at least one of which was spontaneous), a single spontaneous episode of thrombosis with a first-degree relative also affected, or onset of thrombosis at age <45 years. Ten of the 12 thrombophilic probands had onset at age <45 years, 8 had multiple episodes of thrombosis, and 2 probands were ascertained on the basis of family history. Diagnoses of the thrombophilic probands were verified by objective methods. Thrombosis in these individuals was considered idiopathic because of exclusion of all biological causes of thrombosis, including antithrombin deficiency, Protein S and C deficiencies, activated protein C resistance, plasminogen deficiency, heparin cofactor II deficiency, Factor V Leiden, dysfibrogenemia, lupus anticoagulant, and antiphospholipid antibodies, known at the time of recruitment (1995–97).

A total of 398 individuals (with a mean of 19 individuals per family) were examined. Most pedigrees contained three generations, although eight families had four generations and one family had five. Subjects had a mean age at examination of 37.7 years, and there were approximately equal numbers of males and females. The composition of the families and the collection of lifestyle, medical, and family-history data have been described elsewhere (Souto et al. 2000). Reported history of thrombosis in family members was verified by examination of medical records, when available. Although some deceased family members had a history of thrombosis, only individuals interviewed and examined in person were included in the analyses. The primary residence of each subject was also determined, to assess the contribution of shared environmental influences (such as diet) common to members of a household. The study was performed according to the Declaration of Helsinki of 1975, and all adult patients provided informed consent for themselves and for their minor children.

Laboratory Measurements and Techniques

A total of 27 quantitative phenotypes were measured in the plasma of each individual. None of the participants was being treated with anticoagulant therapy at the time of blood drawing. Activated partial thromboplastin time (APTT), prothrombin time (PT), coagulation factors (FII, FV, FVII, FVIII, FIX, FX, FXI, and FXII), functional protein S, and the activated protein C-sensitivity ratio (APCR) were measured by automated coagulometry. Antithrombin, protein C, heparin cofactor II, plasminogen, and plasminogen activator inhibitor were measured by chromogenic methods. Fibrinogen was measured by the Clauss method (Clauss 1957). Total and free protein S, tissue plasminogen activator (t-PA), D-dimer (DD), tissue factor (TF), and von Willebrand factor (vWF) were assayed by use of commercially available ELISA kits. Histidine-rich glycoprotein (HRG) was measured by electroimmunoassay, tissue factor pathway inhibitor (TFPI) by a functional method (Sandset et al. 1991), and homocysteine by a fluorimetric method (Hyland and Bottiglieri 1992). ABO blood groups and Factor V Leiden genotypes were assessed by means of standard techniques. Details of phenotype assays are available in Souto et al. (2000).

Statistical Genetic Analysis

The heritability (the proportion of the total phenotypic variability attributable to genetic effects) of susceptibility to thrombosis was evaluated by means of a pedigree-based maximum-likelihood method that models affection status as a threshold process (Duggirala et al. 1997, 1999*a*; Williams et al. 1999*b*). Although disease status is usually operationalized as a discrete trait, with individuals scored as unaffected or affected, it is generally assumed that there is an unobservable continuous trait, termed "liability" or "susceptibility," that determines affection status. If an individual's liability score exceeds some specified threshold, disease results; if it is below the threshold, the individual is unaffected. The threshold is placed in an age- and sex-specific manner, to produce the appropriate population prevalence. A specific individual's liability is only known to be above or below the threshold, depending on the individual's affection status, and an integral over the appropriate region of the curve is used to estimate each person's liability value. Since such continuous processes determine most biological phenomena, it is useful to make inferences on the underlying continuous scale, which is more consistent with current models of gene action. Threshold models permit such inferences regarding the latent underlying quantitative scale to be made. To use a threshold model, some weak assumptions regarding the form of the underlying continuous process are necessary. For genetic modeling, we assume that the underlying liability distribution is normal, and we calculate the joint probability of observing the disease statuses of family members by using a multivariate normal distribution that allows for correlations among family members.

The analysis of heritability of thrombosis susceptibility was performed using the variance component method. The total phenotypic variance in thrombosis susceptibility was partitioned into three components: (1) an additive genetic variance, caused by the sum of the average effects of all the genes that influence thrombosis; (2) a shared environmental variance, caused by the effects of environmental factors that are common to households; and (3) a random environmental variance specific to each individual. The random environmental variance also absorbs nonadditive genetic effects, such as interactions between alleles within loci (dominance effects), interactions between alleles at different loci (epistatic effects), and effects caused by gene-environment interactions. Therefore, such models will generally underestimate the role of genetics in the determination of the trait.

With this approach, the relative components of variance can be estimated by use of maximum-likelihood estimation. Evaluation of the likelihood function for a pedigree involves high dimensional integration of a multivariate normal distribution. The limits of integration may be different for each individual, depending on affection status as well as on any covariates that are introduced as fixed effects in the model for the mean liability. In the current analyses, these covariates included age and sex.

To study the genetic relationships between thrombosis susceptibility and quantitative variation in hemostatic parameters, we used a new mixed discrete/continuous trait variance component analysis (Williams et al. 1999*b*). This analysis used a modified variance component method to accommodate a mixture of discrete and continuous data and allows the phenotypic correlations between these traits to be decomposed into factors caused by common genetic influences and common environmental influences on the two traits. Examination of the underlying determinants of phenotypic correlations provides information on the role of pleiotropic genetic effects.

All the extant epidemiological evidence for the relationship between thrombosis and hemostatic parameters is based on the evaluation of phenotypic correlations. However, the decomposition of phenotypic correlations into genetic and environmental components is potentially valuable, since hidden relationships between traits can be revealed (Comuzzie et al. 1996). For example, if trait $y_1 = g_1 + e_1$ and trait $y_2 = g_2 + e_2$, where g and e denote genetic and environmental effects, the observed correlations between the phenotypic traits are determined by the latent genetic and environmental correlations between the component variables. By studying both traits in extended families, we can estimate both the genetic (ρ_g) and the environmental (ρ_e) correlations between traits. The phenotypic correlation (ρ_p) is derived from these two constituent correlations and the heritabilities of the traits:

$$\rho_p = \sqrt{(h_1^2 h_2^2)}\rho_g + \sqrt{(1-h_1^2)}\sqrt{(1-h_2^2)}\rho_e \, .$$

We have incorporated the threshold model (Duggirala et al. 1997, 1999*a*) and the mixed discrete/continuous trait variance component method (Williams et al. 1999*b*) into our statistical genetic computer package, *SOLAR* (Almasy and Blangero 1998). All statistical genetic analyses were performed using *SOLAR,* with these modifications. Estimates of variance component parameters, including the heritabilities of thrombosis and the quantitative measures and all the phenotypic, genetic, and environmental correlations between thrombosis and the quantitative phenotypes, were obtained by use of maximum-likelihood estimation. All hypothesis tests were performed using likelihood-ratio test statistics (Kendall and Stuart 1972; Self and Liang 1987).

Because 12 of the 21 pedigrees were ascertained

through a thrombophilic proband, all analyses included an ascertainment correction, to allow unbiased estimation of parameters relevant to the general population. To achieve this, the likelihood for each family ascertained through a thrombophilic proband was conditioned on the phenotype of the proband (Hopper and Mathews 1982; Boehnke and Lange 1984). Since two families were ascertained, in part, because of the family history of the proband, analyses were repeated conditioning on both the original proband and the affected first-degree relative in these two families. However, the results of the analyses were unchanged.

Results

Characteristics of Affected Individuals

A total of 53 people with venous or arterial thrombosis were identified, 47 in the families ascertained through thrombophilic probands and 6 in the randomly ascertained families. The number of affected individuals per family ascertained through a thrombophilic proband was 2–8, with a mean of 3.9. The distribution of thrombotic subjects in these extended families included many instances of affected first-degree relatives (siblings or parents and children) but also grandparents, aunts or uncles, and first cousins. Eight of these families contained cases of both arterial and venous thrombosis. Two of the randomly ascertained families each had two individuals with thrombophlebitis. One of these was a parent-child pair, but the other consisted of two unrelated individuals (in-laws). One randomly ascertained family had a single individual with deep-vein thrombosis, and one had an individual with transient ischemic attacks.

There were slightly more affected females ($n = 31$, 58.5%) than males ($n = 22$, 41.5%), and the age at diagnosis of first thrombosis was 12–76 years, with a mean of 44.5 (table 1). When venous and arterial thrombosis were considered separately, 40 individuals, with an average age at first diagnosis of 39.7 years, had one or more diagnoses of venous thrombosis; 17 individuals, with an average age at first diagnosis of 61.0 years, had one or more arterial thromboses. The early observed age at diagnosis for venous thrombosis is partially a function of the ascertainment criteria. Deep-vein thrombosis was the most common condition ($n = 28$) and superficial thrombophlebitis (SFT) the second most common ($n = 14$). Fifteen (28%) of the 53 affected people had multiple thrombotic diagnoses, and five (9.4%) of these people had both venous and arterial events. Twelve individuals had deep-vein thrombosis and one to three other venous or arterial thromboses; one person had ischemic stroke and transient ischemic attacks; one person had SFT and pulmonary embolism; and one had SFT and other venous thrombosis.

Table 1

Number and Percent of Individuals in Each Diagnostic Category of Thrombosis and Age at Diagnosis

Diagnosis	No. (and %) of Individuals with Thrombosis	Mean Age at Diagnosis (years)
Venous thrombosis:		
Deep-vein thrombosis	28 (52.8)	40.3
Pulmonary embolism	9 (17.0)	45.6
SFT	14 (26.4)	41.2
Other venous thrombosis	3 (5.7)	58.0
Any venous thrombosis	40 (75.5)	39.7
Arterial thrombosis:		
Myocardial infarction	4 (7.5)	66.5
Angina pectoris	4 (7.5)	57.3
Ischemic stroke	6 (11.3)	61.0
Transient ischemic attack	5 (9.4)	55.4
Any arterial thrombosis	17 (32.1)	61.0
Any thrombosis	53 (100.0)	44.5

NOTE.—Some individuals are represented in multiple diagnostic categories.

Genetic Determinants of Liability to Thrombosis

The evidence for a strong genetic influence on risk of thrombosis was striking. Liability to thrombosis exhibited an additive genetic heritability of 0.61 ± 0.16 ($P = 9 \times 10^{-5}$), indicating that, after correction for the effects of age and sex, 61% of the variation in liability to thrombosis at the population level can be attributed to genetic factors. No shared environmental effects were found among members of a household for liability to thrombosis. Therefore, the above heritability estimates are unlikely to be inflated by nongenetic correlations among family members, and environmental factors shared by members of a household, such as diet, do not have major effects on thrombosis susceptibility. When the diagnoses considered are restricted to venous thrombosis, excluding arterial thrombotic events, the additive genetic heritability is not significantly different from that obtained with any thrombosis. Similarly, when venous and arterial thrombosis are analyzed jointly as two distinct traits, the phenotypic correlation between these two manifestations of thrombosis is .333 ($P = .0126$), and the genetic correlation is .55 ($P = .09$). Additionally, the genetic correlation is not significantly different from 1. Both the robustness of the heritability when combining across venous and arterial diagnoses and the fact that the genetic correlation is not significantly different from one strongly suggest that arterial and venous thromboses are highly genetically correlated and that our broad phenotypic characterization will be useful to increase the power to detect genetic effects.

Ref. 12

Correlations between Thrombosis Liability and Quantitative Risk Factors

Table 2 shows the results of bivariate genetic analyses of thrombosis, with each of the quantitative physiological traits considered. Only the nine quantitative traits showing at least one significant correlation ($P < .05$) are presented. Of these, seven exhibit significant phenotypic correlations with thrombosis susceptibility, eight demonstrate significant genetic correlations with thrombosis, and only two exhibit significant environmental correlations. The largest phenotypic correlations ($|\rho_p| > 0.2$) are seen between FVIII, vWF, APCR, FXI, homocysteine, and thrombosis.

The genetic correlations provide strong evidence for significant pleiotropy underlying the covariation between several of the quantitative traits and thrombosis risk. Those quantitative measures exhibiting the largest genetic correlations ($|\rho_g| > 0.6$) with thrombosis include vWF, t-PA, FVIII, homocysteine, and APCR. The only traits to exhibit significant environmental correlations with thrombosis were APCR and FVII. Table 2 provides a good demonstration of how low-phenotypic correlations may misrepresent the true underlying relationships. Both FIX and FVII failed to show significant phenotypic correlations with thrombosis. However, both provide strong evidence for correlations between genetic effects (FIX) and environmental effects (FVII) with thrombosis. Similarly, the genetic and environmental correlations between APCR and thrombosis are of similar magnitudes but exhibit different directions. When such differences in sign appear, the phenotypic correlation is attenuated, although the underlying components suggest much stronger correlations. Relationships between APCR and thrombosis were unchanged when the presence of the Factor V Leiden mutation (there were nine heterozygotes in the sample) was statistically controlled. Similarly, the correlations between FVIII and thrombosis were unchanged when ABO blood type was incorporated into the model.

Discussion

This is the first study that formally documents the large genetic component for risk of thrombosis. By gathering and analyzing data on extended pedigrees that have been methodically ascertained to allow general population inferences, we have begun to fill a critical gap in the study designs used in thrombosis genetics. Researchers in hemostasis/thrombosis generally have not actively pursued family studies, except for the occasional serendipitous collection of unusual families with high densities of affected individuals. Therefore, most of our knowledge regarding the genetic factors involved in common thrombosis has been limited to association studies that use case-control designs to look at known polymorphic variations in candidate genes (Poort et al. 1996; Rosendaal 1997; Rosendaal et al. 1997; Iacoviello et al. 1998). Although such studies provide important indirect evidence for the presence of genetic effects, they have a number of weaknesses. These include their limitation to known candidate genes, their propensity for type I errors caused by hidden population stratification, the lack of direct evaluation of familial transmission, and their general inability to reliably estimate the relative importance of genetic factors in determining within-population variation in thrombosis risk. Family-based studies eliminate these problems, although their costs tend to be greater.

The high additive genetic heritability that we estimated suggests that whole-genome approaches to localizing and characterizing QTLs that underlie thrombosis susceptibility will be feasible. The magnitude of the additive genetic heritability is greater than or equal to that seen in other common complex diseases such as type II diabetes (Duggirala et al. 1999*a*), gallbladder disease (Duggirala et al. 1999*b*), alcoholism (Williams et al. 1999*a*), and obesity (Comuzzie et al. 1997), whose contributing QTLs are currently being pursued through genome scans.

This is also the first study that attempts to decompose the phenotypic correlations between quantitative physiological risk factors and thrombosis into genetic and environmental components. Evidence for strong genetic correlations between FVIII, vWF, APCR, FIX, FXI, homocysteine, t-PA, and thrombosis indicate that there are sets of genes that jointly influence both disease risk and quantitative physiological variation. The detection of genetic effects that act jointly on both quantitative risk factors and disease liability is critically important for subsequent genetic analyses. When evidence of pleiotropy is detected, the correlational structure between the quantitative phenotypes and risk of thrombosis can be exploited to improve the power of joint linkage anal-

Table 2

Phenotypic, Genetic, and Environmental Correlations of Quantitative Risk Factors with Thrombosis

Phenotype[a]	ρ_p	P[b]	ρ_g	P	ρ_e	P[b]
APCR	−.230	.0003	−.650	1×10^{-6}	.669	.0006
FVII	.025	NS	−.354	.0564	.568	.0091
FVIII	.288	.0002	.689	.0005	−.126	NS
FIX	.151	.0787	.597	.0131	−.198	NS
FXI	.209	.0180	.564	.0245	.070	NS
FXII	.172	.0339	.351	.0500	−.145	NS
Homocysteine	.227	.0018	.652	.0015	−.028	NS
t-PA	.180	.0002	.752	.0070	−.099	NS
vWF	.261	.0010	.729	.0005	−.181	NS

[a] Only phenotypes with one or more correlations having $P < .05$ are shown.

[b] NS = nonsignificant ($P > .10$).

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yses to detect QTLs contributing to thrombotic risk (Almasy et al. 1997).

Most of our observed phenotypic correlations are consistent with known epidemiological results. For example, there is previous evidence for a positive relationship between both vWF and FVIII levels and risk of venous (Koster et al. 1995) and arterial thrombosis (Folsom et al. 1997). High plasma homocysteine levels have been associated with deep-vein thrombosis (den Heijer et al. 1996) and with arterial thrombosis (Nygard et al. 1997). The quantitative measure of APCR is correlated with risk of venous thrombosis, even when the Factor V Leiden polymorphism is taken into account (De Visser et al. 1999). Similarly, levels of FXII (Kohler et al. 1998) and t-PA (Ridker et al. 1993; Carter et al. 1998) have been correlated with arterial thrombosis. Evidence regarding the association of FVII levels with thrombosis has been equivocal (Doggen et al. 1998; Iacoviello et al. 1998). Very recently, results from the LETS study have implicated high plasma levels of factor IX (Vlieg et al. 2000) and factor XI (Meijers et al. 2000) as risk factors for venous thrombosis.

The unique aspects of our correlational analyses lie in the ability to disentangle genetic and environmental sources of correlation. This technique allows us, for the first time, to conclude that most of the phenotypic correlations between thrombosis susceptibility and the quantitative physiological measures are due to pleiotropic effects of genes. There is little evidence that environmental effects induce much of the observed phenotypic correlations. In the two cases where we did observe significant environmental correlations, they were opposite in sign to the genetic correlations. Other investigators have reported similar results from bivariate genetic analyses of a wide variety of traits (e.g., Comuzzie et al. 1996; Brooks 2000; Mahaney et al. 2000; Stern et al. 2000). One interpretation of the difference in sign is that the genetic and environmental sources of variation on these traits act through different physiological mechanisms.

In this study, we have chosen a broad definition of thrombosis that includes both venous and arterial forms. Our justification for this is both empirical and theoretical. Pooling two genetically heterogeneous traits would decrease the genetic signal-to-noise ratio of the composite trait. However, our heritability analyses provided no evidence for such a depression in genetic signal, indicating that there must exist substantial overlap in the genetic determinants of venous and arterial forms of thrombosis. Similarly, the bivariate analysis of venous and arterial thrombosis yielded a genetic correlation not significantly different from 1 and suggests that many of the same genes are involved in the pathogenesis of venous and arterial events. Additionally, there is epidemiological evidence that similar pathways are involved in venous and arterial thrombosis, as evidenced by the correlation between critical risk factors (such as homocysteine, vWF, and FVIII) and both venous and arterial thrombosis. Although unique local environmental factors can separately influence thrombogenesis in veins and arteries, the evidence suggests that much of the underlying process is driven by a common set of genes. Pooling of these two categories of thrombosis clearly improved the power of the present study. However, even if we disaggregate these components and analyze only venous thrombosis, our results are effectively unchanged (data not shown) except for predictable alterations in observed significance values resulting from the decreased overall prevalence of disease.

Finally, these results provide strong support for using genome scans to localize and evaluate the specific QTLs involved in thrombosis susceptibility. We hope to use the information on the genetic correlations between thrombosis and quantitative phenotypes obtained in this study to maximize our potential for mapping the responsible QTLs in a genome scan currently under way.

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Venous thromboembolism risk assessment with a multilocus genetic risk score

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Background

In the development of venous thromboembolism (VTE) genetics contribute in a relevant manner. In clinical routine the presence of two mutations Factor V Leiden (FVL) and G20210A Prothrombin (PT) are analysed to evaluate this genetic contribution. However, new and relevant genetic variants have been associated with VTE. Though these genetic variants have not been properly validated neither translated to clinical practise.

Aim

To evaluate whether the use of Genetic Risk Scores (GRS) with these new variants provides a better assessment of the VTE risk than a model based only on FVL-PT .

Methods

Three panels of genetic variants were compared: FVL+PT; TIC panel (FVL, PT, ABO group A1 carriers, FV Cambridge, FV Hong Kong, rs2232698 SerpinA10 gene, rs121909548 SerpinC1 gene, 46C>T F12, and rs5985 F13 gene); and TIC panel plus F11 (TIC panel, rs2289252 and rs2036914 in F11 gene). All these variants influence the coagulation pathway.

For each panel a multi-locus GRS was computed for each individual as the sum of the number of risk alleles, after weighting them by its effect size published in the literature. The GRS was validated in two case-control studies. MARTHA: 1,150 cases (347 males, 803 females; 38.0±13.9 years old), 801 controls (383 males, 418 females; 47.4±14.0 years old) designated to assess the association of FVL and PT with other risk factors; and Sant Pau (SP): a study with Spanish population with 249 cases (111 males, 138 females; 47.1±14.0 years old), 248 controls (109 males, 139 females; 49.0±14.9 years old).

All models were adjusted by age and sex. The predictive capacity was assessed by the discrimination of the different GRS calculating the c-statistic (AUC-ROC); and by the reclassification when using the new GRSs compared with FVL+PT calculating the NRI (net reclassification improvement) and IDI (integrated discrimination improvement). Informed consent was obtained and the studies were

Results

When compared to FVL+PT, the use of TIC or TIC+F11 panels significantly improved the capacity to discriminate VTE in both populations (AUC-ROC: 0.57 vs 0.68 and 0.67 respectively, in SP and 0.56 vs 0.57 and 0.58 respectively, in MARTHA population,). When compared to the risk stratification by FVL+PT, only the use of TIC panel improved the capacity to reclassify both cases and controls in both populations (NRI, 19.2, p<0.005 and 4.9, p-value >0.05, IDI 5.5, p<0.001 and 3,2, p-value<0.01 for TIC panel vs FVL+PT in SP and MARTHA populations, respectively). Moreover, clinical sensitivity (number of cases where a genetic thrombophilia was demonstrated) increases very significantly in relation to FVL+PT (19.7% and 50.4% of the cases for FVL+PT, 87.3% and 95.1% for TIC in SP and MARTHA populations, respectively). TIC+F11 did not further improve the reclassification.

Conclusions

TIC panel significantly improves the predictive capacity of VTE risk, by improving discrimination and reclassification when compared to FVL+PT. Thus, our study suggests that the use of algorithms with a set of confirmed susceptibility loci (TIC) improves disease risk assessment and could be also an aid in the prevention, diagnosis and treatment of VTE disease.

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FAMILY HISTORY AND GENETIC RISK SCORES IN VENOUS THROMBOEMBOLISM

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INTRODUCTION

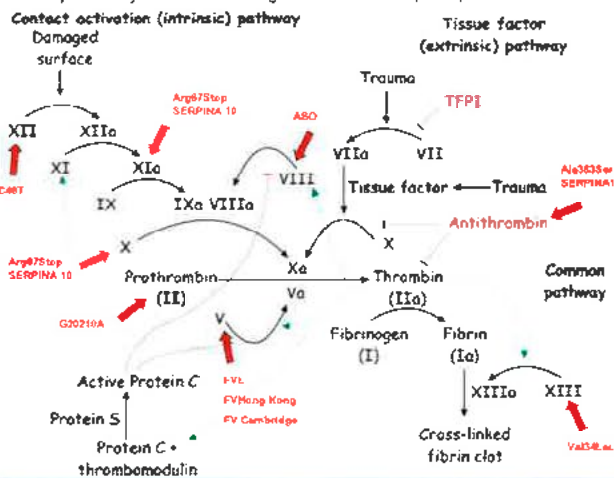
Venous thromboembolism (VTE) is an important cause of mortality and morbidity worldwide and the main preventable cause of mortality in hospitals[1]. Each year around 600.000 VTE-related deaths happens in the United States and Europe, many cases of them being only diagnosed at autopsy[1,2]. Deep vein thrombosis (DVT) affects approximately two million Americans annually, while pulmonary embolism (PE) is the most common cause of preventable hospital death accounting for 60.000 deaths in the United States every year[3].

VTE is a multifactorial disease with many known genetic and acquired risk factors. Family history (FH) of VTE increases the risk in first-degree relatives and it has been promoted as a very useful tool for risk assessment, even more than genetic testing[4]. In clinical routine the presence of two mutations Factor V Leiden (FVL) and G20210A Prothrombin (PT) are analysed to evaluate the genetic contribution to VTE. However, new and relevant genetic variants have been associated with VTE. The measurement of these new genetic factors could provide a better method in the assessment of VTE risk.

Given the high incidence and recurrence rates of VTE, a better identification of patients at risk of thrombosis might improve the preventive strategy and decrease the incidence of DVT and associated complications.

OBJECTIVES

The objective of this study was to evaluate whether the VTE risk assessment capability of FH can be improved by the addition of genetic risk score (GRS).



METHODS

Case Control Population

The study was conducted in a population of 249 VTE cases (111 males, 138 females; 47.1±14.0 years old) and 248 controls (109 males, 139 females; 49.0±14.9 years old). Informed consent was obtained and the study was approved by the Ethics Committee.

Genetic profile analyzed

Two multi-locus GRSs were used (Figure 1): a) FVL+PT; b) TiC panel: FVL, PT, ABO group A1 carriers, FVC, FVHK, Serpin A10, Serpin C1, 46C>T factor XII, and Val34Leu factor XIII. All these variants influence the coagulation pathway.

For each panel a multi-locus GRS was computed for each individual as the sum of the number of risk alleles, after weighting them by its effect size published in the literature.

Genotyping of FVL+PT was performed by TaqMan SNP Genotyping Assay

Genotyping of TiC panel was performed by using THROMBOINCODE test kit. THROMBOINCODE is and IVD-CE marked kit for the simultaneous allele determination of TiC panel in genomic DNA, extracted from either saliva or blood samples. The calculated sensibility and specificity of the kit is ≥98% per SNP.

Statistical Analysis

All models were adjusted by age and sex. The predictive capacity was assessed by the discrimination of the different GRS calculating the c-statistic (AUC-ROC); and by the reclassification when using the GRSs compared with FH calculating the NRI (net reclassification improvement) and IDI (integrated discrimination improvement).

RESULTS

When compared to FH, the addition of FVL+PT or TIC panels significantly improved the capacity to discriminate VTE (Table: AUC-ROC: 0.59 vs 0.65 and 0.7, respectively).

Both GRSs, TIC to a higher degree, improved the capacity to reclassify both cases and controls (NRI: 16, p<0.001 and 29.4, p-value <0.01, IDI: 3.4 p<0.001 and 6.6 p-value<0.001 for FVL+PT and TIC, respectively), when compared to the risk stratification by FH.

Table: c-statistic and reclassification [NRI (net reclassification improvement) and IDI (integrated discrimination improvement)] comparing the use of the GRSs to FH

	c-statistic	NRI	IDI
FH	0.59	ref	ref
FVL+PT	0.65	16	3.4
TiC panel	0.70	29.4	6.6
P-value	<0.001	<0.001	<0.001

CONCLUSIONS

TIC panel significantly improves the predictive capacity of VTE risk, by improving discrimination and reclassification when compared to FH or FH+FVL+PT. Thus, our study suggests that the use of algorithms with a set of confirmed susceptibility loci (TIC) improves disease risk assessment and could be also an aid in the prevention, diagnosis and treatment of VTE disease.

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Human Genome Epidemiology (HuGE) Review

Factor XIII Val34Leu Variant Is Protective against Venous Thromboembolism: A HuGE Review and Meta-Analysis

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It has been suggested that a *G*-to-*T* transition in exon 2 of the *factor XIIIA* gene resulting in a substitution of leucine for valine at amino acid 34 (*FXIII Val34Leu*) protects against venous thromboembolism (VTE). However, the evidence to date is insufficient to incorporate testing for the *FXIII Val34Leu* variant into clinical practice. To determine whether genotypes with the *FXIII Val34Leu* variant are protective against VTE, the authors performed a meta-analysis of 12 studies with genotyping for the *FXIII Val34Leu* variant (3,165 objectively diagnosed VTE cases and 4,909 controls). When a random-effects model was used, the combined odds ratios for VTE were 0.63 (95% confidence interval: 0.46, 0.86) for the homozygotes of the *FXIII Val34Leu* variant, 0.89 (95% confidence interval: 0.80, 0.99) for the heterozygotes, and 0.85 (95% confidence interval: 0.77, 0.95) for the homozygotes and heterozygotes combined. Potential sources of heterogeneity and potential bias were explored. The meta-analysis provided evidence that the *FXIII Val34Leu* variant has a small, but significant protective effect against VTE. Since VTE is a complex disorder, this information, along with results of ongoing studies to identify additional genetic factors underlying VTE, will be crucial in developing accurate risk profiles to identify individuals at higher risk of VTE.

epidemiology; factor XIII; *FXIII Val34Leu*; genetics; leucine; meta-analysis; valine; venous thrombosis

Abbreviations: CI, confidence interval; FXIII, factor XIII; FXIIIA, factor XIII A subunit; *FXIII Val34Leu*, *factor XIII Valine34Leucine* variant; Leu, leucine; Val, valine; VTE, venous thromboembolism.

Editor's note: *This paper is also available on the website of the Human Genome Epidemiology Network (http://www.cdc.gov/genomics/hugenet/).*

GENE

Factor XIII (FXIII), also called fibrin stabilizing factor, has a crucial role in the blood coagulation and fibrinolytic pathways. Plasma FXIII is an inactive enzyme precursor that circulates in plasma in the form of two pairs of non-identical A and B subunits. The gene encoding for the B subunit of FXIII has been assigned to chromosome 1q31-32 (1) and helps stabilize and transport the A subunit in plasma (2). The gene coding for the A subunit of FXIII (*FXIIIA*) is localized on chromosome 6p24-25. FXIIIA exhibits the transglutaminase activity (i.e., the activated form of FXIII), which, along with thrombin-activated fibrinolysis inhibitor, is involved in stabilizing the fibrin clot and making the clot more lysis resistant (3).

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Mutations in the *FXIIIA* gene have been associated with FXIII deficiency, which in turn is associated with the tendency toward spontaneous bleeding and several related morbid events (4, 5). Despite being associated with severe bleeding in some cases, several of these mutations have little impact at the population level because they are rare (4, 6). The *FXIII Val34Leu* variant is common, however, and has also been associated with higher transglutaminase activity, leading to decreased clot formation (7–10).

GENE VARIANT

The *FXIII Val34Leu* variant is a *G*-to-*T* transition in exon 2 of the gene encoding for FXIIIA, leading to a valine (Val)-to-leucine (Leu) substitution at amino acid 34 (5). This variant is common in White populations, with a frequency of approximately 0.25–0.30 (11–13). However, the frequency varies among ethnic groups, with the lowest (0.01) in Japanese and the highest (0.40) in Pima Indians (2).

The biochemical consequences of the *FXIII Val34Leu* variant are not well understood. The *FXIII Val34Leu* variant does not result in a change in the plasma concentration of FXIII, but the amino acid change may modify FXIII activity (7, 9, 14, 15). Activation of FXIIIA by thrombin was found to proceed two- to threefold more rapidly in plasma of *FXIII Val34Leu* variant carriers (8–10, 16). This action has an effect on clot stability since the catalytic efficiency of thrombin-induced cleavage of FXIIIA alters the structure of the cross-linked fibrin such that fibrin fully cross-linked by *FXIII Val34Leu* product has a finer structure with thinner fibers and smaller pores. Lateral aggregation of fibrin fibers is impaired (17).

DISEASE

A disruption in normal hemostasis that maintains blood fluidity and prevents blood loss could result in the formation and growth of thrombi that can obstruct venous circulation and may embolize. These common diseases are referred to as deep vein thrombosis and pulmonary embolism, collectively known as venous thromboembolism (VTE). Clinical implications of VTE include morbidity from pain, swelling, or dyspnea from pulmonary embolism; bleeding risk from anticoagulant treatment to prevent recurrent events; post-phlebitic syndrome; and death from pulmonary embolism, which, in many cases, is sudden. Although a number of acquired and inherited predisposing factors are known, we cannot accurately identify which persons will experience a VTE. Accurate identification of patients at risk would be of great clinical utility since one third of cases of VTE are the more serious pulmonary emboli and approximately 22 percent of patients with pulmonary embolism die before being diagnosed (18–20).

Inherited thrombophilia is the term applied to the genetic predisposition to VTE. Thrombophilia is a highly prevalent problem, with more than 10 percent of the population affected by one of the currently known genetic risk factors, the common ones being *factor V Leiden* and *Prothrombin G20210A*, and it is identified in at least 50 percent of cases of VTE (21). However, approximately 50 percent of familial cases are unexplained despite accounting for known genetic or acquired thrombophilias. Furthermore, many patients with inherited thrombophilia never develop a VTE, suggesting the existence of protective factors. Thus, the mechanisms underlying VTE are still largely unknown, so the individual risk profile remains largely unpredictable.

Several case-control studies have suggested a protective effect of the *FXIII Val34Leu* variant against VTE (22–25). However, most studies had sample sizes insufficient to detect statistically significant differences, while a few others reported no effect of this variant on the risk of VTE (9, 26, 27). Variants with a protective effect are of interest because they provide insight into the biochemical pathways conferring the beneficial effect against VTE. Identifying such variants is also useful in establishing risk profiles to identify those at risk of developing VTE.

We conducted a meta-analysis to determine whether genotypes with the *FXIII Val34Leu* variant confer protection against VTE. Results from this meta-analysis suggest that incorporation of the *FXIII Val34Leu* variant will be useful in developing risk-profiling tools to assess the risk of VTE.

META-ANALYSIS METHODS

Identification and eligibility of relevant studies

We considered all studies (published in full or in abstract form) that examined the association between the *FXIII Val34Leu* variant and VTE. Electronic databases were searched by using the OVID search engine (Ovid Technologies, Inc., New York, New York) for MEDLINE (National Library of Medicine, Bethesda, Maryland) (1966 to week 2 of June 2004) and EMBASE (Elsevier B.V., the Netherlands). The search strategy was based on combinations of the terms "venous thromboembolism," "factor XIII," "valine," and "leucine." This electronic search was followed by a manual search of reference lists from retrieved articles, symposia proceedings, and abstracts from major thrombosis conferences. The search was not limited to the English language.

Studies were included if the distribution of genotypes for the *FXIII Val34Leu* variant was reported for patients with objectively diagnosed VTE and for a control population (i.e., without VTE). Objective diagnosis of VTE was based on results of ultrasonography, phlebography, high-probability ventilation-perfusion lung scan with a moderate-to-high pretest clinical probability, spiral computed tomographic scan with contrast medium, or pulmonary angiography.

Data extraction

Two examiners independently extracted data from the studies by using a standardized form. Any disagreements were resolved by discussion or in consultation with a third examiner. The collected information from each report included the authors, journal and year of publication, country of origin, selection and characteristics of VTE cases and controls, demographics, ethnic group of the study

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population, and number of cases and controls for each *FXIII* genotype.

Meta-analysis

Prior to pooling the studies for the meta-analysis, Hardy-Weinberg equilibrium was assessed in the control groups of individual studies by using the goodness-of-fit χ^2 statistic with one degree of freedom. A two-sided p value of >0.05 was considered consistent with Hardy-Weinberg equilibrium. The choice of the comparison groups for the meta-analysis was based on the hypothesis that the *Leu* allele (i.e., *factor XIII Val34Leu*) has a protective effect against VTE. Therefore, we performed three comparisons, with the *Val/Val* genotype as the reference group: *Leu/Leu* genotype, *Leu/Val* genotype, and both groups together (*Leu/Leu* + *Leu/Val* combined). We used the odds ratio with a 95 percent confidence interval as the metric of risk.

For each comparison, we tested the between-study heterogeneity by using the Breslow-Day χ^2 statistic (28). Because tests for heterogeneity may have low power when the sample sizes are small, as in several of the studies to be included in our meta-analysis, we chose to be conservative by using a two-sided p value of <0.10 as the threshold for considering the test significant for heterogeneity, as suggested by Attia et al. (29). We also used the I^2 statistic, which describes the percentage of variability in point estimates due to sample heterogeneity rather than sampling error (30, 31). I^2 values from 31 percent to 56 percent have been defined as indicating "low" to "moderate" heterogeneity, whereas values greater than 56 percent are considered indicative of "notable" heterogeneity (31). We chose to use the DerSimonian-Laird random-effects model (32) to combine the data because the selection criteria were not entirely identical among the eligible studies and because heterogeneity tests may lack power in some contexts. This approach incorporates an estimate of the between-study variance; therefore, the confidence intervals tend to be wider when the studies differ among themselves. Again, this approach leads to more conservative estimates (33).

Because the frequency of the *FXIII Val34Leu* variant differs substantially across ethnic groups (2), and because the large majority of the study subjects were White, we also performed a subgroup analysis that excluded the two studies that clearly enrolled non-Whites (23, 34), and, for one paper, we included the data generated for Whites only (35). In studies that did not describe the ethnicity of the population clearly, we contacted the authors. Furthermore, two studies ascertained their cases quite differently from the other studies; for example, the cases were further ascertained for known thrombophilias (9, 27), and, in one study, the genotype distribution in the control group was not in Hardy-Weinberg equilibrium (23). To verify whether this affected the results, we reanalyzed the data by also excluding these studies. Funnel plots were generated to assess publication bias, particularly whether large studies lead to different results than smaller studies (36). Statistical analysis was performed with SAS software (version 8.2; SAS Institute, Inc., Cary, North Carolina).

META-ANALYSIS RESULTS

Eligible studies

Characteristics of the 12 studies included in the meta-analysis (9, 17, 22–27, 34, 35, and 37; P. Wells, University of Ottawa, unpublished manuscript) are shown in table 1. The studies were conducted on three continents: Europe, North America, and South America; six studies were reported to be multicenter. More than 80 percent of the subjects in each individual study were White. These studies were not entirely uniform with respect to case and control selection. Five studies were limited to cases with isolated deep vein thrombosis (9, 17, 23, 26, 34), and seven studies included cases with deep vein thrombosis and/or pulmonary embolism (22, 24, 25, 27, 35, and 37; P. Wells, unpublished manuscript). Only one study limited cases to idiopathic VTE (P. Wells, unpublished manuscript), while an additional three studies excluded patients with malignancy (9, 23, 34). Six studies used upper age limits in the selection of cases; these studies included only those patients with VTE prior to age 45 years (9) or patients less than age 70 years (17, 34, 35). Compared with the rest of the studies, those by Balogh et al. (9) and Margaglione et al. (27) are the most different with regard to the ascertainment of cases because they focused on groups that may have been "extreme" VTE cases.

Meta-analysis database

The distribution of the *FXIII* genotypes among VTE cases and controls in the eligible studies is presented in table 2. A total of 8,074 subjects with genotype data were available, with 3,165 VTE cases and 4,909 controls. The distribution of genotypes in control groups was consistent with Hardy-Weinberg equilibrium in 11 of the 12 studies. The control group genotypes in the Franco et al. (23) study were not in Hardy-Weinberg equilibrium, with $\chi^2 = 6.42$ and $p < 0.02$.

Overall effects

Our results suggest that the *FXIII Val34Leu* variant confers a small, but significant protective effect against VTE (figures 1–3). When a random-effects model was used, the *Val/Val* genotype being the reference group, the combined odds ratios for VTE were 0.63 (95 percent confidence interval (CI): 0.46, 0.86) for the homozygotes (*Leu/Leu* vs. *Val/Val*) (figure 1), 0.89 (95 percent CI: 0.80, 0.99) for the heterozygotes (*Leu/Val* vs. *Val/Val*) (figure 2), and 0.85 (95 percent CI: 0.77, 0.95) for *Leu/Val* and *Leu/Leu* (figure 3) vs. *Val/Val*. There was no statistically significant between-study heterogeneity for any of the comparison groups as assessed by the Breslow-Day test, with p values of >0.4. The I^2 statistic was consistent with these results, suggesting only "very low" (10 percent) to "moderate" heterogeneity.

Bias diagnostics

Removing the two studies that clearly enrolled non-Whites (23, 34) did not affect the results. The odds ratios remained consistent with a protective effect against VTE

TABLE 1. Characteristics of studies included in the meta-analysis

First author, year (reference no.)	Country	Mean age (years)		Subjects (no.)		Selection	
		Cases	Controls	Cases	Controls	Cases	Controls
Catto, 1999 (22)	United Kingdom	62.0*	60.0*	217	252	Clinical VTE† diagnosis	No personal or family history of VTE
Franco, 1999 (23)	Brazil	41.0	41.0	189	187	Aged <65 years at first DVT† episode	Unrelated; asymptomatic; healthy; no history of VTE
Corral, 2000 (26)	Spain	60.9	60.8	97	97	Confirmed diagnosis of DVT	No history of vascular disease
Renner, 2000 (25)	Austria	53.2	53.2	154	308	Admitted with a documented DVT	No history of VTE or arterial disease
Balogh, 2000 (9)	Hungary	35.2	30.3	273	288	Patients with VTE before age 45 years; family history of VTE or recurrent thrombosis or unusual location	Healthy volunteers; no personal or family history of VTE, arterial disease, or malignancy
Margaglione, 2000 (27)	Italy	45.0*	36.0*	427	1,045	Referred for thrombophilia workup; DVT and PE† diagnosed objectively	Healthy; no clinical history of VTE
Alhenc-Gelas, 2000 (24)	France	42.6	42.7	354	1,229	Aged <61 years; objectively diagnosed DVT and/or PE	No history of VTE, arterial disease, or malignancy
van Hylckama Vlieg, 2002 (17)	The Netherlands	N/R†	N/R	471	474	Aged <70 years; referred for anticoagulation treatment; first objectively diagnosed DVT	No history of VTE or malignancy; no use of OACs† for at least the prior 3 months; same geographic area
Dowling, 2003 (35)	United States	49.2‡	49.5‡	190	157	Aged 18–70 years; hospitalized for VTE	No history of VTE, mental/physical problems, or anticoagulant use
Zidane, 2003 (37)	The Netherlands	54.9	47.4	66	148	Objectively confirmed PE	Suspected PE confirmed as absent (internal control group)
Wells, 2004 (unpublished manuscript)	Canada	56.2	56.4	309	306	Objectively confirmed, idiopathic VTE	Healthy friends; no history of VTE, malignancy, or use of OACs for at least the prior 3 months
Pintao, 2004 (34)	Brazil	42.0	42.0	418	418	Aged <70 years; no history of malignancy; objectively diagnosed DVT	Healthy blood donors; no history of VTE, arterial thrombosis, or malignancy

* Median age.
† VTE, venous thromboembolism; DVT, deep vein thrombosis; PE, pulmonary embolism; N/R, not reported; OACs, oral anticoagulants.
‡ Mean age reported for the total population.

associated with the *FXIII Val34Leu* variant. Compared with those for the *Val/Val* genotype group, the odds ratios were 0.75 (95 percent CI: 0.58, 0.97) for the *Leu/Leu* genotype group, 0.88 (95 percent CI: 0.78, 0.99) for the *Leu/Val* genotype group, and 0.86 (95 percent CI: 0.76, 0.98) for the combined *Leu/Leu* and *Leu/Val* genotype groups. Between-study homogeneity remained when these two studies were excluded from the meta-analysis. Finally, reanalysis of the data excluding the two studies mentioned above (23, 34) and the two studies that ascertained cases by using either very low upper age limits and/or persons with known thrombophilia (9, 27), or those studies not in Hardy-Weinberg equilibrium (23), did not change our conclusion. Again, the odds ratios remained consistent with a protective effect against VTE associated with the *FXIII Val34Leu* variant. Compared with those for the *Val/Val* genotype group, the odds ratios were 0.61 (95 percent CI: 0.46, 0.80) for the *Leu/Leu* genotype group, 0.84 (95 percent CI: 0.72, 0.97) for the *Leu/Val* genotype group, and 0.80 (95 percent CI: 0.71, 0.92) for the combined *Leu/Leu* and *Leu/Val* genotype groups. Between-study homogeneity remained when these studies were excluded from the meta-analysis, with the I^2 statistic much below 30 percent. Publication bias was also unlikely as demonstrated by the funnel plot analysis, which showed symmetric odds ratios against study sample size (data not shown).

DISCUSSION

To our knowledge, our meta-analysis of 12 studies, involving over 3,000 genotyped cases and about 5,000 controls, provides the most comprehensive assessment so far of the association of the *FXIII Val34Leu* variant with VTE. It

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TABLE 2. Distribution of the *factor XIII* genotypes* among venous thromboembolism cases and controls in the included studies

First author, year (reference)	*Val/Val*				*Val/Leu*				*Leu/Leu*			
	Cases		Controls		Cases		Controls		Cases		Controls	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Catto, 1999 (22)	137	29	123	26	68	15	107	23	12	2	22	5
Franco, 1999 (23)	116	31	110	29	70	18	59	16	3	1	18	5
Corral, 2000 (26)	61	31	66	34	34	18	28	14	2	1	3	2
Renner, 2000 (25)	97	21	163	35	49	11	121	26	8	2	24	5
Balogh, 2000 (9)	144	26	158	28	111	20	111	20	18	3	19	3
Margaglione, 2000 (27)	276	19	677	46	123	8	319	22	28	2	49	3
Alhenc-Gelas, 2000 (24)	225	14	728	46	115	7	437	28	14	1	64	4
van Hylckama Vlieg, 2002 (17)	286	30	273	29	165	18	174	18	20	2	27	3
Dowling, 2003 (35)	111	32	86	25	69	20	53	15	10	3	18	5
Zidane, 2003 (37)	36	17	80	37	27	13	55	26	3	1	13	6
Wells, 2004 (unpublished manuscript)	184	30	164	27	110	18	122	20	15	2	20	3
Pintao, 2004 (34)	261	31	236	28	148	18	157	19	9	1	25	3
Total	1,934		2,864		1,089		1,743		142		302	

* *Val*, valine; *Leu*, leucine.

supplies evidence for a protective effect of the *FXIII Val34-Leu* variant against VTE in both homozygotes and heterozygotes for the variant, with significantly lower odds ratios. The protective effect was stronger for the homozygotes alone than for the heterozygotes alone or combined with the homozygotes. This conclusion was further supported

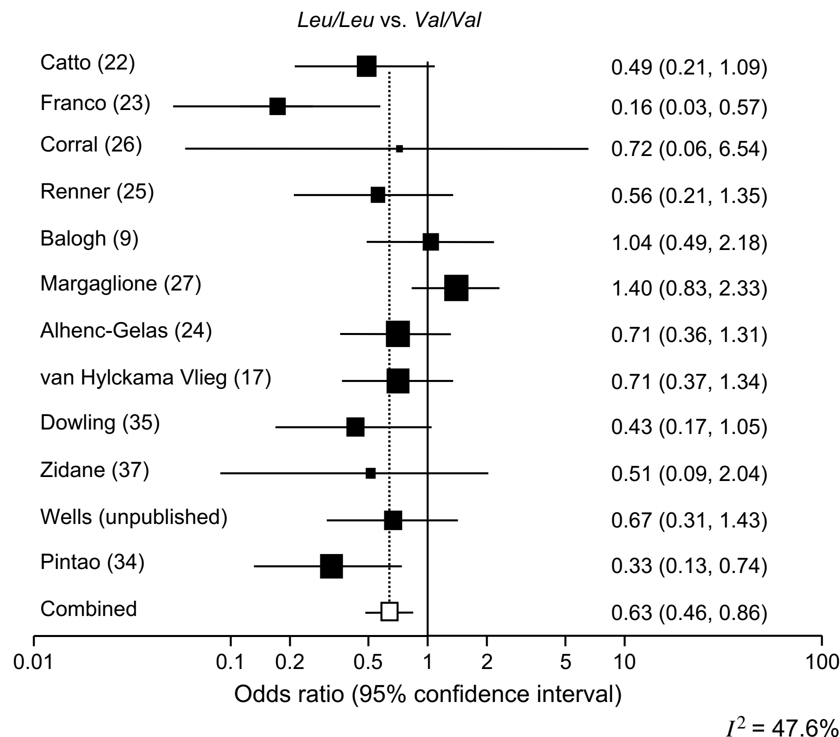

FIGURE 1. Odds ratio (95% confidence interval) for the association between the *Leu/Leu* genotype vs. the *Val/Val* genotype of the *FXIIIA* gene and venous thromboembolism. On the left, the first author of the study is followed by the reference number in parentheses. The size of the black box corresponding to each study is proportional to the sample size; the horizontal lines show the 95% confidence interval of the odds ratio. The combined estimate is based on a random-effects model shown by the dashed vertical line and white box. The solid vertical line represents the null result: an odds ratio of 1. The I^2 statistic is shown in the bottom right corner.

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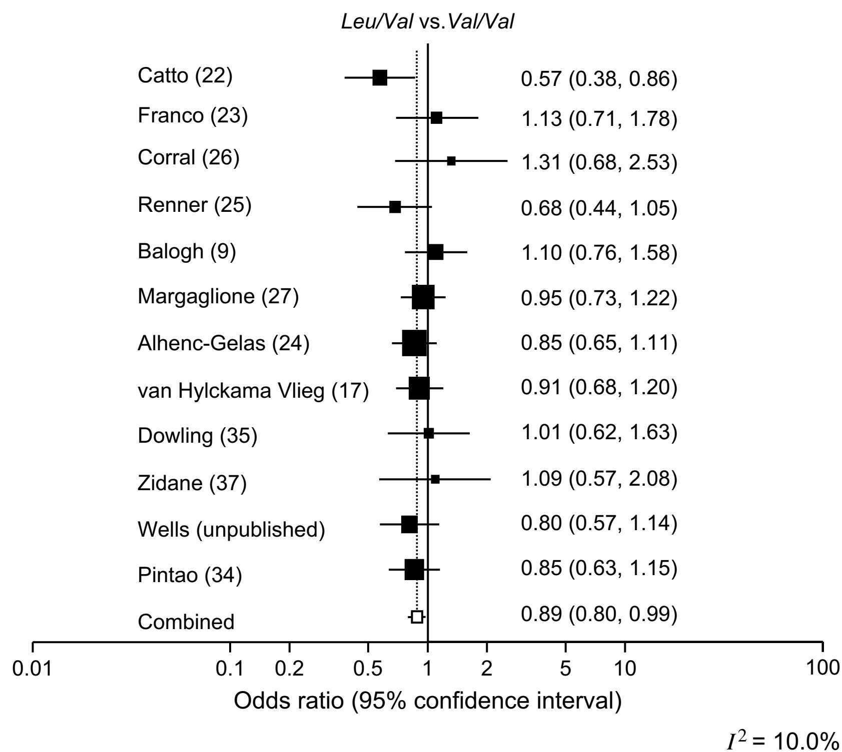

FIGURE 2. Odds ratio (95% confidence interval) for the association between the *Leu/Val* genotype vs. the *Val/Val* genotype of the *FXIIIA* gene and venous thromboembolism. On the left, the first author of the study is followed by the reference number in parentheses. The size of the black box corresponding to each study is proportional to the sample size; the horizontal lines show the 95% confidence interval of the odds ratio. The combined estimate is based on a random-effects model shown by the dashed vertical line and white box. The solid vertical line represents the null result: an odds ratio of 1. The I^2 statistic is shown in the bottom right corner.

when the four studies with selection criteria likely to bias the results were excluded from the analysis, leading to even stronger protective effects of the *FXIII Val34Leu* variant against VTE and somewhat narrower confidence intervals.

Our meta-analysis included an additional 983 cases and 1,135 controls compared with the largest of the two meta-analyses of studies examining the *FXIII Val34Leu* variant in relation to VTE (17, 24). The meta-analysis by Alhenc-Gelas et al. (24) combined the results of five studies (1,340 cases and 2,211 controls) and found a statistically significant protective effect of the *FXIII Val34Leu* variant against VTE, with odds ratios of 0.58 (95 percent CI: 0.41, 0.82) for homozygotes (*Leu/Leu*), 0.86 (95 percent CI: 0.74, 0.99) for heterozygotes (*Leu/Val*), and 0.80 (95 percent CI: 0.69, 0.94) for homozygotes and heterozygotes combined (*Leu/Leu* + *Leu/Val*). The meta-analysis by van Hylckama Vlieg et al. (17), which included three additional studies, reported a smaller, nonstatistically significant effect despite an additional 842 cases and 1,563 controls compared with the meta-analysis by Alhenc-Gelas et al. In the meta-analysis by van Hylckama Vlieg et al., the odds ratios for the homozygotes were 0.80 (95 percent CI: 0.60, 1.00), 0.80 (95 percent CI: 0.74, 0.99) for the heterozygotes, and 0.90 (95 percent CI: 0.80, 1.00) for the homozygotes and heterozygotes combined. These authors also analyzed their data by excluding two of the new studies in which cases were further ascertained for known thrombophilias (i.e., Balogh et al. (9) and Margaglione et al. (27)). When these studies were excluded from the analysis, the effects became more prominent and statistically significant, with odds ratios of 0.60 (95 percent CI: 0.40, 0.80) for the homozygotes, 0.80 (95 percent CI: 0.70, 1.00) for the heterozygotes, and 0.80 (95 percent CI: 0.70, 0.90) for the homozygotes and heterozygotes combined. The Margaglione et al. cases were all derived from referral for thrombophilic workup; therefore, the most critically ill subjects may have been referred. In the Balogh et al. study, the cases had their first thrombotic episode at an unusually young age; other inclusion criteria were positive family history of VTE or recurrent thrombosis or thrombosis at unusual sites.

These observations suggest that the two studies may have selected cases with more than one determinant for VTE, including additional genetic determinants, which introduced heterogeneity, possibly biasing the estimated odds ratios. It was not possible to specifically assess the risk of VTE in this subgroup—that is, cases with a known predisposition to VTE—because the two studies did not follow the same ascertainment scheme. For example, the mean age of cases in the Balogh et al. study (9) seemed to be younger than that in the Margaglione et al. study (27). However, the age comparison is not simple here. Balogh et al. presented the mean age of cases, whereas Margaglione et al. presented the

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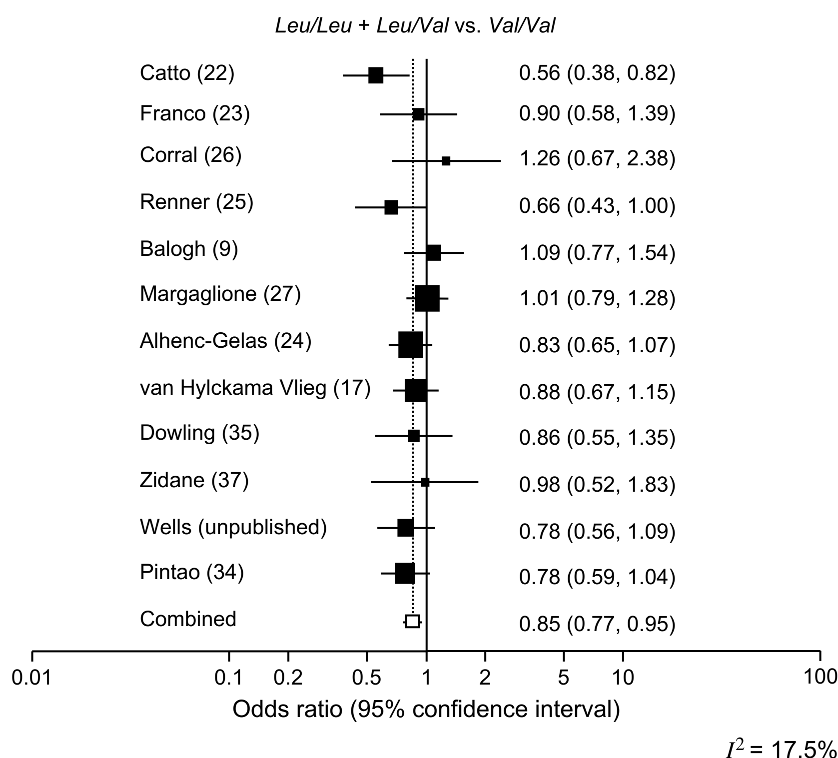

FIGURE 3. Odds ratio (95% confidence interval) for the association between the *Leu/Leu* + *Leu/Val* genotypes combined vs. the *Val/Val* genotype of the *FXIIIA* gene and venous thromboembolism. On the left, the first author of the study is followed by the reference number in parentheses. The size of the black box corresponding to each study is proportional to the sample size; the horizontal lines show the 95% confidence interval of the odds ratio. The combined estimate is based on a random-effects model shown by the dashed vertical line and white box. The solid vertical line represents the null result: an odds ratio of 1. The I^2 statistic is shown in the bottom right corner.

median age. This problem, along with the small sample size of the subset, did not allow subgroup analysis.

Potential problems in conducting and reporting genetic association studies of complex traits have been well described by several authors (38–41). One such problem, population stratification, is always a concern in genetic association studies that use data on unrelated persons. However, to minimize the risk of this potential confounder, in a subgroup analysis we included data samples predominantly composed of White subjects. Work by Ioannidis et al. (42) suggests that such an approach is likely to be sufficient to avoid confounding effects due to population structure and that indeed genetic variants vary in frequency across populations, but the biologic impact usually is consistent across racial boundaries. We found no change in our results with this subgroup analysis. The absence of deviation from Hardy-Weinberg equilibrium of almost all individual studies included in the meta-analysis supports the absence of population stratification. Hardy-Weinberg equilibrium is also consistent with a good selection of genomic controls.

Potential problems in applying meta-analysis methods are also of concern (29, 43). We made attempts to deal with them in the current study. Our meta-analysis used methods and statistical significance thresholds that provided conservative estimates. We did not detect publication bias or heterogeneity between studies. Since we did not have access to individual subjects' data that would have enabled us to use an approach based on individuals, heterogeneity may have remained masked. We identified two potential sources of heterogeneity by carefully inspecting the selection criteria of the individual studies selected for our meta-analysis. The two studies identified as a potential source of heterogeneity were those previously identified by van Hylckama Vlieg et al. (17). We reanalyzed the data by excluding the two studies and the study not in Hardy-Weinberg equilibrium. Doing so did not change our conclusion; rather, their exclusion increased the protective effect measured for the *FXIII Val34Leu* variant against VTE. The odds ratios, particularly for the homozygotes, were lower and the confidence intervals were narrower.

Of particular note here is the vague description of the ethnic background of the study sample and the limited information on the procedures related to determining the genotypes in the vast majority of studies. Several studies did not describe the ethnic background of their samples in percentages or absolute values. Moreover, although most studies referred to a polymerase chain reaction approach for genotyping, only about half of the published studies specifically named the primers used (9, 17, 23, 26, 37), two others referred to a previous paper, and the remainder did not mention anything regarding the primers used. Only two studies mentioned whether the laboratory technicians were blinded

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to the case/control status of the samples. Genotyping error rates were not mentioned in any of the studies. Although we acknowledge that the genotyping error rate may be difficult to estimate in the context of biallelic markers, such as for the *FXIII Val34Leu* variant and in the absence of parental data, more attention to this and other information related to the genotyping section of the methodology would be useful in comparing the studies and in evaluating their quality.

Another problem that complicates the comparison of genetic association studies, and the pooling of data for meta-analysis purposes, is the nonstandardized definition of cases. For example, the majority of studies ascertained the cases with either first or recurrent VTE, and it was not noted whether VTE was secondary to transient risk factors. Furthermore, in several studies, patients with cancer were also included. It is well known that cancer represents a hypercoagulable state. Conversely, experiencing VTE secondary to transient risk factors is associated with low risk for recurrence and has not been well linked to genetic predisposition. Since the data from individual studies were not presented in a way that enabled this type of subgroup analysis, the implications of these factors could not be verified in our analysis. Ideally, future association studies, either on *factor XIII Val34Leu* or other variants, should be more specific regarding the phenotype definition.

The implication of the *FXIII Val34Leu* variant as a factor conferring protection against VTE is biologically plausible. It has been demonstrated that, in carriers of the *FXIII Val34-Leu* variant, activation and depletion of the plasma FXIIIA subunits is more rapid (2). This action results in less stable clots and indeed may result in a decrease in FXIIIA available for stabilization of the clot and thus could provide a protective effect against VTE. VTE, as other complex and multifactorial diseases are, is likely influenced by several genes as well as environmental factors. Simultaneously considering genetic and nongenetic risk and protective factors will be necessary to develop accurate risk profiles to identify persons at risk of VTE. For example, Lim et al. (44) suggest that the protective effect of the *FXIII Val34Leu* variant is specific to conditions in which fibrinogen levels are high, that is, levels known to be associated with an increased risk of VTE. At higher fibrinogen levels, clots in *Leu/Leu* persons were more permeable and looser than in *Val/Val* persons, characteristics associated with more breakable clots. Similar changes in permeability were also observed for a *fibrinogen* variant (44). Thus, the joint effect of genetic variants and hemostatic trait concentrations is likely to be important in predicting the risk of VTE. This is only one example of the complexity likely to be underlying venous thromboembolic disorders. While it may be premature to routinely test for the *FXIII Val34Leu* variant, our results suggest that this variant will be a useful component of risk profiles in the future.

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